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(54) **ECM-COMPLEX ANTIBODY COMPOSITIONS AND METHODS OF USE**

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(57) **ABSTRACT**

The invention provides isolated anti-ECM-complex antibodies that bind to ECM-complexes. The invention also encompasses compositions comprising an anti-ECM-complex antibody and a carrier. These compositions can be provided in an article of manufacture or a kit. Another aspect of the invention is an isolated nucleic acid encoding an anti-ECM-complex antibody, as well as an expression vector comprising the isolated nucleic acid. Also provided are cells that produce the anti-ECM-complex antibodies. The invention encompasses a method of producing the anti-ECM-complex antibodies. Other aspects of the invention are a method of detecting an ECM-complex expressing cancer, a method of inhibiting growth of an ECM-complex-expressing tumor, and a method of alleviating or treating an ECM-complex-expressing cancer in a mammal, comprising administering a therapeutically effective amount of the anti-ECM-complex antibody to the mammal.

ECM-COMPLEX ANTIBODY COMPOSITIONS AND METHODS OF USE

[0001] This patent application claims the benefit of priority from U.S. Provisional Application Ser. No. 61/123,689 filed Apr. 10, 2008, teachings of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

Background of the Invention

Secretoglobins

[0002] Using microarray and quantitative RT-PCR analysis, it has been previously shown that mRNA corresponding to several members of the human secretoglobin family is highly expressed in various cancer tissues, as compared to the corresponding normal tissues. ECM2, known in the literature as Lipophilin B (LipB), was found to be upregulated in endometrial, uterine, breast and prostate cancer tissues. ECM3, also known as Mammaglobin B (MamB) or Lipophilin C (LipC), was found to be upregulated in endometrial and uterine cancer tissues.

[0003] There is considerable evidence suggesting that members of the secretoglobin family exist predominantly as heterodimers and higher order complexes, referred to herein as ECM-complexes. Mammaglobin A (MamA) protein has been found to be present in breast tissue in a complex with LipB protein. However, other heterodimer and homodimer complexes of secretoglobin family members have not been described, nor have their detection in serum or relative levels in individuals with cancer.

Breast Cancer

[0004] Breast cancer, also referred to as mammary tumor cancer, is the second most common cancer among women, accounting for a third of the cancers diagnosed in the United States. One in nine women will develop breast cancer in her lifetime and about 192,000 new cases of breast cancer are diagnosed annually with about 42,000 deaths. Bevers, *Primary Prevention of Breast Cancer*, in *Breast Cancer,* 20-54 (Kelly K Hunt et al., ed., 2001); Kochanek et al., 49 Nat'l Vital Statistics Reports 1, 14 (2001). Breast cancer is extremely rare in women younger than 20 and is very rare in women under 30. The incidence of breast cancer rises with age and becomes significant by age 50. White Non-Hispanic women have the highest incidence rate for breast cancer and Korean women have the lowest. Increased prevalence of the genetic mutations BRCA1 and BRCA2 that promote breast and other cancers are found in Ashkenazi Jews. African American women have the highest mortality rate for breast cancer among these same groups (31 per 100,000), while Chinese women have the lowest at 11 per 100,000. Although men can get breast cancer, this is extremely rare. In the United States it is estimated there will be 240,510 new cases of breast cancer (62,030 in situ and 178,480 invasive cases) and 40,460 deaths due to breast cancer in 2007. (American Cancer Society Website: cancer with the extension .org of the world wide web). With the exception of those cases with associated genetic factors, precise causes of breast cancer are not known.

[0005] In the treatment of breast cancer, there is considerable emphasis on detection and risk assessment because early and accurate staging of breast cancer has a significant impact on survival. For example, breast cancer detected at an early stage (stage T0, discussed below) has a five-year survival rate of 92%. Conversely, if the cancer is not detected until a late stage (i.e., stage T4 (IV)), the five-year survival rate is reduced to 13%. *AJCC Cancer Staging Handbook* pp. 164-65 (Irvin D. Fleming et al. eds., 5th ed. 1998). Some detection techniques, such as mammography and biopsy, involve increased discomfort, expense, and/or radiation, and are prescribed only to patients with an increased risk of breast cancer.

[0006] Current methods for predicting or detecting breast cancer risk are not optimal. One method for predicting the relative risk of breast cancer is by examining a patient's risk factors and pursuing aggressive diagnostic and treatment regimes for high risk patients. A patient's risk of breast cancer has been positively associated with increasing age, nulliparity, family history of breast cancer, personal history of breast cancer, early menarche, late menopause, late age of first full term pregnancy, prior proliferative breast disease, irradiation of the breast at an early age and a personal history of malignancy. Lifestyle factors such as fat consumption, alcohol consumption, education, and socioeconomic status have also been associated with an increased incidence of breast cancer, although a direct cause and effect relationship has not been established. While these risk factors are statistically significant, their weak association with breast cancer limits their usefulness. Most women who develop breast cancer have none of the risk factors listed above, other than the risk that comes with growing older. NIH Publication No. 00-1556 (2000).

[0007] Current screening methods for detecting cancer, such as breast self exam, ultrasound, and mammography have drawbacks that reduce their effectiveness or prevent their widespread adoption. Breast self exams, while useful, are unreliable for the detection of breast cancer in the initial stages where the tumor is small and difficult to detect by palpation. Ultrasound measurements require skilled operators at an increased expense. Mammography, while sensitive, is subject to over diagnosis in the detection of lesions that have questionable malignant potential. There is also the fear of the radiation used in mammography because prior chest radiation is a factor associated with an increased incidence of breast cancer.

[0008] At this time, there are no adequate methods of breast cancer prevention. The current methods of breast cancer prevention involve prophylactic mastectomy (mastectomy performed before cancer diagnosis) and chemoprevention (chemotherapy before cancer diagnosis). These are drastic measures that limit their adoption even among women with increased risk of breast cancer. Bevers, supra.

[0009] A number of genetic markers have been associated with breast cancer. Examples of these markers include carcinoembryonic antigen (CEA) (Mughal et al., *JAMA* 249:1881 (1983)), MUC-1 (Frische and Liu, *J. Clin. Ligand* 22:320 (2000)), HER-2/neu (Haris et al., Proc. Am. Soc. Clin. Oncology 15:A96 (1996)), uPA, PAI-1, LPA, LPC, RAK and BRCA (Esteva and Fritsche, Serum and Tissue Markers for Breast Cancer, in Breast Cancer, 286-308 (2001)). These markers have problems with limited sensitivity, low correlation, and false negatives which limit their use for initial diagnosis. For example, while the BRCA1 gene mutation is useful as an indicator of an increased risk for breast cancer, it has limited use in cancer diagnosis because only 6.2% of breast

cancers are BRCA1 positive. Malone et al., JAMA 279:922 (1998). See also, Mewman et al., JAMA 279:915 (1998) (correlation of only 3.3%).

[0010] There are four primary classifications of breast cancer varying by the site of origin and the extent of disease development.

[0011] I. Ductal carcinoma in situ (DCIS): Malignant transformation of ductal epithelial cells that remain in their normal position. DCIS is a purely localized disease, incapable of metastasis.

[0012] II. Invasive ductal carcinoma (IDC): Malignancy of the ductal epithelial cells breaking through the basal membrane and into the supporting tissue of the breast. IDC may eventually spread elsewhere in the body.

[0013] III. Lobular carcinoma in situ (LCIS): Malignancy arising in a single lobule of the breast that fails to extend through the lobule wall, it generally remains localized.

[0014] IV. Infiltrating lobular carcinoma (ILC): Malignancy arising in a single lobule of the breast and invading directly through the lobule wall into adjacent tissues. By virtue of its invasion beyond the lobule wall, ILC may penetrate lymphatics and blood vessels and spread to distant sites.

[0015] For purpose of determining prognosis and treatment, these four breast cancer types have been staged according to the size of the primary tumor (T), the involvement of lymph nodes (N), and the presence of metastasis (M). Although DCIS by definition represents localized stage I disease, the other forms of breast cancer may range from stage II to stage IV. There are additional prognostic factors that further serve to guide surgical and medical intervention. The most common ones are total number of lymph nodes involved, ER (estrogen receptor) status, Her2/neu receptor status and histologic grades. Breast cancers are diagnosed into the appropriate stage categories recognizing that different treatments are more effective for different stages of cancer. Stage TX indicates that primary tumor cannot be assessed (i.e., tumor was removed or breast tissue was removed). Stage T0 is characterized by abnormalities such as hyperplasia but with no evidence of primary tumor. Stage T is is characterized by carcinoma in situ, intraductal carcinoma, lobular carcinoma in situ, or Paget's disease of the nipple with no tumor. Stage T1 (I) is characterized as having a tumor of 2 cm or less in the greatest dimension. Within stage T1, Tmic indicates microinvasion of 0.1 cm or less, T1a indicates a tumor of between 0.1 to 0.5 cm, T1b indicates a tumor of between 0.5 to 1 cm, and T1c indicates tumors of between 1 cm to 2 cm. Stage T2 (II) is characterized by tumors from 2 cm to 5 cm in the greatest dimension. Tumors greater than 5 cm in size are classified as stage T3 (III). Stage T4 (IV) indicates a tumor of any size with extension to the chest wall or skin. Within stage T4, T4a indicates extension of the tumor to the chest wall, T4b indicates edema or ulceration of the skin of the breast or satellite skin nodules confined to the same breast, T4c indicates a combination of T4a and T4b, and T4d indicates inflammatory carcinoma. AJCC Cancer Staging Handbook pp. 159-70 (Irvin D. Fleming et al. eds., 5th ed. 1998). In addition to standard staging, breast tumors may be classified according to their estrogen receptor and progesterone receptor protein status. Fisher et al., *Breast Cancer Research and Treatment* 7:147 (1986). Additional pathological status, such as HER2/neu status may also be useful. Thor et al., *J. Nat'l. Cancer Inst.* 90:1346 (1998); Paik et al., *J. Nat'l. Cancer Inst.* 90:1361 (1998); Hutchins et al., *Proc. Am. Soc. Clin. Oncology* 17:A2 (1998); and Simpson et al., *J. Clin. Oncology* 18:2059 (2000).

[0016] In addition to the staging of the primary tumor, breast cancer metastases to regional lymph nodes may be staged. Stage NX indicates that the lymph nodes cannot be assessed (e.g., previously removed). Stage N0 indicates no regional lymph node metastasis. Stage N1 indicates metastasis to movable ipsilateral axillary lymph nodes. Stage N2 indicates metastasis to ipsilateral axillary lymph nodes fixed to one another or to other structures. Stage N3 indicates metastasis to ipsilateral internal mammary lymph nodes. Id.

[0017] Stage determination has potential prognostic value and provides criteria for designing optimal therapy. Simpson et al., *J. Clin. Oncology* 18:2059 (2000). Generally, pathological staging of breast cancer is preferable to clinical staging because the former gives a more accurate prognosis. However, clinical staging would be preferred if it were as accurate as pathological staging because it does not depend on an invasive procedure to obtain tissue for pathological evaluation. Staging of breast cancer would be improved by detecting new markers in cells, tissues, or bodily fluids which could differentiate between different stages of invasion. Progress in this field will allow more rapid and reliable methods for treating breast cancer patients.

[0018] Treatment of breast cancer is generally decided after an accurate staging of the primary tumor. Primary treatment options include breast conserving therapy (lumpectomy, breast irradiation, and surgical staging of the axilla), and modified radical mastectomy. Additional treatments include chemotherapy, regional irradiation, and, in extreme cases, terminating estrogen production by ovarian ablation.

[0019] Until recently, the customary treatment for all breast cancer was mastectomy. Fonseca et al., *Annals of Internal Medicine* 127:1013 (1997). However, recent data indicate that less radical procedures may be equally effective, in terms of survival, for early stage breast cancer. Fisher et al., *J. of Clinical Oncology* 16:441 (1998). The treatment options for a patient with early stage breast cancer (i.e., stage Tis) may be breast-sparing surgery followed by localized radiation therapy at the breast. Alternatively, mastectomy optionally coupled with radiation or breast reconstruction may be employed. These treatment methods are equally effective in the early stages of breast cancer.

[0020] Patients with stage I and stage I breast cancer require surgery with chemotherapy and/or hormonal therapy. Surgery is of limited use in stage III and stage IV patients. These patients are better candidates for chemotherapy and radiation therapy with surgery limited to biopsy to permit initial staging or subsequent restaging since cancer is rarely curative at this stage of the disease. *AJCC Cancer Staging Handbook* 84, 164-65 (Irvin D. Fleming et al. eds., 5th ed. 1998).

[0021] In an effort to provide more treatment options to patients, efforts are underway to define an earlier stage of breast cancer with low recurrence which could be treated with lumpectomy without postoperative radiation treatment. While a number of attempts have been made to classify early stage breast cancer, no consensus recommendation on postoperative radiation treatment has been obtained from these studies. Page et al., *Cancer* 75:1219 (1995); Fisher et al., *Cancer* 75:1223 (1995); Silverstein et al., *Cancer* 77:2267 (1996).

Ovarian Cancer

[0022] Cancer of the ovaries is the fourth-most common cause of cancer death in women in the United States, with

more than 23,000 new cases and roughly 14,000 deaths predicted for the year 2001. Shridhar, V. et al., *Cancer Res.* 61(15): 5895-904 (2001); Memarzadeh, S. & Berek, J. S., *J. Reprod. Med.* 46(7): 621-29 (2001). The American Cancer Society (ACS) estimated that there would be about 25,580 new cases of ovarian cancer in 2004 and ovarian cancer will cause about 16,090 deaths in the United States. ACS Website: cancer with the extension .org of the world wide web. More women die annually from ovarian cancer than from all other gynecologic malignancies combined. The incidence of ovarian cancer in the United States is estimated to be 14.2 women per 100,000 women per year and 9 women per 100,000 die every year from ovarian cancer. In 2004, approximately 70-75% of new diagnoses were predicted to be stage III and IV carcinoma with a predicted 5-year survival of ~15%. Jemal et al., Annual Report to the Nation on the Status of Cancer, 1975-2001, with a Special Feature Regarding Survival. Cancer 2004; 101: 3-27. The incidence of ovarian cancer is of serious concern worldwide, with an estimated 191, 000 new cases predicted annually. Runnebaum, I. B. & Stickeler, E., *J. Cancer Res. Clin. Oncol.* 127(2): 73-79 (2001). Unfortunately, women with ovarian cancer are typically asymptomatic until the disease has metastasized. Because effective screening for ovarian cancer is not available, roughly 70% of women diagnosed have an advanced stage of the cancer with a five-year survival rate of ~25-30%. Memarzadeh, S. & Berek, J. S., supra; Nunns, D. et al., *Obstet. Gynecol. Surv.* 55(12): 746-51. Conversely, women diagnosed with early stage ovarian cancer enjoy considerably higher survival rates. Werness, B. A. & Eltabbakh, G. H., *Int'l. J. Gynecol. Pathol.* 20(1): 48-63 (2001). Although our understanding of the etiology of ovarian cancer is incomplete, the results of extensive research in this area point to a combination of age, genetics, reproductive, and dietary/environmental factors. Age is a key risk factor in the development of ovarian cancer: while the risk for developing ovarian cancer before the age of 30 is slim, the incidence of ovarian cancer rises linearly between ages 30 to 50, increasing at a slower rate thereafter, with the highest incidence being among septagenarian women. Jeanne M. Schilder et al., *Heriditary Ovarian Cancer: Clinical Syndromes and Management*, in *Ovarian Cancer* 182 (Stephen C. Rubin & Gregory P. Sutton eds., 2d ed. 2001).

[0023] With respect to genetic factors, a family history of ovarian cancer is the most significant risk factor in the development of the disease, with that risk depending on the number of affected family members, the degree of their relationship to the woman, and which particular first degree relatives are affected by the disease. Id. Mutations in several genes have been associated with ovarian cancer, including BRCA1 and BRCA2, both of which play a key role in the development of breast cancer, as well as hMSH2 and hMLH1, both of which are associated with heriditary non-polyposis colon cancer. Katherine Y. Look, *Epidemiology, Etiology, and Screening of Ovarian Cancer*, in *Ovarian Cancer* 169, 171-73 (Stephen C. Rubin & Gregory P. Sutton eds., 2d ed. 2001). BRCA1, located on chromosome 17, and BRCA2, located on chromosome 13, are tumor suppressor genes implicated in DNA repair; mutations in these genes are linked to roughly 10% of ovarian cancers. Id. at 171-72; Schilder et al., supra at 185-86. hMSH2 and hMLH1 are associated with DNA mismatch repair, and are located on chromsomes 2 and 3, respectively; it has been reported that roughly 3% of heriditary ovarian carcinomas are due to mutations in these genes. Look, supra at 173; Schilder et al., supra at 184, 188-89.

[0024] Reproductive factors have also been associated with an increased or reduced risk of ovarian cancer. Late menopause, nulliparity, and early age at menarche have all been linked with an elevated risk of ovarian cancer. Schilder et al., supra at 182. One theory hypothesizes that these factors increase the number of ovulatory cycles over the course of a woman's life, leading to "incessant ovulation," which is thought to be the primary cause of mutations to the ovarian epithelium. Id.; Laura J. Havrilesky & Andrew Berchuck, *Molecular Alterations in Sporadic Ovarian Cancer*, in *Ovarian Cancer* 25 (Stephen C. Rubin & Gregory P. Sutton eds., 2d ed. 2001). The mutations may be explained by the fact that ovulation results in the destruction and repair of that epithelium, necessitating increased cell division, thereby increasing the possibility that an undetected mutation will occur. Id. Support for this theory may be found in the fact that pregnancy, lactation, and the use of oral contraceptives, all of which suppress ovulation, confer a protective effect with respect to developing ovarian cancer. Id.

[0025] Among dietary/environmental factors, there would appear to be an association between high intake of animal fat or red meat and ovarian cancer, while the antioxidant Vitamin A, which prevents free radical formation and also assists in maintaining normal cellular differentiation, may offer a protective effect. Look, supra at 169. Reports have also associated asbestos and hydrous magnesium trisilicate (talc), the latter of which may be present in diaphragms and sanitary napkins, which ovarian cancer. Id. at 169-70.

[0026] Current screening procedures for ovarian cancer, while of some utility, are quite limited in their diagnostic ability, a problem that is particularly acute at early stages of cancer progression when the disease is typically asymptomatic yet is most readily treated. Walter J. Burdette, *Cancer: Etiology, Diagnosis, and Treatment* 166 (1998); Memarzadeh & Berek, supra; Runnebaum & Stickeler, supra; Werness & Eltabbakh, supra. Commonly used screening tests include biannual rectovaginal pelvic examination, radioimmunoassay to detect the CA-125 serum tumor marker, and transvaginal ultrasonography. Burdette, supra at 166. Currently, CA-125 is the only clinically approved serum marker for use in ovarian cancer. CA-125 is found elevated in the majority of serous cancers, but is elevated in only half of those women with early stage disease. The major clinical application of CA125 is in monitoring treatment success or detection of recurrence in women undergoing treatment for ovarian cancer. Markman M. *The Oncologist;* 2: 6-9 (1997). The use of CA125 as a screening marker is limited because it is frequently elevated in women with benign diseases such as endometriosis. Hence, there is a critical need for serum markers that are more sensitive and specific for the detection of ovarian cancer when used alone, or in combination with CA125. Bast R C. Et al., *Early Detection of Ovarian Cancer: Promise and Reality* in *Ovarian Cancer. Cancer Research and Treatment* Vol 107 (Stack M S, Fishman, D A, eds., 2001).

[0027] Pelvic examination has failed to yield adequate numbers of early diagnoses, and the other methods are not sufficiently accurate. Id. One study reported that only 15% of patients who suffered from ovarian cancer were diagnosed with the disease at the time of their pelvic examination. Look, supra at 174. Moreover, the CA-125 test is prone to giving false positives in pre-menopausal women and has been

reported to be of low predictive value in post-menopausal women. Id. at 174-75. Although transvaginal ultrasonography is now the preferred procedure for screening for ovarian cancer, it is unable to distinguish reliably between benign and malignant tumors, and also cannot locate primary peritoneal malignancies or ovarian cancer if the ovary size is normal. Schilder et al., supra at 194-95. While genetic testing for mutations of the BRCA1, BRCA2, hMSH2, and hMLH1 genes is now available, such testing may be too costly for some patients and may also yield false negative or indeterminate results. Schilder et al., supra at 191-94.

[0028] Current efforts focus on the identification of panels of biomarkers that can be used in combination. Bast R C Jr., J Clin Oncol 2003; 21: 200-205. Currently, other markers being evaluated as potential ovarian serum markers which may serve as members of a multi-marker panel to improve detection of ovarian cancer are HE4; mesothelin; kallikrein 5, 8, 10 and 11; and prostasin. Urban et al. Ovarian cancer screening Hematol Oncol Clin North Am. 2003 August; 17(4):989-1005; Hellstrom et al. The HE4 (WFDC2) protein is a biomarker for ovarian carcinoma, Cancer Res. 2003 Jul. 1; 63(13):3695-700; Ordonez, Application of mesothelin immunostaining in tumor diagnosis, Am J Surg Pathol. 2003 November; 27(11):1418-28; Diamandis E P et al., Cancer Research 2002; 62: 295-300; Yousef G M et al., Cancer Research 2003; 63: 3958-3965; Kishi T et al., Cancer Research 2003; 63: 2771-2774; Luo L Y et al., Cancer Research 2003; 63: 807-811; Mok S C et al., J Natl Cancer Inst 2001; 93 (19): 1437-1439.

[0029] The staging of ovarian cancer, which is accomplished through surgical exploration, is crucial in determining the course of treatment and management of the disease. *AJCC Cancer Staging Handbook* 187 (Irvin D. Fleming et al. eds., 5th ed. 1998); Burdette, supra at 170; Memarzadeh & Berek, supra; Shridhar et al., supra. Staging is performed by reference to the classification system developed by the International Federation of Gynecology and Obstetrics. David H. Moore, *Primary Surgical Management of Early Epithelial Ovarian Carcinoma*, in *Ovarian Cancer* 203 (Stephen C. Rubin & Gregory P. Sutton eds., 2d ed. 2001); Fleming et al. eds., supra at 188. Stage I ovarian cancer is characterized by tumor growth that is limited to the ovaries and is comprised of three substages. Id. In substage IA, tumor growth is limited to one ovary, there is no tumor on the external surface of the ovary, the ovarian capsule is intact, and no malignant cells are present in ascites or peritoneal washings. Id. Substage IB is identical to A1, except that tumor growth is limited to both ovaries. Id. Substage IC refers to the presence of tumor growth limited to one or both ovaries, and also includes one or more of the following characteristics: capsule rupture, tumor growth on the surface of one or both ovaries, and malignant cells present in ascites or peritoneal washings. Id.

[0030] Stage II ovarian cancer refers to tumor growth involving one or both ovaries, along with pelvic extension. Id. Substage IIA involves extension and/or implants on the uterus and/or fallopian tubes, with no malignant cells in the ascites or peritoneal washings, while substage IIB involves extension into other pelvic organs and tissues, again with no malignant cells in the ascites or peritoneal washings. Id. Substage IIC involves pelvic extension as in IIA or IIB, but with malignant cells in the ascites or peritoneal washings. Id.

[0031] Stage III ovarian cancer involves tumor growth in one or both ovaries, with peritoneal metastasis beyond the pelvis confirmed by microscope and/or metastasis in the regional lymph nodes. Id. Substage IIIA is characterized by microscopic peritoneal metastasis outside the pelvis, with substage IIIB involving macroscopic peritoneal metastasis outside the pelvis 2 cm or less in greatest dimension. Id. Substage IIIC is identical to IIIB, except that the metastasis is greater than 2 cm in greatest dimension and may include regional lymph node metastasis. Id. Lastly, Stage IV refers to the presence of distant metastasis, excluding peritoneal metastasis. Id.

[0032] While surgical staging is currently the benchmark for assessing the management and treatment of ovarian cancer, it suffers from considerable drawbacks, including the invasiveness of the procedure, the potential for complications, as well as the potential for inaccuracy. Moore, supra at 206-208, 213. In view of these limitations, attention has turned to developing alternative staging methodologies through understanding differential gene expression in various stages of ovarian cancer and by obtaining various biomarkers to help better assess the progression of the disease. Vartiainen, J. et al., *Int'l J. Cancer,* 95(5): 313-16 (2001); Shridhar et al. supra; Baekelandt, M. et al., *J. Clin. Oncol.* 18(22): 3775-81.

[0033] The treatment of ovarian cancer typically involves a multiprong attack, with surgical intervention serving as the foundation of treatment. Dennis S. Chi & William J. Hoskins, Primary Surgical Management of Advanced Epithelial Ovarian Cancer, in *Ovarian Cancer* 241 (Stephen C. Rubin & Gregory P. Sutton eds., 2d ed. 2001). For example, in the case of epithelial ovarian cancer, which accounts for ~90% of cases of ovarian cancer, treatment typically consists of: (1) cytoreductive surgery, including total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and lymphadenectomy, followed by (2) adjuvant chemotherapy with paclitaxel and either cisplatin or carboplatin. Eltabbakh, G. H. & Awtrey, C. S., *Expert Op. Pharmacother.* 2(10): 109-24. Despite a clinical response rate of 80% to the adjuvant therapy, most patients experience tumor recurrence within three years of treatment. Id. Certain patients may undergo a second cytoreductive surgery and/or second-line chemotherapy. Memarzadeh & Berek, supra.

[0034] From the foregoing, it is clear that procedures used for detecting, diagnosing, monitoring, staging, prognosticating, and preventing the recurrence of ovarian cancer are of critical importance to the outcome of the patient. Moreover, current procedures, while helpful in each of these analyses, are limited by their specificity, sensitivity, invasiveness, and/or their cost. As such, highly specific and sensitive procedures that would operate by way of detecting markers in cells, tissues, or bodily fluids, with minimal invasiveness and at a reasonable cost, are highly desirable.

[0035] Accordingly, there is a great need for more sensitive and accurate methods for predicting whether a person is likely to develop ovarian cancer, for diagnosing ovarian cancer, for monitoring the progression of the disease, for staging the ovarian cancer, for determining whether the ovarian cancer has metastasized, and for imaging the ovarian cancer. There is also a need for better treatment of ovarian cancer.

Angiogenesis in Cancer

[0036] Growth and metastasis of solid tumors are also dependent on angiogenesis. Folkman, J., 1986, *Cancer Research,* 46, 467-473; Folkman, J., 1989, *Journal of the National Cancer Institute,* 82, 4-6. It has been shown, for example, that tumors which enlarge to greater than 2 mm must obtain their own blood supply and do so by inducing the

growth of new capillary blood vessels. Once these new blood vessels become embedded in the tumor, they provide a means for tumor cells to enter the circulation and metastasize to distant sites such as liver, lung or bone. Weidner, N., et al., 1991, *The New England Journal of Medicine,* 324(1), 1-8.

[0037] Angiogenesis, defined as the growth or sprouting of new blood vessels from existing vessels, is a complex process that primarily occurs during embryonic development. The process is distinct from vasculogenesis, in that the new endothelial cells lining the vessel arise from proliferation of existing cells, rather than differentiating from stem cells. The process is invasive and dependent upon proteolyisis of the extracellular matrix (ECM), migration of new endothelial cells, and synthesis of new matrix components. Angiogenesis occurs during embryogenic development of the circulatory system; however, in adult humans, angiogenesis only occurs as a response to a pathological condition (except during the reproductive cycle in women).

[0038] Under normal physiological conditions in adults, angiogenesis takes place only in very restricted situations such as hair growth and wounding healing. Auerbach, W. and Auerbach, R., 1994, *Pharmacol Ther.* 63(3):265-3 11; Ribatti et al., 1991, *Haematologica* 76(4):3 11-20; Risau, 1997, *Nature* 386(6626):67 1-4. Angiogenesis progresses by a stimulus which results in the formation of a migrating column of endothelial cells. Proteolytic activity is focused at the advancing tip of this "vascular sprout", which breaks down the ECM sufficiently to permit the column of cells to infiltrate and migrate. Behind the advancing front, the endothelial cells differentiate and begin to adhere to each other, thus forming a new basement membrane. The cells then cease proliferation and finally define a lumen for the new arteriole or capillary.

[0039] Unregulated angiogenesis has gradually been recognized to be responsible for a wide range of disorders, including, but not limited to, cancer, cardiovascular disease, rheumatoid arthritis, psoriasis and diabetic retinopathy. Folkman, 1995, *Nat Med* 1(1):27-31; Isner, 1999, *Circulation* 99(13): 1653-5; Koch, 1998, *Arthritis Rheum* 41(6):951-62; Walsh, 1999, Rheumatology (Oxford) 38(2):103-12; Ware and Simons, 1997, Nat Med 3(2): 158-64.

[0040] Of particular interest is the observation that angiogenesis is required by solid tumors for their growth and metastases. Folkman, 1986 supra; Folkman 1990, *J Natl. Cancer Inst.,* 82(1) 4-6; Folkman, 1992, *Semin Cancer Biol* 3(2):65-71; Zetter, 1998, *Annu Rev Med* 49:407-24. A tumor usually begins as a single aberrant cell which can proliferate only to a size of a few cubic millimeters due to the distance from available capillary beds. It can stay 'dormant' without further growth and dissemination for a long period of time. Some tumor cells then switch to the angiogenic phenotype to activate endothelial cells, which proliferate and mature into new capillary blood vessels. These newly formed blood vessels not only allow for continued growth of the primary tumor, but also for the dissemination and recolonization of metastatic tumor cells. The precise mechanisms that control the angiogenic switch is not well understood, but it is believed that neovascularization of tumor mass results from the net balance of a multitude of angiogenesis stimulators and inhibitors Folkman, 1995, supra.

[0041] One of the most potent angiogenesis inhibitors is endostatin identified by O'Reilly and Folkunan. O'Reilly et al., 1997, *Cell* 88(2):277-85; O'Reilly et al., 1994, *Cell* 79(2):3 15-28. Its discovery was based on the phenomenon that certain primary tumors can inhibit the growth of distant metastases. O'Reilly and Folkman hypothesized that a primary tumor initiates angiogenesis by generating angiogenic stimulators in excess of inhibitors. However, angiogenic inhibitors, by virtue of their longer half life in the circulation, reach the site of a secondary tumor in excess of the stimulators. The net result is the growth of primary tumor and inhibition of secondary tumor. Endostatin is one of a growing list of such angiogenesis inhibitors produced by primary tumors. It is a proteolytic fragment of a larger protein: endostatin is a 20 kDa fragment of collagen XVIII (amino acid H1132-K1315 in murine collagen XVIII). Endostatin has been shown to specifically inhibit endothelial cell proliferation in vitro and block angiogenesis in vivo. More importantly, administration of endostatin to tumor-bearing mice leads to significant tumor regression, and no toxicity or drug resistance has been observed even after multiple treatment cycles. Boehm et al., 1997, Nature 390(6658):404-407. The fact that endostatin targets genetically stable endothelial cells and inhibits a variety of solid tumors makes it a very attractive candidate for anticancer therapy. Fidler and Ellis, 1994, Cell 79(2):185-8; Gastl et al., 1997, Oncology 54(3):177-84; Hinsbergh et al., 1999, Ann Oncol 10 Suppl 4:60-3. In addition, angiogenesis inhibitors have been shown to be more effective when combined with radiation and chemotherapeutic agents. Klement, 2000, J. Clin Invest, 105(8) R15-24. Browder, 2000, Cancer Res. 6-(7) 1878-86, Arap et al., 1998, Science 279(5349):377-80; Mauceri et al., 1998, Nature 394 (6690):287-91.

[0042] As discussed above, each of the methods for diagnosing and staging breast or ovarian cancer is limited by the technology employed. Accordingly, there is need for sensitive molecular and cellular markers for the detection of breast or ovarian cancer. There is a need for molecular markers for the accurate staging, including clinical and pathological staging, of breast or ovarian cancers to optimize treatment methods. In addition, there is a need for sensitive molecular and cellular markers to monitor the progress of cancer treatments, including markers that can detect recurrence of breast or ovarian cancers following remission.

[0043] The present invention provides alternative methods of treating breast or ovarian cancer that overcome the limitations of conventional therapeutic methods as well as offer additional advantages that will be apparent from the detailed description below.

SUMMARY OF THE INVENTION

[0044] This invention is directed to an isolated ECM-complex antibody that binds to an ECM-complex. The antibody may be a monoclonal antibody. Alternatively, the antibody is an antibody fragment, or a chimeric, a human or a humanized antibody.

[0045] The invention is also directed to labeled and conjugated antibodies. They may be conjugated to a growth inhibitory agent or a cytotoxic agent. The cytotoxic agent may be selected from the group consisting of toxins, antibiotics, radioactive isotopes and nucleolytic enzymes and toxins. Examples of toxins include, but are not limited to, maytansin, maytansinoids, saporin, gelonin, ricin and calicheamicin.

[0046] The antibodies may bind to an ECM-complex in a mammalian cell. The mammalian cell may be a cancer cell. Preferably, the anti-ECM-complex monoclonal antibody inhibits the growth of ECM-complex-expressing cancer cells.

Preferably, the cancer is selected from the group consisting of breast cancer, ovarian cancer, metastatic breast cancer and metastatic ovarian cancer.

[0047] The invention is also directed to a method of producing the antibodies comprising culturing an appropriate cell and recovering the antibody from the cell culture.

[0048] The invention is also directed to compositions comprising the antibodies and a carrier.

[0049] The invention is also directed to a method of inhibiting growth of an ECM-complex-expressing tumor, comprising contacting the tumor with the antibodies of this invention, thereby inhibiting growth of the tumor. The tumor may be selected from the group consisting of breast and ovarian tumors. The breast or ovarian tumor may be metastatic tumors.

[0050] The invention is also directed to a method of alleviating an ECM-complex-expressing cancer in a mammal, comprising administering a therapeutically effective amount of the antibodies to the mammal.

[0051] In addition, the invention is directed to an article of manufacture comprising a container and a composition contained therein, wherein the composition comprises an antibody as described herein. The article of manufacture may also comprise an additional component, e.g., a package insert indicating that the composition can be used to detect or treat breast or ovarian cancer.

[0052] The invention is also directed to a method for determining the presence of an ECM-complex in a sample comprising contacting a sample with an ECM-complex antibody and determining the amount of binding of the antibody, wherein binding indicates the presence of the ECM-complex in a sample.

[0053] The invention is further directed to a method for detecting the presence of breast or ovarian cancer in a subject comprising determining the level of ECM-complex in a sample from the subject and comparing the level of ECM-complex in the sample to a control, wherein an increase in the level in the sample from the subject is indicative of breast or ovarian cancer.

[0054] The invention is further directed to a screening method for antibodies that bind to same epitope as antibodies described herein.

DETAILED DESCRIPTION OF THE INVENTION

Definitions and General Techniques

[0055] Human "ECM-complex" as used herein, refers to heterodimers, homodimers, and higher order complexes (such as tetramers comprising dimers of heterodimer and homodimers) of members of the secretoglobin family. Secretoglobin family members include: ECM2, known in the literature as Lipophilin B (LipB); ECM3, known in the literature as Mammaglobin B (MamB) or Lipophilin C (LipC); Mammaglobin A (MamA); and Lipophilin A (LipA). Secretoglobin family members are secreted from cells and ECM-complexes are detectable in bodily fluids. ECM-complex as used herein includes allelic variants and conservative substitution mutants of the complex which have ECM-complex biological activity. Members of the secretoglobin family are described below. Information referenced from RefSeq and other sources is hereby incorporated by reference.

[0056] ECM2, known in the literature as Lipophilin B (LipB) is identified in the RefSeq database as accessions NM_006551 and NP_006542 (accessible at ncbi with the extension .nlm.nih.gov of the world wide web) and titled "*Homo sapiens* secretoglobin, family 1D, member 2". Other synonyms for ECM2 include: SCGB1D2, Lipophilin B (LipB, LPHB), prostatein-like lipophilin B, lipophilin B (uteroglobin family member), and prostatein-like. The refseq database includes the following summary of ECM2:

[0057] The protein encoded by this gene is a member of the lipophilin subfamily, part of the uteroglobin superfamily, and is an ortholog of prostatein, the major secretory glycoprotein of the rat ventral prostate gland. Lipophilin gene products are widely expressed in normal tissues, especially in endocrine-responsive organs. Assuming that human lipophilins are the functional counterparts of prostatein, they may be transcriptionally regulated by steroid hormones, with the ability to bind androgens, other steroids and possibly bind and concentrate estramustine, a chemotherapeutic agent widely used for prostate cancer. Although the gene has been reported to be on chromosome 10, this sequence appears to be from a cluster of genes on chromosome 11 that includes mammaglobin 2.

[0058] ECM3, known in the literature as Mammaglobin B (MamB) or Lipophilin C (LipC) is identified in the RefSeq database as accessions NM_002407 and NP_002398 (accessible at ncbi with the extension .nlm.nih.gov of the world wide web) and titled "*Homo sapiens* secretoglobin, family 2A, member 1". Other synonyms for ECM3 include: SCGB2A1, LPHC, MGB2, UGB3, MGC71973, lipophilin C, mammaglobin 2, and mammaglobin B.

[0059] Mammaglobin A (MamA) is identified in the RefSeq database as accessions NM_002411 and NP_002402 (accessible at ncbi with the extension .nlm.nih.gov of the world wide web) and titled "*Homo sapiens* secretoglobin, family 2A, member 2". Other synonyms for MamA include: SCGB2A2, MGB1, UGB2, MGC71974, mammaglobin 1, mammaglobin A.

[0060] Lipophilin A (LipA) is identified in the RefSeq database as accessions NM_006552 and NP_006543 (accessible at ncbi with the extension .nlm.nih.gov of the world wide web) and titled "*Homo sapiens* secretoglobin, family 1D, member 1". Other synonyms for LipA include: SCGB1D1, LIPA, LPHA, MGC71958, lipophilin A, prostatein-like lipophilin A, and lipophilin A (uteroglobin family member). The refseq database includes the following summary of LipA:

[0061] The protein encoded by this gene is a member of the lipophilin subfamily, part of the uteroglobin superfamily, and is an ortholog of prostatein, the major secretory glycoprotein of the rat ventral prostate gland. This gene product represents one component of a heterodimeric molecule present in human tears whose elution profile is consistent with prostatein, a tetrameric molecule composed of three peptide components in heterodimers. Assuming that human lipophilins are the functional counterparts of prostatein, they may be transcriptionally regulated by steroid hormones, with the ability to bind androgens, other steroids and possibly bind and concentrate estramustine, a chemotherapeutic agent widely used for prostate cancer. Although the gene has been reported to be on chromosome 15, this sequence appears to be from a cluster of genes on chromosome 11 that includes mammaglobin 2.

[0062] Publications have described the identification, characterization, association with disease, and clinical develop-

ment of secretoglobin family members as a molecular target for disease detection, therapy and vaccination including the following which are hereby incorporated by reference in their entirety.

Colpitts TL, et al. Biochemistry. 2001 Sep 18; 40(37): 11048-59.
Carter D, et al. Biochemistry. 2002 May 28; 41(21): 6714-22.
Fanger GR et al. Tumour Biol. 2002 Jul-Aug; 23(4): 212-21.
Zehentner BK et al. Clin Chem. 2004 Nov; 50(11): 2069-76. Epub 2004 Sep 16.
Bernstein JL et al. Clin Cancer Res. 2005 Sep 15; 11(18): 6528-35.
Zafrakas M et al. BMC Cancer. 2006 Apr 9; 6: 88.
Pawlik TM et al. BMC Cancer. 2006 Mar 16; 6: 68.
Bignotti E et al. Gynecol Oncol. 2006 Nov; 103(2): 405-16. Epub 2006 May 24.
Brown NM et al. Breast Cancer Res Treat. 2006 May; 97(1): 41-7. Epub 2005 Dec 1.
Culleton J et al. Int J Cancer. 2007 Mar 1; 120(5): 1087-92.
Tassi RA et al. Gynecol Oncol. 2007 Jun; 105(3): 578-85. Epub 2007 Mar 6.
Sasaki E et al. Mod Pathol. 2007 Feb; 20(2): 208-14. Epub 2006 Dec 22.

[0063] The antibodies of the instant invention, and assays which employ the antibodies, specifically bind ECM-complexes. Binding of naturally occurring ECM-complexes instead of individual secretoglobin family members make these antibodies ideal diagnostic reagents. As shown herein, determining levels of ECM-complexes allows for detection of cancer in an individual.

[0064] Additionally, the antibodies of the instant invention specifically bind ECM-complexes and have demonstrated characteristics which make them ideal therapeutic agents for modulating ECM-complex activity or functions. Modulation of these functions is achieved by binding of an antibody to the functional domain and antagonistically preventing the activity of the functional domain. Inhibition of ECM-complex function may be also achieved by preventing or inhibiting formation of the secretoglobin family members into the functional mature ECM-complex. Alternatively, inhibition of ECM-complex function may be achieved by disrupting, dissolving, or preventing formation of ECM-complexes with an anti-ECM-complex antibody.

[0065] Inhibition of ECM-complex function results in inhibition or reduction of ECM-complex biological functions. Anti-ECM-complex antibodies which bind ECM-complex inhibit or reduce ECM-complex biological functions.

[0066] Furthermore, the antibodies of the instant invention are useful as therapeutic agents for individuals suffering from breast or ovarian carcinomas. The antibodies may have therapeutic effect by killing ECM-complex expressing cancer cells, inhibiting growth of ECM-complex expressing tumors, shrinking ECM-complex expressing tumors, extending survival time of individuals with ECM-complex expressing tumors, reducing metastases of ECM-complex expressing tumors, inducing immune response against ECM-complex expressing tumors, reducing inhibition of immune response against ECM-complex expressing tumors and/or reducing angiogenesis or vascularization of ECM-complex expressing tumors.

[0067] Taken together, the differential expression in cancer and role in regulation of cellular processes make ECM-complexes a promising target for diagnosis and immunotherapy of various tumor types. Anti-ECM-complex antibodies are useful in diagnostic or therapeutic applications alone or in combination with antibodies against other secretoglobin family members.

[0068] The term "antibody" (Ab) as used herein includes monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity. The term "immunoglobulin" (Ig) is used interchangeably with "antibody" herein.

[0069] An "isolated antibody" is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. Preferably, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or non-reducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

[0070] The basic 4-chain antibody unit is a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains (an IgM antibody consists of 5 of the basic heterotetramer units along with an additional polypeptide called J chain, and therefore contains 10 antigen binding sites, while secreted IgA antibodies can polymerize to form polyvalent assemblages comprising 2-5 of the basic 4-chain units along with J chain). In the case of IgGs, the 4-chain unit is generally about 150,000 daltons. Each L chain is linked to an H chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype. Each H and L chain also has regularly spaced intrachain disulfide bridges. Each H chain has at the N-terminus a variable domain (VH) followed by three constant domains (CH) for each of the α and γ chains and four CH domains for L and F isotypes. Each L chain has at the N-terminus, a variable domain (VL) followed by a constant domain (CL) at its other end.

[0071] The VL is aligned with the VH and the CL is aligned with the first constant domain of the heavy chain (CHI).

[0072] Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains. The pairing of a VH and VL together forms a single antigen-binding site. For the structure and properties of the different classes of antibodies, see, e.g., Basic and Clinical Immunology, 8th edition, Daniel P. Stites, Abba I. Teff and Tristram G. Parslow (eds.), Appleton & Lange, Norwalk, Conn., 1994, page 71 and Chapter 6.

[0073] The L chain from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains (CH), immunoglobulins can be assigned to different classes or isotypes. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, having heavy chains designated α, δ, ϵ, γ and μ, respectively.

The γ and α classes are further divided into subclasses on the basis of relatively minor differences in C_H sequence and function, e.g., humans express the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2.

[0074] The term "variable" refers to the fact that certain segments of the variable domains differ extensively in sequence among antibodies. The V domain mediates antigen binding and defines specificity of a particular antibody for its particular antigen. However, the variability is not evenly distributed across the 1-10-amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches called framework regions (FRs) of 15-30 amino acids separated by shorter regions of extreme variability called "hypervariable regions" that are each 9-12 amino acids long. The variable domains of native heavy and light chains each comprise four FRs, largely adopting a P-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the P-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

[0075] The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. around about residues 24-34 (LI), 5056 (L2) and 89-97 (L3) in the VL, and around about 1-35 (HI), 50-65 (H2) and 95-102 (113) in the VH; Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues 26-32 (LI), 50-52 (L2) and 91-96 (U) in the VL, and 26-32 (HI), 53-55 (1-12) and 96-101 (H3) in the VH; Chothia and Lesk J. Mol. Biol. 196: 901-917 (1987)).

[0076] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies useful in the present invention may be prepared by the hybridoma methodology first described by Kohler et al., Nature, 256:495 (1975), or may be made using recombinant DNA methods in bacterial, eukaryotic animal or plant cells (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature, 352:624-628 (1991) and Marks et al., J. Mol. Biol., 222:581-597 (1991), for example.

[0077] The monoclonal antibodies herein include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (see U.S. Pat. No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, Ape etc), and human constant region sequences.

[0078] An "intact" antibody is one which comprises an antigen-binding site as well as a CL and at least heavy chain constant domains, CHI, CH2 and CH3. The constant domains may be native sequence constant domains (e.g. human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

[0079] An "antibody fragment" comprises a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (see U.S. Pat. No. 5,641,870, Example 2; Zapata et al., Protein Eng. 8(10): 1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. The Fab fragment consists of an entire L chain along with the variable region domain of the H chain (VH), and the first constant domain of one heavy chain (CHI). Each Fab fragment is monovalent with respect to antigen binding, i.e., it has a single antigen-binding site. Pepsin treatment of an antibody yields a single large F(ab')2 fragment which roughly corresponds to two disulfide linked Fab fragments having divalent antigen-binding activity and is still capable of cross-linking antigen. Fab' fragments differ from Fab fragments by having additional few residues at the carboxy terminus of the CHI domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of 8 Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0080] The Fc fragment comprises the carboxy-terminal portions of both H chains held together by disulfides. The effector functions of antibodies are determined by sequences in the Fc region, which region is also the part recognized by Fc receptors (FcR) found on certain types of cells.

[0081] "Fv" is the minimum antibody fragment which contains a complete antigen recognition and binding site. This fragment consists of a dimer of one heavy chain and one light chain variable region domain in tight, non-covalent association. From the folding of these two domains emanate six hypervariable loops (3 loops each from the H and L chain)

that contribute the amino acid residues for antigen binding and confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0082] "Single-chain Fv" also abbreviated as "sFv" or "scFv" are antibody fragments that comprise the VH and VL antibody domains connected into a single polypeptide chain. Preferably, the sFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv, see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); Borrebaeck 1995, infra.

[0083] The term "diabodies" refers to small antibody fragments prepared by constructing sFv fragments (see preceding paragraph) with short linkers (about 5-10 residues) between the VH and VL domains such that inter-chain but not intra-chain pairing of the V domains is achieved, resulting in a bivalent fragment, i.e., a fragment having two antigen-binding sites. Bispecific diabodies are heterodimers of two "crossover" sFv fragments in which the VH and VL domains of the two antibodies are present on different polypeptide chains. Diabodies are described more fully in, for example, EP 404, 097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993). Furthermore, effects of linker sequence alterations in engineering bispecific tandem diabodies are described in Le Gall et al., Protein Eng Des Sel. 17(4):357-66 (2004).

[0084] A "native sequence" polypeptide is one which has the same amino acid sequence as a polypeptide (e.g., antibody) derived from nature. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of a naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

[0085] The term "amino acid sequence variant" refers to a polypeptide that has amino acid sequences that differ to some extent from a native sequence polypeptide. Ordinarily, amino acid sequence variants of ECM-complex will possess at least about 70% homology with the native sequence ECM-complex, preferably, at least about 80%, more preferably at least about 85%, even more preferably at least about 90% homology, and most preferably at least 95%. The amino acid sequence variants can possess substitutions, deletions, insertions and/or alterations due to allelic variation or Single Nucleotide Polymorphisms (SNPs) within the native nucleic acid sequence encoding the amino acid sequence.

[0086] Several definitions of SNPs exist. See, e.g., Brooks, 235 *Gene* 177-86 (1999). As used herein, the term "single nucleotide polymorphism" or "SNP" includes all single base variants, thus including nucleotide insertions and deletions in addition to single nucleotide substitutions and any resulting amino acid variants due to codon alteration. There are two types of nucleotide substitutions. A transition is the replacement of one purine by another purine or one pyrimidine by another pyrimidine. A transversion is the replacement of a purine for a pyrimidine, or vice versa.

[0087] Numerous methods exist for detecting SNPs within a nucleotide sequence. A review of many of these methods can be found in Landegren et al., 8 *Genome Res.* 769-76 (1998). For example, a SNP in a genomic sample can be detected by preparing a Reduced Complexity Genome (RCG) from the genomic sample, then analyzing the RCG for the presence or absence of a SNP. See, e.g., WO 00/18960. Multiple SNPs in a population of target polynucleotides in parallel can be detected using, for example, the methods of WO 00/50869. Other SNP detection methods include the methods of U.S. Pat. Nos. 6,297,018 and 6,322,980. Furthermore, SNPs can be detected by restriction fragment length polymorphism (RFLP) analysis. See, e.g., U.S. Pat. Nos. 5,324,631; 5,645,995. RFLP analysis of SNPs, however, is limited to cases where the SNP either creates or destroys a restriction enzyme cleavage site. SNPs can also be detected by direct sequencing of the nucleotide sequence of interest. In addition, numerous assays based on hybridization have been developed to detect SNPs and mismatch distinction by polymerases and ligases. Several web sites provide information about SNPs including Ensembl (ensemble with the extension .org of the world wide web), Sanger Institute (sanger with the extension ac.uk/genetics/exon of the world wide web), and National Center for Biotechnology Information (NCBI) (ncbi with the extension nlm.nih.gov/SNP/of the world wide web), The SNP Consortium Ltd. (snp with the extension .cshl.org). The chromosomal locations for the compositions disclosed herein are provided below. In addition, one of ordinary skill in the art could perform a search against the genome or any of the databases cited above using BLAST to find the chromosomal location or locations of SNPs. Another preferred method to find the genomic coordinates and associated SNPs is to use the BLAT tool (genome with the extension .ucsc.edu, Kent et al. 2001, The Human Genome Browser at UCSC, Genome Research 996-1006 or Kent 2002 BLAT, The BLAST-Like Alignment Tool Genome Research, 1-9). All web sites above were accessed Dec. 3, 2003.

[0088] Preferred amino acid sequence variants of ECM-complexes are described in the databases above. The nucleic acid and amino acid sequences of ECM-complex are disclosed in the references cited above, which are incorporated by reference in their entirety. The polynucleotides encoding the amino acids of the present invention contain single nucleotide polymorphism (SNP) attributes. Specifically identified are SNPs present in the coding region of the nucleotide, the alleles of the SNP, the nucleotide ambiguity code for the SNP, the position in the codon of the SNP if within the Open Reading Frame (1, 2, 3 or UTR for untranslated regions), and the SNP type (synonymous or non-synonymous to the protein translation). In addition to the attributes above, the SNP rs# ID for the NCBI SNP database (dbSNP) which is accessible at ncbi with the extension .nlm.nih.gov/SNP/ of the world wide web is referenced for each SNP. Additional single nucleotide polymorphism (SNP) information can be accessed at the databases listed above. Variants of ECM-complexes as described above and antibodies which bind to these variants individually or in combination are part of the invention described herein. Antibodies of instant invention may have diagnostic or therapeutic utility for the variants of ECM-complexes outlined above.

[0089] The phrase "functional fragment or analog" of an antibody is a compound having qualitative biological activity in common with a full-length antibody. For example, a functional fragment or analog of an anti-IgE antibody is one which can bind to an IgE immunoglobulin in such a manner so as to

prevent or substantially reduce the ability of such molecule from having the ability to bind to the high affinity receptor, FccRI.

[0090] "Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. Sequence similarity may be measured by any common sequence analysis algorithm, such as GAP or BESTFIT or other variation Smith-Waterman alignment. See, T. F. Smith and M. S. Waterman, J. Mol. Biol. 147:195-197 (1981) and W. R. Pearson, Genomics 11:635-650 (1991).

[0091] "Humanized" forms of non-human (e.g., rodent) antibodies are chimeric antibodies that contain minimal sequence derived from the non-human antibody. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or non-human primate having the desired antibody specificity, affinity, and capability. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332: 323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992).

[0092] As used herein, an anti-ECM-complex antibody that "internalizes" is one that is taken up by (i.e., enters) the cell upon binding to ECM-complex on a mammalian cell (i.e. cell surface ECM-complex). The internalizing antibody will of course include antibody fragments, human or humanized antibody and antibody conjugate. For therapeutic applications, internalization in vivo is contemplated. The number of antibody molecules internalized will be sufficient or adequate to kill an ECM-complex-expressing cell, especially an ECM-complex-expressing cancer cell. Depending on the potency of the antibody or antibody conjugate, in some instances, the uptake of a single antibody molecule into the cell is sufficient to kill the target cell to which the antibody binds. For example, certain toxins are highly potent in killing such that internalization of one molecule of the toxin conjugated to the antibody is sufficient to kill the tumor cell.

[0093] Whether an anti-ECM-complex antibody internalizes upon binding ECM-complex on a mammalian cell can be determined by various assays including those described in the experimental examples herein. For example, to test internalization in vivo, the test antibody is labeled and introduced into an animal known to have ECM-complex expressed on the surface of certain cells. The antibody can be radiolabeled or labeled with fluorescent or gold particles, for instance. Animals suitable for this assay include a mammal such as a NCR nude mouse that contains a human ECM-complex-expressing tumor transplant or xenograft, or a mouse into which cells transfected with human ECM-complex have been introduced, or a transgenic mouse expressing the human ECM-complex transgene. Appropriate controls include animals that did not receive the test antibody or that received an unrelated antibody, and animals that received an antibody to another antigen on the cells of interest, which antibody is known to be internalized upon binding to the antigen. The antibody can be administered to the animal, e.g., by intravenous injection. At suitable time intervals, tissue sections of the animal can be prepared using known methods or as described in the experimental examples below, and analyzed by light microscopy or electron microscopy, for internalization as well as the location of the internalized antibody in the cell. For internalization in vitro, the cells can be incubated in tissue culture dishes in the presence or absence of the relevant antibodies added to the culture media and processed for microscopic analysis at desired time points. The presence of an internalized, labeled antibody in the cells can be directly visualized by microscopy or by autoradiography if radiolabeled antibody is used. Alternatively, in a quantitative biochemical assay, a population of cells comprising ECM-complex-expressing cells are contacted in vitro or in vivo with a radiolabeled test antibody and the cells (if contacted in vivo, cells are then isolated after a suitable amount of time) are treated with a protease or subjected to an acid wash to remove uninternalized antibody on the cell surface. The cells are ground up and the amount of protease resistant, radioactive counts per minute (cpm) associated with each batch of cells is measured by passing the homogenate through a scintillation counter. Based on the known specific activity of the radiolabeled antibody, the number of antibody molecules internalized per cell can be deduced from the scintillation counts of the ground-up cells. Cells are "contacted" with antibody in vitro preferably in solution form such as by adding the cells to the cell culture media in the culture dish or flask and mixing the antibody well with the media to ensure uniform exposure of the cells to the antibody. Instead of adding to the culture media, the cells can be contacted with the test antibody in an isotonic solution such as PBS in a test tube for the desired time period. In vivo, the cells are contacted with antibody by any suitable method of administering the test antibody such as the methods of administration described below when administered to a patient.

[0094] The faster the rate of internalization of the antibody upon binding to the ECM-complex-expressing cell in vivo, the faster the desired killing or growth inhibitory effect on the target ECM-complex-expressing cell can be achieved, e.g., by a cytotoxic immunoconjugate. Preferably, the kinetics of internalization of the anti-ECM-complex antibodies are such that they favor rapid killing of the ECM-complex-expressing target cell. Therefore, it is desirable that the anti-ECM-complex antibody exhibit a rapid rate of internalization preferably, within 24 hours from administration of the antibody in vivo, more preferably within about 12 hours, even more preferably within about 30 minutes to 1 hour, and most preferably, within about 30 minutes. The present invention provides antibodies that internalize as fast as about 15 minutes from the time of introducing the anti-ECM-complex antibody in vivo. The antibody will preferably be internalized into the cell within a few hours upon binding to ECM-complex on the cell surface, preferably within 1 hour, even more preferably within 15-30 minutes.

[0095] To determine if a test antibody can compete for binding to the same epitope as the epitope bound by the anti-ECM-complex antibodies of the present invention including the antibodies produced by the hybridomas deposited with the ATCC, a cross-blocking assay e.g., a competitive ELISA assay can be performed. In an exemplary competitive ELISA assay, ECM-complex-coated wells of a microtiter plate, or ECM-complex-coated sepharose beads, are pre-incubated with or without candidate competing antibody and then a biotin-labeled anti-ECM-complex antibody of the invention is added. The amount of labeled anti-ECM-complex antibody bound to the ECM-complex antigen in the wells or on the beads is measured using avidin-peroxidase conjugate and appropriate substrate.

[0096] Alternatively, the anti-ECM-complex antibody can be labeled, e.g., with a radioactive or fluorescent label or some other detectable and measurable label. The amount of labeled anti-ECM-complex antibody that binds to the antigen will have an inverse correlation to the ability of the candidate competing antibody (test antibody) to compete for binding to the same epitope on the antigen, i.e., the greater the affinity of the test antibody for the same epitope, the less labeled anti-ECM-complex antibody will be bound to the antigen-coated wells. A candidate competing antibody is considered an antibody that binds substantially to the same epitope or that competes for binding to the same epitope as an anti-ECM-complex antibody of the invention if the candidate competing antibody can block binding of the anti-ECM-complex antibody by at least 20%, preferably by at least 20-50%, even more preferably, by at least 50% as compared to a control performed in parallel in the absence of the candidate competing antibody (but may be in the presence of a known noncompeting antibody). It will be understood that variations of this assay can be performed to arrive at the same quantitative value.

[0097] An antibody having a "biological characteristic" of a designated antibody, such as any of the monoclonal antibodies ECM.D1, ECM.D2, ECM.D3, ECM.D4, ECM.D5, ECM.D6, ECM.D7, ECM.D8, ECM.D9, ECM.D10, ECM.D11, ECM.D12, ECM.D13, ECM.D14, ECM.D15, ECM.D16, ECM.D17, ECM.D18, ECM.D19, ECM.D20, ECM.D21, ECM.D22, ECM.D23, ECM.D24, ECM.D25, ECM.D26, ECM.D27, ECM.D28, ECM.D29, ECM.D30, ECM.D31, ECM.D32, ECM.D33, ECM.D34, ECM.D35, ECM.D36, ECM.D37, ECM.D38, ECM.D39, ECM.D40, ECM.D41, ECM.D42, ECM3.G1, ECM3.G3, ECM3.G4, ECM3.G5, ECM3.G6, ECM3.G8, ECM3.G9, ECM3.G11, ECM3.G12, ECM3.G13, ECM3.G14, ECM3.G15, ECM3.G16, ECM3.G17, MamA.H1, MamA.H2, MamA.H3, MamA.H4, MamA.H5, MamA.H6, MamA.H7, MamA.H8, MamA.H9, MamA.H10, MamA.H11, MamA.H12, MamA.H13, ECM3.J1, ECM3.J3, ECM3.J4, ECM3.J5, ECM3.J6, ECM3.J7, ECM3.J8, ECM3.J9, ECM3.J10, ECM3.J11, ECM3.J12, ECM3.J13, ECM3.J14, ECM3.J15, ECM3.J17, ECM3.J18, ECM3.J19, ECM3.J21, ECM3.J23, ECM3.J24, ECM3.J25, ECM3.J26, LipA.J1, LipA.J2, LipA.J3, and/or LipA.J4, is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen, ECM.D1, ECM.D2, ECM.D3, ECM.D4, ECM.D5, ECM.D6, ECM.D7, ECM.D8, ECM.D9, ECM.D10, ECM.D11, ECM.D12, ECM.D13, ECM.D14, ECM.D15, ECM.D16, ECM.D17, ECM.D18, ECM.D19, ECM.D20, ECM.D21, ECM.D22, ECM.D23, ECM.D24, ECM.D25, ECM.D26, ECM.D27, ECM.D28, ECM.D29, ECM.D30, ECM.D31, ECM.D32, ECM.D33, ECM.D34, ECM.D35, ECM.D36, ECM.D37, ECM.D38, ECM.D39, ECM.D40, ECM.D41, ECM.D42, ECM3.G1, ECM3.G3, ECM3.G4, ECM3.G5, ECM3.G6, ECM3.G8, ECM3.G9, ECM3.G11, ECM3.G12, ECM3.G13, ECM3.G14, ECM3.G15, ECM3.G16, ECM3.G17, MamA.H1, MamA.H2, MamA.H3, MamA.H4, MamA.H5, MamA.H6, MamA.H7, MamA.H8, MamA.H9, MamA.H10, MamA.H11, MamA.H12, MamA.H13, ECM3.J1, ECM3.J3, ECM3.J4, ECM3.J5, ECM3.J6, ECM3.J7, ECM3.J8, ECM3.J9, ECM3.J10, ECM3.J11, ECM3.J12, ECM3.J13, ECM3.J14, ECM3.J15, ECM3.J17, ECM3.J18, ECM3.J19, ECM3.J21, ECM3.J23, ECM3.J24, ECM3.J25, ECM3.J26, LipA.J1, LipA.J2, LipA.J3, and/or LipA.J4, will bind the same epitope as that bound by ECM.D1, ECM.D2, ECM.D3, ECM.D4, ECM.D5, ECM.D6, ECM.D7, ECM.D8, ECM.D9, ECM.D10, ECM.D11, ECM.D12, ECM.D13, ECM.D14, ECM.D15, ECM.D16, ECM.D17, ECM.D18, ECM.D19, ECM.D20, ECM.D21, ECM.D22, ECM.D23, ECM.D24, ECM.D25, ECM.D26, ECM.D27, ECM.D28, ECM.D29, ECM.D30, ECM.D31, ECM.D32, ECM.D33, ECM.D34, ECM.D35, ECM.D36, ECM.D37, ECM.D38, ECM.D39, ECM.D40, ECM.D41, ECM.D42, ECM3.G1, ECM3.G3, ECM3.G4, ECM3.G5, ECM3.G6, ECM3.G8, ECM3.G9, ECM3.G11, ECM3.G12, ECM3.G13, ECM3.G14, ECM3.G15, ECM3.G16, ECM3.G17, MamA.H1, MamA.H2, MamA.H3, MamA.H4, MamA.H5, MamA.H6, MamA.H7, MamA.H8, MamA.H9, MamA.H10, MamA.H11, MamA.H12, MamA.H13, ECM3.J1, ECM3.J3, ECM3.J4, ECM3.J5, ECM3.J6, ECM3.J7, ECM3.J8, ECM3.J9, ECM3.J10, ECM3.J11, ECM3.J12, ECM3.J13, ECM3.J14, ECM3.J15, ECM3.J17, ECM3.J18, ECM3.J19, ECM3.J21, ECM3.J23, ECM3.J24, ECM3.J25, ECM3.J26, LipA.J1, LipA.J2, LipA.J3, and/or LipA.J4, (e.g. which competes for binding or blocks binding of monoclonal antibody ECM.D1, ECM.D2, ECM.D3, ECM.D4, ECM.D5, ECM.D6, ECM.D7, ECM.D8, ECM.D9, ECM.D10, ECM.D11, ECM.D12, ECM.D13, ECM.D14, ECM.D15, ECM.D16, ECM.D17, ECM.D18, ECM.D19, ECM.D20, ECM.D21, ECM.D22, ECM.D23, ECM.D24, ECM.D25, ECM.D26, ECM.D27, ECM.D28, ECM.D29, ECM.D30, ECM.D31, ECM.D32, ECM.D33, ECM.D34, ECM.D35, ECM.D36, ECM.D37, ECM.D38, ECM.D39, ECM.D40, ECM.D41, ECM.D42, ECM3.G1, ECM3.G3, ECM3.G4, ECM3.G5, ECM3.G6, ECM3.G8, ECM3.G9, ECM3.G11, ECM3.G12, ECM3.G13, ECM3.G14, ECM3.G15, ECM3.G16, ECM3.G17, MamA.H1, MamA.H2, MamA.H3, MamA.H4, MamA.H5, MamA.H6, MamA.H7, MamA.H8, MamA.H9, MamA.H10, MamA.H11, MamA.H12, MamA.H13, ECM3.J1, ECM3.J3, ECM3.J4, ECM3.J5, ECM3.J6, ECM3.J7, ECM3.J8, ECM3.J9, ECM3.J10, ECM3.J11, ECM3.J12, ECM3.J13, ECM3.J14, ECM3.J15, ECM3.J17, ECM3.J18, ECM3.J19, ECM3.J21, ECM3.J23, ECM3.J24, ECM3.J25, ECM3.J26, LipA.J1, LipA.J2, LipA.J3, and/or LipA.J4), be able to target an ECM-complex-expressing tumor in vivo and/or may internalize upon binding to ECM-complex on a mammalian cell in vivo. Likewise, an antibody with the biological characteristic of the ECM.D1, ECM.D2, ECM.D3, ECM.D4, ECM.D5, ECM.D6, ECM.D7, ECM.D8, ECM.D9, ECM.D10, ECM.D11, ECM.D12, ECM.D13, ECM.D14, ECM.D15, ECM.D16, ECM.D17, ECM.D18, ECM.D19, ECM.D20, ECM.D21, ECM.D22, ECM.D23, ECM.D24, ECM.D25, ECM.D26, ECM.D27, ECM.D28, ECM.D29, ECM.D30, ECM.D31, ECM.D32, ECM.D33, ECM.D34, ECM.D35, ECM.D36,

ECM.D37, ECM.D38, ECM.D39, ECM.D40, ECM.D41, ECM.D42, ECM3.G1, ECM3.G3, ECM3.G4, ECM3.G5, ECM3.G6, ECM3.G8, ECM3.G9, ECM3.G11, ECM3.G12, ECM3.G13, ECM3.G14, ECM3.G15, ECM3.G16, ECM3.G17, MamA.H1, MamA.H2, MamA.H3, MamA.H4, MamA.H5, MamA.H6, MamA.H7, MamA.H8, MamA.H9, MamA.H10, MamA.H11, MamA.H12, MamA.H13, ECM3.J1, ECM3.J3, ECM3.J4, ECM3.J5, ECM3.J6, ECM3.J7, ECM3.J8, ECM3.J9, ECM3.J10, ECM3.J11, ECM3.J12, ECM3.J13, ECM3.J14, ECM3.J15, ECM3.J17, ECM3.J18, ECM3.J19, ECM3.J21, ECM3.J23, ECM3.J24, ECM3.J25, ECM3.J26, LipA.J1, LipA.J2, LipA.J3, and/or LipA.J4 antibody will have the same epitope binding, targeting, internalizing, tumor growth inhibitory and/or cytotoxic properties of the antibody.

[0098] The term "antagonist" antibody is used in the broadest sense, and includes an antibody that partially or fully blocks, inhibits, or neutralizes a biological activity of a native ECM-complex protein disclosed herein. Methods for identifying antagonists of an ECM-complex polypeptide may comprise contacting an ECM-complex polypeptide or a cell expressing ECM-complex on the cell surface, with a candidate antagonist antibody and measuring a detectable change in one or more biological activities normally associated with the ECM-complex polypeptide.

[0099] The term 'agonistic" antibody is used in the broadest sense, and includes an antibody that partially or fully promotes, activates, or increases biological activity of ECM-complex. Additionally, an agonistic antibody may mimic an ECM-complex binding partner (e.g. receptor or ligand) when binding of the ECM-complex antibody has substantially the same effect on biologic activity of ECM-complex as binding of the binding partner. Methods for identifying agonists of an ECM-complex polypeptide may comprise contacting an ECM-complex polypeptide or a cell expressing ECM-complex on the cell surface, with a candidate agonistic antibody and measuring a detectable change in one or more biological activities normally associated with the ECM-complex polypeptide.

[0100] An "antibody that inhibits the growth of tumor cells expressing ECM-complex" or a "growth inhibitory" antibody is one which binds to and results in measurable growth inhibition of cancer cells expressing or overexpressing ECM-complex. Preferred growth inhibitory anti-ECM-complex antibodies inhibit growth of ECM-complex-expressing tumor cells (e.g., breast or ovarian cancer cells) by greater than 20%, preferably from about 20% to about 50%, and even more preferably, by greater than 50% (e.g. from about 50% to about 100%) as compared to the appropriate control, the control typically being tumor cells not treated with the antibody being tested. Growth inhibition can be measured at an antibody concentration of about 0.1 to 30 pg/ml or about 0.5 nM to 200 nM in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the antibody. Growth inhibition of tumor cells in vivo can be determined in various ways such as is described in the examples herein. The antibody is growth inhibitory in vivo if administration of the anti-ECM-complex antibody at about 1 pg/kg to about 100 mg/kg body weight results in reduction in tumor size or tumor cell proliferation within about 5 days to 3 months from the first administration of the antibody, preferably within about 5 to 30 days.

[0101] An antibody which "induces apoptosis" is one which induces programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which overexpresses ECM-complex. Preferably the cell is a tumor cell, e.g. an ovarian, colon, prostate, or lung cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cells in an annexin binding assay.

[0102] Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); complement dependent cytotoxicity (CDC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

[0103] "Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. PNAS (USA) 95:652-656 (1998).

[0104] "Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRI1B contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daeron, Annu. Rev. Immunol. 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991); Capel et al., Immu-

nomethods 4:25-34 (1994); and de Haas et al., J. Lab. Clin. Med. 126.330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FeRn, which is responsible for the transfer, of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)).

[0105] "Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source, e.g. from blood.

[0106] "Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), may be performed.

[0107] The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, cancer of the urinary tract, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, melanoma, multiple myeloma and B-cell lymphoma, brain, as well as head and neck cancer, and associated metastases.

[0108] An "ECM-complex-expressing cell" is a cell which expresses endogenous or transfected ECM-complex on the cell surface or secretes endogenous or transfected ECM-complex. An "ECM-complex-expressing cancer" is a cancer comprising cells that have ECM-complex protein present on the cell surface or secretes ECM-complex from the cell. An "ECM-complex-expressing cancer" produces sufficient levels of ECM-complex on the surface of cells thereof or secretes ECM-complex from the cells thereof, such that an anti-ECM-complex antibody can bind thereto and have a therapeutic effect with respect to the cancer. A cancer which "overexpresses" ECM-complex is one which has significantly higher levels of ECM-complex at the cell surface thereof or secretes ECM-complex from the cells thereof, compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. ECM-complex overexpression may be determined in a diagnostic or prognostic assay by evaluating increased levels of the ECM-complex protein present on the surface of a cell (e.g. via an immunohistochemistry assay; FACS analysis). Alternatively, or additionally, one may measure levels of ECM-complex-encoding nucleic acid or mRNA in the cell, e.g. via fluorescent in situ hybridization; (FISH; see W098/45479 published October, 1998), Southern blotting, Northern blotting, or polymerase chain reaction (PCR) techniques, such as real time quantitative PCR (RT-PCR). One may also study ECM-complex overexpression by measuring shed antigen in a biological fluid such as serum, e.g., using antibody-based assays (see also, e.g., U.S. Pat. No. 4,933,294 issued Jun. 12, 1990; WO91/05264 published Apr. 18, 1991; U.S. Pat. No. 5,401,638 issued Mar. 28, 1995; and Sias et al. J. Immunol. Methods 132: 73-80 (1990)). Aside from the above assays, various in vivo assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, e.g. a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, e.g. by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody. An ECM-complex-expressing cancer includes breast or ovarian cancer.

[0109] A "mammal" for purposes of treating a cancer or alleviating the symptoms of cancer, refers to any mammal, including, but not limited to, humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, etc. Preferably, the mammal is human.

[0110] "Treating" or "treatment" or "alleviation" or "alleviating" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to having the disorder or those in whom the disorder is to be prevented. A subject or mammal is successfully "treated" for an ECM-complex-expressing cancer if, after receiving a therapeutic amount of an anti-ECM-complex antibody according to the methods of the present invention, the subject or mammal shows observable and/or measurable reduction in or absence of one or more of the following: reduction in the number of cancer cells or absence of the cancer cells; reduction in the tumor size; inhibition (i.e., slow to some extent and preferably stop) of cancer cell infiltration into peripheral organs including the spread of cancer into soft tissue and bone; inhibition (i.e., slow to some extent and preferably stop) of tumor metastasis; inhibition, to some extent, of tumor growth; and/or relief to some extent, of one or more of the symptoms associated with the specific cancer; reduced morbidity and mortality, and improvement in quality of life issues. To the extent the anti-ECM-complex antibody may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. Reduction of these signs or symptoms may also be felt by the subject or mammal.

[0111] The above parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar to a physician. For cancer therapy, efficacy can be measured, for example, by assessing the time to disease progression (TTP) and/or determining the response rate (RR).

[0112] The term "therapeutically effective amount" refers to an amount of an antibody or a drug effective to "treat" a disease or disorder in a subject or mammal. In the case of cancer, the therapeutically effective amount of the antibody or drug may reduce the number of cancer cells; reduce the

tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. See preceding definition of "treating". To the extent the antibody or drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic.

[0113] "Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time.

[0114] "Intermittent" administration is treatment that is not consecutively done without interruption, but rather is cyclic in nature.

[0115] Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

[0116] "Carriers" as used herein include physiologically acceptable carriers, pharmaceutically acceptable carriers, excipients, and stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution.

[0117] Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™.

[0118] The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, and radioactive isotopes of Lu), chemotherapeutic agents e.g. methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, e.g., gelonin, ricin, saporin, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

[0119] A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ECM-complex-expressing cancer cell, either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of ECM-complex-expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

[0120] "Label" as used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody so as to generate a "labeled" antibody. The label may be detectable by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

[0121] The term "epitope tagged" used herein refers to a chimeric polypeptide comprising an anti-ECM-complex antibody polypeptide fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Ig polypeptide to which it is fused. The tag polypeptide is also preferably fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 and 50 amino acid residues (preferably, between about 10 and 20 amino acid residues).

[0122] A "small molecule" is defined herein to have a molecular weight below about 500 Daltons.

[0123] The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products. A package insert is also used to refer to instructions customarily included in commercial packages of diagnostic products that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such diagnostic products.

[0124] An "isolated nucleic acid molecule" is a nucleic acid molecule, e.g., an RNA, DNA, or a mixed polymer, which is substantially separated from other genome DNA sequences as well as proteins or complexes such as ribosomes and polymerases, which naturally accompany a native sequence. The term embraces a nucleic acid molecule which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogues or analogues biologically synthesized by heterologous systems. A substantially pure nucleic acid molecule includes isolated forms of the nucleic acid molecule.

[0125] "Vector" includes shuttle and expression vectors and includes, e.g., a plasmid, cosmid, or phagemid. Typically, a plasmid construct will also include an origin of replication (e.g., the ColEl origin of replication) and a selectable marker

(e.g., ampicillin or tetracycline resistance), for replication and selection, respectively, of the plasmids in bacteria. An "expression vector" refers to a vector that contains the necessary control sequences or regulatory elements for expression of the antibodies including antibody fragments of the invention, in prokaryotic, e.g., bacterial, or eukaryotic cells. Suitable vectors are disclosed herein.

[0126] Cells that produce an anti-ECM-complex antibody of the invention include the parent hybridoma cell e.g., the hybridomas that are deposited with the ATCC, as well as bacterial and eukaryotic host cells into which nucleic acid encoding the antibodies have been introduced. Suitable host cells are disclosed herein.

[0127] RNA interference refers to the process of sequence-specific post transcriptional gene silencing in animals mediated by short interfering RNAs (siRNA) (Fire et al., 1998, Nature, 391, 806). The corresponding process in plants is commonly referred to as post transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post transcriptional gene silencing is thought to be an evolutionarily conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire et al., 1999, Trends Genet., 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double stranded RNAs (dsRNA) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

[0128] The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNA) (Berstein et al., 2001, Nature, 409, 363). Short interfering RNAs derived from dicer activity are typically about 21-23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21 and 22 nucleotide small temporal RNAs (stRNA) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner et al., 2001, Science, 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir et al., 2001, Genes Dev., 15, 188).

[0129] Short interfering RNA mediated RNAi has been studied in a variety of systems. Fire et al. (Nature, 1998, 391, 806) were the first to observe RNAi in *C. Elegans*. Wianny and Goetz (Nature Cell Biol., 1999, 2, 70), describe RNAi mediated by dsRNA in mouse embryos. Hammond et al. (Nature, 2000, 404, 293) describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir et al. (Nature, 2001, 411, 494) describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in *Drosophila* embryonic lysates (Elbashir et al., 2001, EMBO J., 20, 6877) has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two nucleotide 3'-overhangs.

[0130] Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of the 3'-terminal siRNA overhang nucleotides with deoxy nucleotides (2'-H) was shown to be tolerated. Single mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. These studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir et al., 2001, EMBO J., 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen et al., 2001, Cell, 107, 309).

[0131] Studies have shown that replacing the 3'-overhanging segments of a 21-mer siRNA duplex having 2 nucleotide 3' overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to 4 nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir et al., 2001, EMBO J., 20, 6877). Elbashir et al., supra, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li et al., International PCT Publication No. WO 00/44914, and Beach et al., International PCT Publication No. WO 01/68836 both suggest that siRNA "may include modifications to either the phosphate-sugar back bone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom", however neither application teaches to what extent these modifications are tolerated in siRNA molecules nor provide any examples of such modified siRNA. Kreutzer and Limmer, Canadian Patent Application No. 2,359,180, also describe certain chemical modifications for use in dsRNA constructs in order to counteract activation of double stranded-RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-o or 4'-C methylene bridge. However, Kreutzer and Limmer similarly fail to show to what extent these modifications are tolerated in siRNA molecules nor do they provide any examples of such modified siRNA.

[0132] Parrish et al. (Molecular Cell, 2000, 6, 1977-1087) tested certain chemical modifications targeting the unc-22 gene in *C. elegans* using long (>25 nt) siRNA transcripts. The authors describe the introduction of thiophosphate residues into these siRNA transcripts by incorporating thiophosphate nucleotide analogs with T7 and T3 RNA polymerase and observed that "RNAs with two (phosphorothioate) modified bases also had substantial decreases in effectiveness as RNAi triggers (data not shown); (phosphorothioate) modification of more than two residues greatly destabilized the RNAs in vitro and we were not able to assay interference activities." Id. at 1081. The authors also tested certain modifications at the 2'-position of the nucleotide sugar in the long siRNA transcripts and observed that substituting deoxynucleotides for ribonucleotides "produced a substantial decrease in interference activity", especially in the case of Uridine to Thymidine

and/or Cytidine to deoxy-Cytidine substitutions. Id. In addition, the authors tested certain base modifications, including substituting 4-thiouracil, 5-bromouracil, 5-iodouracil, 3-(aminoallyl)uracil for uracil, and inosine for guanosine in sense and antisense strands of the siRNA, and found that whereas 4-thiouracil and 5-bromouracil were all well tolerated, inosine "produced a substantial decrease in interference activity" when incorporated in either strand. Incorporation of 5-iodouracil and 3-(aminoallyl)uracil in the antisense strand resulted in substantial decrease in RNAi activity as well.

[0133] Beach et al., International PCT Publication No. WO 01/68836, describes specific methods for attenuating gene expression using endogenously derived dsRNA. Tuschl et al., International PCT Publication No. WO 01/75164, describes a *Drosophila* in vitro RNAi system and the use of specific siRNA molecules for certain functional genomic and certain therapeutic applications; although Tuschl, 2001, Chem. Biochem., 2, 239-245, doubts that RNAi can be used to cure genetic diseases or viral infection due "to the danger of activating interferon response". Li et al., International PCT Publication No. WO 00/44914, describes the use of specific dsRNAs for use in attenuating the expression of certain target genes. Zernicka-Goetz et al., International PCT Publication No. WO 01/36646, describes certain methods for inhibiting the expression of particular genes in mammalian cells using certain dsRNA molecules. Fire et al., International PCT Publication No. WO 99/32619, describes particular methods for introducing certain dsRNA molecules into cells for use in inhibiting gene expression. Plaetinck et al., International PCT Publication No. WO 00/01846, describes certain methods for identifying specific genes responsible for conferring a particular phenotype in a cell using specific dsRNA molecules. Mello et al., International PCT Publication No. WO 01/29058, describes the identification of specific genes involved in dsRNA mediated RNAi. Deschamps Depaillette et al., International PCT Publication No. WO 99/07409, describes specific compositions consisting of particular dsRNA molecules combined with certain anti-viral agents. Driscoll et al., International PCT Publication No. WO 01/49844, describes specific DNA constructs for use in facilitating gene silencing in targeted organisms. Parrish et al. (Molecular Cell, 2000, 6, 1977-1087), describes specific chemically modified siRNA constructs targeting the unc-22 gene of *C. elegans*. Tuschl et al., International PCT Publication No. WO 02/44321, describe certain synthetic siRNA constructs.

Compositions and Methods of the Invention

[0134] The invention provides anti-ECM-complex antibodies. Preferably, the anti-ECM-complex antibodies internalize upon binding to cell surface ECM-complex on a mammalian cell. The anti-ECM-complex antibodies may also destroy or lead to the destruction of tumor cells expressing ECM-complex.

[0135] It was not apparent that ECM-complex was internalization-competent. In addition the ability of an antibody to internalize depends on several factors including the affinity, avidity, and isotype of the antibody, and the epitope that it binds. We have demonstrated herein that the cell surface ECM-complex is internalization competent upon binding by the anti-ECM-complex antibodies of the invention. Additionally, it was demonstrated that the anti-ECM-complex antibodies of the present invention can specifically target ECM-complex-expressing tumor cells. These tumor targeting, internalization and growth inhibitory properties of the anti-ECM-complex antibodies make these antibodies very suitable for therapeutic uses, e.g., in the treatment of various cancers including breast or ovarian cancer. Internalization of the anti-ECM-complex antibody is preferred, e.g., if the antibody or antibody conjugate has an intracellular site of action and if the cytotoxic agent conjugated to the antibody does not readily cross the plasma membrane (e.g., the toxin calicheamicin). Internalization is not necessary if the antibodies or the agent conjugated to the antibodies do not have intracellular sites of action, e.g., if the antibody can kill the tumor cell by ADCC or some other mechanism.

[0136] The anti-ECM-complex antibodies of the invention also have various non-therapeutic applications. The anti-ECM-complex antibodies of the present invention can be used for diagnosis and staging of ECM-complex-expressing cancers (e.g., in radioimaging). They may be used alone or in combination with other ovarian cancer markers, including, but not limited to, CA125, HE4 and mesothelin. The antibodies are also useful for purification or immunoprecipitation of ECM-complex from cells, for detection and quantitation of ECM-complex in vitro, e.g. in an ELISA or a Western blot, and to kill and eliminate ECM-complex-expressing cells from a population of mixed cells as a step in the purification of other cells. The internalizing anti-ECM-complex antibodies of the invention can be in the different forms encompassed by the definition of "antibody" herein. Thus, the antibodies include full length or intact antibody, antibody fragments, native sequence antibody or amino acid variants, humanized, chimeric or fusion antibodies, immunoconjugates, and functional fragments thereof. In fusion antibodies, an antibody sequence is fused to a heterologous polypeptide sequence. The antibodies can be modified in the Fc region to provide desired effector functions. As discussed in more detail herein, with the appropriate Fc regions, the naked antibody bound on the cell surface can induce cytotoxicity, e.g., via antibody-dependent cellular cytotoxicity (ADCC) or by recruiting complement in complement dependent cytotoxicity, or some other mechanism. Alternatively, where it is desirable to eliminate or reduce effector function, so as to minimize side effects or therapeutic complications, certain other Fc regions may be used.

[0137] The antibody may compete for binding, or bind substantially to, the same epitope bound by the antibodies of the invention. Antibodies having the biological characteristics of the present anti-ECM-complex antibodies of the invention are also contemplated, e.g., an anti-ECM-complex antibody which has the biological characteristics of a monoclonal antibody produced by the hybridomas described herein, specifically including the in vivo tumor targeting, internalization and any cell proliferation inhibition or cytotoxic characteristics. Specifically provided are anti-ECM-complex antibodies that bind to an epitope present on human ECM-complexes.

[0138] Methods of producing these antibodies are described in detail herein.

[0139] The present anti-ECM-complex antibodies are useful for treating a ECM-complex-expressing cancer or alleviating one or more symptoms of the cancer in a mammal. Such a cancer includes breast or ovarian cancer, cancer of the urinary tract, lung cancer, breast cancer, colon cancer, pancreatic cancer, and ovarian cancer, more specifically, prostate adenocarcinoma, renal cell carcinomas, colorectal adenocarcinomas, lung adenocarcinomas, lung squamous cell carcinomas, and pleural mesothelioma. The cancers encompass

metastatic cancers of any of the preceding, e.g., breast or ovarian cancer metastases. The antibody is able to bind to at least a portion of the cancer cells that express ECM-complex in the mammal and preferably is one that does not induce or that minimizes HAMA response. Preferably, the antibody is effective to destroy or kill ECM-complex-expressing tumor cells or inhibit the growth of such tumor cells, in vitro or in vivo, upon binding to ECM-complex on the cell. Such an antibody includes a naked anti-ECM-complex antibody (not conjugated to any agent). Naked anti-ECM-complex antibodies having tumor growth inhibition properties in vivo include the antibodies described in the examples herein. Naked antibodies that have cytotoxic or cell growth inhibition properties can be further conjugated with a cytotoxic agent to render them even more potent in tumor cell destruction. Cytotoxic properties can be conferred to an anti-ECM-complex antibody by, e.g., conjugating the antibody with a cytotoxic agent to form an immunoconjugate as described herein. The cytotoxic agent or a growth inhibitory agent is preferably a small molecule. Toxins such as maytansin, maytansinoids, saporin, gelonin, ricin or calicheamicin and analogs or derivatives thereof, are preferable.

[0140] The invention provides a composition comprising an anti-ECM-complex antibody of the invention, and a carrier. For the purposes of treating cancer, compositions can be administered to a subject in need of such treatment, wherein the composition can comprise one or more anti-ECM-complex antibodies present as an immunoconjugate or as the naked antibody. Further, the compositions can comprise these antibodies in combination with other therapeutic agents such as cytotoxic or growth inhibitory agents, including chemotherapeutic agents. The invention also provides formulations comprising an anti-ECM-complex antibody of the invention, and a carrier. The formulation may be a therapeutic formulation comprising a pharmaceutically acceptable carrier.

[0141] Another aspect of the invention is isolated nucleic acids encoding the internalizing anti-ECM-complex antibodies. Nucleic acids encoding both the H and L chains and especially the hypervariable region residues, chains which encode the native sequence antibody as well as variants, modifications and humanized versions of the antibody, are encompassed.

[0142] The invention also provides methods useful for treating an ECM-complex-expressing cancer or alleviating one or more symptoms of the cancer in a mammal, comprising administering a therapeutically effective amount of an internalizing anti-ECM-complex antibody to the mammal. The antibody therapeutic compositions can be administered short term (acute) or chronically, or intermittently as directed by a physician. Also provided are methods of inhibiting the growth of or killing an ECM-complex expressing cell. Finally, the invention also provides kits and articles of manufacture comprising at least one antibody of this invention, preferably at least one internalizing anti-ECM-complex antibody of this invention. Kits containing anti-ECM-complex antibodies find use in detecting ECM-complex expression, and in therapeutic or diagnostic assays, e.g., for ECM-complex cell killing assays or for purification and/or immunoprecipitation of ECM-complex from cells, tissues or bodily fluids. For example, for isolation and purification of ECM-complex, the kit can contain an anti-ECM-complex antibody coupled to a solid support, e.g., a tissue culture plate or beads (e.g., sepharose beads). Kits can be provided which contain antibodies for detection and quantitation of ECM-complex in vitro, e.g. in an ELISA or a Western blot. Such antibody useful for detection may be provided with a label such as a fluorescent or radiolabel.

Production of Anti-ECM-Complex Antibodies

[0143] The following describes exemplary techniques for the production of the antibodies useful in the present invention. Some of these techniques are described further in Example 1. The ECM-complex antigen to be used for production of antibodies may be, e.g., the full length polypeptide or a portion thereof, including a soluble form of ECM-complex lacking the membrane spanning sequence, or synthetic peptides to selected portions of the protein.

[0144] Alternatively, cells expressing ECM-complex at their cell surface (e.g. CHO or NIH-3T3 cells transformed to overexpress ECM-complex; ovarian, pancreatic, lung, breast or other ECM-complex-expressing tumor cell line), or membranes prepared from such cells can be used to generate antibodies. The nucleotide and amino acid sequences of human and murine ECM-complex are available as provided herein. ECM-complex can be produced recombinantly in and isolated from, prokaryotic cells, e.g., bacterial cells, or eukaryotic cells using standard recombinant DNA methodology. ECM-complex can be expressed as a tagged (e.g., epitope tag) or other fusion protein to facilitate its isolation as well as its identification in various assays.

[0145] Antibodies or binding proteins that bind to various tags and fusion sequences are available as elaborated herein. Other forms of ECM-complex useful for generating antibodies will be apparent to those skilled in the art.

[0146] Tags

[0147] Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 (Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky et al., Protein Engineering, 3(6):547-553 (1990)). The FLAG-peptide (Hopp et al., BioTechnology, 6:1204-1210 (1988)) is recognized by an anti-FLAG M2 monoclonal antibody (Eastman Kodak Co., New Haven, Conn.). Purification of a protein containing the FLAG peptide can be performed by immunoaffinity chromatography using an affinity matrix comprising the anti-FLAG M2 monoclonal antibody covalently attached to agarose (Eastman Kodak Co., New Haven, Conn.). Other tag polypeptides include the KT3 epitope peptide (Martin et al., Science, 255:192-194 (1992)); an α-tubulin epitope peptide (Skinner et al., J. Biol. Chenz., 266:15163-15166 (1991)); and the T7 gene protein peptide tag (Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)).

[0148] Polyclonal Antibodies

[0149] Polyclonal antibodies are preferably raised in animals, preferably non-human animals, by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen (especially when synthetic peptides are used) to a protein that is immunogenic in the species to be immunized. For example, the antigen can be conjugated to keyhole limpet hemocyanin (KLH), serum, bovine thyroglobulin, or soybean trypsin inhibitor, using a bifunctional or derivatizing agent, e.g., maleimidobenzoyl sulfosuccinimide ester (conjugation

through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups. Conjugates also can be made in recombinant cell culture as protein fusions.

[0150] Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 5-100 pg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later, the animals are boosted with ⅕ to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later, the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Also, aggregating agents such as alum are suitably used to enhance the immune response.

[0151] Monoclonal Antibodies

[0152] Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., Nature, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567). In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as described herein to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. After immunization, lymphocytes are isolated and then fused with a "fusion partner", e.g., a myeloma cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies. Principles and Practice, pp 103 (Academic Press, 1986)).

[0153] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium which medium preferably contains one or more substances that inhibit the growth or survival of the unfused, fusion partner, e.g., the parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the selective culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[0154] Preferred fusion partner myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a selective medium that selects against the unfused parental cells. Preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-II mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 and derivatives e.g., X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); and Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

[0155] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

[0156] The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis described in Munson et al. (Anal. Biochem., 107:220 (1980)). Once hybridoma cells that produce antibodies of the desired specificity, affinity, and/or activity are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies: Principles and Practice, pp 103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal e.g., by i.p. injection of the cells into mice.

[0157] The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, affinity chromatography (e.g., using protein A or protein G-Sepharose) or ion-exchange chromatography, hydroxylapatite chromatography, gel electrophoresis, dialysis, etc.

[0158] DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transformed or transfected into prokaryotic or eukaryotic host cells such as, e.g., *E coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells, that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., Curr. Opinion in Immunol., 5:256-262 (1993) and Phickthun, Immunol. Revs., 130:151-188 (1992).

[0159] Further, the monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990). Clackson et al., Nature, 352: 624-628 (1991) and Marks et al., J. Mol. Biol., 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., Bio/Technology, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., Nuc. Acids. Res., 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

[0160] The DNA that encodes the antibody may be modified to produce chimeric or fusion antibody polypeptides, for example, by substituting human heavy chain and light chain constant domain (CH and CL) sequences for the homologous murine sequences (U.S. Pat. No. 4,816,567; and Morrison, et al., Proc. Natl. Acad. Sci. USA, 81:6851 (1984)), or by fusing the immunoglobulin coding sequence with all or part of the coding sequence for a non-immunoglobulin polypeptide (heterologous polypeptide). The nonimmunoglobulin polypeptide sequences can substitute for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chi-

meric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

[0161] Humanized Antibodies

[0162] Methods for humanizing non-human antibodies have been described in the art.

[0163] Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is nonhuman. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0164] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity and HAMA response (human anti-mouse antibody) when the antibody is intended for human therapeutic use. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human V domain sequence which is closest to that of the rodent is identified and the human framework region (FR) within it accepted for the humanized antibody (Sims et al., J. Immunol., 151:2296 (1993); Chothia et al., J. Mol. Biol., 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)).

[0165] It is further important that antibodies be humanized with retention of high binding affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art.

[0166] Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

[0167] Various forms of a humanized anti-ECM-complex antibody are contemplated. For example, the humanized antibody may be an antibody fragment, such as a Fab, which is optionally conjugated with one or more cytotoxic agent(s) in order to generate an immunoconjugate. Alternatively, the humanized antibody may be an intact antibody, such as an intact IgG1 antibody.

[0168] Human Antibodies

[0169] As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production.

[0170] Transfer of the human germ-line immunoglobulin gene array into such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggemann et al., Year in Immuno., 7:33 (1993); U.S. Pat. Nos. 5,545,806, 5,569,825, 5,591,669 (all of GenPharm); 5,545,807; and alternatively, phage display technology (McCafferty et al., Nature 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as Ml3 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats, reviewed in, e.g., Johnson, Kevin S, and Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., Nature, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., J. Mol. Biol. 222:581-597 (1991), or Griffith et al., EMBO J. 12:725-734 (1993). See, also, U.S. Pat. Nos. 5,565,332 and 5,573,905. As discussed above, human antibodies may also be generated by in vitro activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

[0171] Antibody Fragments

[0172] In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992); and Brennan et al., Science, 229:81

(1985)). However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody fragments can all be expressed in and secreted from *E coli*, thus allowing the facile production of large amounts of these fragments. Antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab)_2$ fragments (Carter et al., Bio/Technology 10: 163-167 (1992)). According to another approach, $F(ab)_2$ fragments can be isolated directly from recombinant host cell culture. Fab and $F(ab)_2$ fragment with increased in vivo half-life comprising a salvage receptor binding epitope residues are described in U.S. Pat. No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. The antibody of choice may also be a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458. Fv and sFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. sFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an sFv. See Antibody Engineering, ed. Borrebaeck, supra. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

[0173] Bispecific Antibodies

[0174] Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ECM-complex protein. Other such antibodies may combine an ECM-complex binding site with a binding site for another protein. Alternatively, an anti-ECM-complex binding arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a Tcell receptor molecule (e.g. C133), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), so as to focus and localize cellular defense mechanisms to the ECM-complex-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ECM-complex. These antibodies possess an ECM-complex-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab)_2$ bispecific antibodies). WO 96/16673 describes a bispecific anti-ErbB2/anti-FcγRIII antibody and U.S. Pat. No. 5,837,234 discloses a bispecific anti-ErbB2/anti-FcγRI antibody. A bispecific anti-ErbB2/Fca antibody is shown in WO98/02463. U.S. Pat. No. 5,821,337 teaches a bispecific anti-ErbB2/anti-CD3 antibody.

[0175] Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., EMBO 10:3655-3659 (1991).

[0176] According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. Preferably, the fusion is with an Ig heavy chain constant domain, comprising at least part of the hinge, C_H2, and C_H3 regions. It is preferred to have the first heavy-chain constant region (CHI) containing the site necessary for light chain bonding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host cell. This provides for greater flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yield of the desired bispecific antibody. It is, however, possible to insert the coding sequences for two or all three polypeptide chains into a single expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios have no significant affect on the yield of the desired chain combination.

[0177] Preferably, the bispecific antibodies in this approach are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

[0178] According to another approach described in U.S. Pat. No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the CH3 domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

[0179] Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

[0180] Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al. (Science, 229: 81 (1985)) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent, sodium arsenite, to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

[0181] Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al. (J. Exp. Med., 175: 217-225 (1992)) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

[0182] Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., J. Immunol., 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers.

[0183] The "diabody" technology described by Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a VH connected to a VL by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., J. Immunol., 152:5368 (1994).

[0184] Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. J. Immunol. 147: 60 (1991).

[0185] Multivalent Antibodies

[0186] A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies of the present invention can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fc region. The preferred multivalent antibody herein comprises (or consists of) three to about eight, but preferably four, antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (and preferably two polypeptide chains), wherein the polypeptide chain (s) comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1(X1n-VD2-(X2)n-Fc, wherein VDI is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, XI and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide chain(s) may comprise: VH-CHI-flexible linker-VH-CHI-Fc region chain; or VH-CHI-VH-CHI-Fc region chain. The multivalent antibody herein preferably further comprises at least two (and preferably four) light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to about eight light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain.

[0187] Other Amino Acid Sequence Modifications

[0188] Amino acid sequence modification(s) of the anti-ECM-complex antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the anti-ECM-complex antibody are prepared by introducing appropriate nucleotide changes into the anti-ECM-complex antibody nucleic acid, or by peptide synthesis.

[0189] Such modifications include, for example, deletions from, and/or insertions into, and/or substitutions of, residues within the amino acid sequences of the anti-ECM-complex antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the anti-ECM-complex antibody, such as changing the number or position of glycosylation sites.

[0190] A useful method for identification of certain residues or regions of the anti-ECM-complex antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells in Science, 244:1081-1085 (1989). Here, a residue or group of target residues within the anti-ECM-complex antibody are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with ECM-complex antigen.

[0191] Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at a target codon or region and the expressed anti-ECM-complex antibody variants are screened for the desired activity.

[0192] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more resi-

dues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-ECM-complex antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the anti-ECM-complex antibody molecule include the fusion to the N- or C-terminus of the anti-ECM-complex antibody to an enzyme (e.g. for ADEPT) or a fusion to a polypeptide which increases the serum half-life of the antibody.

[0193] Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-ECM-complex antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table A under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in the table below, or as further described below in reference to amino acid classes, may be introduced and the products screened for a desired characteristic.

TABLE A

Amino Acid Substitutions

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	Val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gin; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	Phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

[0194] Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile; (2) neutral hydrophilic: cys, ser, thr; (3) acidic: asp, glu; (4) basic: asn, gin, his, lys, arg; (5) residues that influence chain orientation: gly, pro; and (6) aromatic: trp, tyr, phe.

[0195] Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Any cysteine residue not involved in maintaining the proper conformation of the anti-ECM-complex antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

[0196] A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino acid substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human ECM-complex. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

[0197] Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody. Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used. Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

[0198] Nucleic acid molecules encoding amino acid sequence variants of the anti-ECM-complex antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and

cassette mutagenesis of an earlier prepared nucleic acid molecule encoding a variant or a non-variant version of the anti-ECM-complex antibody.

[0199] It may be desirable to modify the antibody of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., J. Exp Med. 176:1191-1195 (1992) and Shopes, B. J. Immunol. 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. Cancer Research 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. Anti-Cancer Drug Design 3:219-230 (1989).

[0200] To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Pat. No. 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of the antibody.

Screening for Antibodies with the Desired Properties

[0201] Techniques for generating antibodies have been described herein. One may further select antibodies with certain biological characteristics, as desired.

[0202] The growth inhibitory effects of an anti-ECM-complex antibody of the invention may be assessed by methods known in the art, e.g., using cells which express ECM-complex either endogenously or following transfection with the ECM-complex gene. For example, the tumor cell lines and ECM-complex-transfected cells provided in Example 1 below may be treated with an anti-ECM-complex monoclonal antibody of the invention at various concentrations for a few days (e.g., 2-7) days and stained with crystal violet or MTT or analyzed by some other colorimetric assay. Another method of measuring proliferation would be by comparing ^{3}H-thymidine uptake by the cells treated in the presence or absence an anti-ECM-complex antibody of the invention. After antibody treatment, the cells are harvested and the amount of radioactivity incorporated into the DNA quantitated in a scintillation counter. Appropriated positive controls include treatment of a selected cell line with a growth inhibitory antibody known to inhibit growth of that cell line. Growth inhibition of tumor cells in vivo can be determined in various ways such as is described in the Experimental Examples section below. Preferably, the tumor cell is one that over-expresses ECM-complex. Preferably, the anti-ECM-complex antibody will inhibit cell proliferation of an ECM-complex-expressing tumor cell in vitro or in vivo by about 25-100% compared to the untreated tumor cell, more preferably, by about 30-100%, and even more preferably by about 50-100% or 70-100%, at an antibody concentration of about 0.5 to 30 μg/ml. Growth inhibition can be measured at an antibody concentration of about 0.5 to 30 μg/ml or about 0.5 nM to 200 nM in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the antibody. The antibody is growth inhibitory in vivo if administration of the anti-ECM-complex antibody at about 1 μg/kg to about 100 mg/kg body weight results in reduction in tumor size or tumor cell proliferation within about 5 days to 3 months from the first administration of the antibody, preferably within about 5 to 30 days.

[0203] To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., propidium iodide (PI), trypan blue or 7AAD uptake may be assessed relative to a control. A PI uptake assay can be performed in the absence of complement and immune effector cells. ECM-complex-expressing tumor cells are incubated with medium alone or medium containing of the appropriate monoclonal antibody at e.g., about 10 μg/ml. The cells are incubated for a 3 day time period. Following each treatment, cells are washed and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake may be selected as cell death-inducing antibodies.

[0204] To screen for antibodies which bind to an epitope on ECM-complex bound by an antibody of interest, e.g., the ECM-complex antibodies of this invention, a routine cross-blocking assay such as that describe in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. This assay can be used to determine if a test antibody binds the same site or epitope as an anti-ECM-complex antibody of the invention. Alternatively or additionally, epitope mapping can be performed by methods known in the art. For example, the antibody sequence can be mutagenized such as by alanine scanning, to identify contact residues. The mutant antibody is initially tested for binding with polyclonal antibody to ensure proper folding. In a different method, peptides corresponding to different regions of ECM-complex can be used in competition assays with the test antibodies or with a test antibody and an antibody with a characterized or known epitope.

[0205] For example, a method to screen for antibodies that bind to an epitope which is bound by an antibody this invention may comprise combining an ECM-complex-containing sample with a test antibody and an antibody of this invention to form a mixture, the level of ECM-complex antibody bound to ECM-complex in the mixture is then determined and compared to the level of ECM-complex antibody bound in the mixture to a control mixture, wherein the level of ECM-complex antibody binding to ECM-complex in the mixture as compared to the control is indicative of the test antibody's binding to an epitope that is bound by the anti-ECM-complex antibody of this invention. The level of ECM-complex antibody bound to ECM-complex is determined by ELISA. The control may be a positive or negative control or both. For example, the control may be a mixture of ECM-complex, ECM-complex antibody of this invention and an antibody known to bind the epitope bound by the ECM-complex antibody of this invention. The anti-ECM-complex antibody labeled with a label such as those disclosed herein. The ECM-complex may be bound to a solid support, e.g., a tissue culture plate or to beads, e.g., sepharose beads.

Immunoconjugates

[0206] The invention also pertains to therapy with immunoconjugates comprising an antibody conjugated to an anti-cancer agent such as a cytotoxic agent or a growth inhibitory agent.

[0207] Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, maytansinoids, a trichothene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

[0208] Maytansine and Maytansinoids

[0209] Preferably, an anti-ECM-complex antibody (full length or fragments) of the invention is conjugated to one or more maytansinoid molecules. Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the cast African shrub Maytenus serrata (U.S. Pat. No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Pat. No. 4,151,042). Synthetic maytansinol and derivatives and analogues thereof are disclosed, for example, in U.S. Pat. Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533, the disclosures of which are hereby expressly incorporated by reference.

[0210] Maytansinoid-Antibody Conjugates

[0211] In an attempt to improve their therapeutic index, maytansine and maytansinoids have been conjugated to antibodies specifically binding to tumor cell antigens. Immunoconjugates containing maytansinoids and their therapeutic use are disclosed, for example, in U.S. Pat. Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of which are hereby expressly incorporated by reference. Liu et al., Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DMI linked to the monoclonal antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic towards cultured colon cancer cells, and showed antitumor activity in an in vivo tumor growth assay. Chari et al. (Cancer Research 52:127-131 (1992)) describe immunoconjugates in which a maytansinoid was conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/neu oncogene. The cytotoxicity of the TA.1-maytansinoid conjugate was tested in vitro on the human breast cancer cell line SK-BR-3, which expresses 3×10 5 HER-2 surface antigens per cell. The drug conjugate achieved a degree of cytotoxicity similar to the free maytansonid drug, which could be increased by increasing the number of maytansinoid molecules per antibody molecule. The A7-maytansinoid conjugate showed low systemic cytotoxicity in mice.

[0212] Anti-ECM-Complex Antibody-Maytansinoid Conjugates (Immunoconjugates)

[0213] Anti-ECM-complex antibody-maytansinoid conjugates are prepared by chemically linking an anti-ECM-complex antibody to a maytansinoid molecule without significantly diminishing the biological activity of either the antibody or the maytansinoid molecule. An average of 3-4 maytansinoid molecules conjugated per antibody molecule has shown efficacy in enhancing cytotoxicity of target cells without negatively affecting the function or solubility of the antibody, although even one molecule of toxin/antibody would be expected to enhance cytotoxicity over the use of naked antibody. Maytansinoids are well known in the art and can be synthesized by known techniques or isolated from natural sources. Suitable maytansinoids are disclosed, for example, in U.S. Pat. No. 5,208,020 and in the other patents and nonpatent publications referred to hereinabove. Preferred maytansinoids are maytansinol and maytansinol analogues modified in the aromatic ring or at other positions of the maytansinol molecule, such as various maytansinol esters.

[0214] There are many linking groups known in the art for making antibody-maytansinoid conjugates, including, for example, those disclosed in U.S. Pat. No. 5,208,020 or EP Patent 0 425 235 B1, and Chari et al. Cancer Research 52: 127-131 (1992). The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred. Conjugates of the antibody and maytansinoid may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl (2-pyridyldithio) propionate (SPDP), succinimidyl-(N-maleimidomethyl)cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as his (p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Particularly preferred coupling agents include N-succinimidyl (2-pyridyldithio)propionate (SPDP) (Carlsson et al., Biochem. J. 173:723-737 (1978)) and N-succinimidyl (2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

[0215] The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. Preferably, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

[0216] Calicheamicin

[0217] Another immunoconjugate of interest comprises an anti-ECM-complex antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, see U.S. Pat. Nos. 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296 (all to American Cyanamid Company). Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ_1^I, (Hinman et al. Cancer Research 53: 3336 (1993), Lode et al. Cancer Research 5 8: 2925-2928 (1998) and the aforementioned U.S. patents to American Cyanamid). Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through antibody mediated internalization greatly enhances their cytotoxic effects.

Other Cytotoxic Agents

[0218] Other antitumor agents that can be conjugated to the anti-ECM-complex antibodies of the invention include

BCNU, streptozoicin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex described in U.S. Pat. Nos. 5,053,394, 5,770,710, as well as esperamicins (U.S. Pat. No. 5,877,296). Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, 1 5 nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), *momordica charantia* inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published Oct. 28, 1993. The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase). For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated anti-ECM-complex antibodies. Examples include At^{211}, I^{131}, I^{125}, In^{111}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, and radioactive isotopes of Lu. When the conjugate is used for diagnosis, it may comprise a radioactive atom for scintigraphic studies, for example Tc^{99M} or I^{123}, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

[0219] The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as Tc^{99M}, I^{123}, In^{111}, Re^{186}, Re^{188}, can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al (1978) Biochem. Biophys. Res. Commun. 80: 49-57) can be used to incorporate iodine. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal, CRC Press 1989) describes other methods in detail.

[0220] Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl(2-pyridyldithio)propionate (SPDP), succinimidyl (N-maleimidomethyl)cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. Science 238: 1098 (1987). Carbon labeled 1-isothiocyanatobenzyl methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al. Cancer Research 52: 127-131 (1992); U.S. Pat. No. 5,208,020) may be used.

[0221] Alternatively, a fusion protein comprising the anti-ECM-complex antibody and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

[0222] In addition, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

[0223] The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see W081/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

[0224] The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form. Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as *serratia* protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as O-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; P-lactamase useful for converting drugs derivatized with P-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, Nature 328: 457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population. The enzymes of this invention can be covalently bound to the anti-ECM-complex antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above.

[0225] Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., Nature, 312: 604-608 (1984).

Other Antibody Modifications

[0226] Other modifications of the antibody are contemplated herein. For example, the antibody may be linked to one

of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A., Ed., (1980).

[0227] The anti-ECM-complex antibodies disclosed herein may also be formulated as immunoliposomes. A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., Proc. Natl. Acad. Sci. USA, 82:3688 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA, 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published Oct. 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556. Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. J. Biol. Chem. 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. J. National Cancer Inst. 81(19)1484 (1989).

Vectors, Host Cells, and Recombinant Methods

[0228] The invention also provides isolated nucleic acid molecule encoding the humanized anti-ECM-complex antibody, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody. For recombinant production of the antibody, the nucleic acid molecule encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or inserted into a vector in operable linkage with a promoter for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to nucleic acid molecules encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

[0229] Signal Sequence Component

[0230] The anti-ECM-complex antibody of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native anti-ECM-complex antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, oc factor leader (including *Saccharomyces* and *Kluyveromyces* cc-factor leaders), or acid phosphatase leader, the *C albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available. The DNA for such precursor region is ligated in reading frame to DNA encoding the anti-ECM-complex antibody.

[0231] Origin of Replication

[0232] Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

[0233] Selection Gene Component

[0234] Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli. One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

[0235] Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the anti-ECM-complex antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -11, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc. For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity (e.g., ATCC CRL-9096).

[0236] Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding anti-ECM-complex antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase

(APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

[0237] A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature, 282:39 (1979)). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4 Jones, Genetics, 85:12 (1977). The presence of the trp**1** lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

[0238] In addition, vectors derived from the 1.6 pm circular plasmid pKDI can be used for transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, Bio/Technology, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer et al., Bio/Technology, 9:968-975 (1991).

[0239] Promoter Component

[0240] Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the anti-ECM-complex antibody nucleic acid. Promoters suitable for use with prokaryotic hosts include the phoA promoter, P-lactamase and lactose promoter systems, alkaline phosphatase promoter, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the anti-ECM-complex antibody.

[0241] Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors. Examples of suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

[0242] Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

[0243] Anti-ECM-complex antibody transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

[0244] The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Reyes et al., Nature 297:598-601 (1982) on expression of human P-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

[0245] Enhancer Element Component

[0246] Transcription of a DNA encoding the anti-ECM-complex antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the anti-ECM-complex antibody-encoding sequence, but is preferably located at a site 5' from the promoter.

[0247] Transcription Termination Component

[0248] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding anti-ECM-complex antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO 94/11026 and the expression vector disclosed therein.

[0249] Selection and Transformation of Host Cells

[0250] Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella*, e.g., *Salmonella typhimurium, Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 Apr. 1989),

Pseudomonas such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W31 10 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

[0251] Full length antibody, antibody fragments, and antibody fusion proteins can be produced in bacteria, in particular when glycosylation and Fc effector function are not needed, such as when the therapeutic antibody is conjugated to a cytotoxic agent (e.g., a toxin) and the immunoconjugate by itself shows effectiveness in tumor cell destruction. Full length antibodies have greater half life in circulation. Production in *E. coli* is faster and more cost efficient. For expression of antibody fragments and polypeptides in bacteria, see, e.g., U.S. Pat. No. 5,648,237 (Carter et. al.), U.S. Pat. No. 5,789,199 (Joly et al.), and U.S. Pat. No. 5,840,523 (Simmons et al.) which describes translation initiation region (TIR) and signal sequences for optimizing expression and secretion, these patents incorporated herein by reference. After expression, the antibody is isolated from the *E. coli* cell paste in a soluble fraction and can be purified through, e.g., a protein A or G column depending on the isotype. Final purification can be carried out similar to the process for purifying antibody expressed e.g., in CHO cells.

[0252] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-ECM-complex antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe; Kluyveromyces* hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa; Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

[0253] Suitable host cells for the expression of glycosylated anti-ECM-complex antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruit fly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bin-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

[0254] Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, *Arabidopsis* and tobacco can also be utilized as hosts. Cloning and expression vectors useful in the production of proteins in plant cell culture are known to those of skill in the art. See e.g. Hiatt et al., Nature (1989) 342: 76-78, Owen et al. (1992) Bio/Technology 10: 790-794, Artsaenko et al. (1995) The Plant J 8: 745-750, and Fecker et al. (1996) Plant Mol Biol 32: 979-986.

[0255] However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol. 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/−DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, 1413 8065); mouse mammary tumor (MMT 060562, ATCC CCL5 1); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

[0256] Host cells are transformed with the above-described expression or cloning vectors for anti-ECM-complex antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

[0257] Culturing Host Cells

[0258] The host cells used to produce the anti-ECM-complex antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's FIO (Sigma), Minimal Essential Medium (MEM) (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58:44 (1979), Barnes et al., Anal. Biochem. 102: 255 (1980), U.S. Pat. No. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[0259] Purification of Anti-ECM-Complex Antibody

[0260] When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Carter et al., Bio/Technology 10: 163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants

from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0261] The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., J. Immunol. Meth. 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., EMBO J. 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a CH3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SIDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

[0262] Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

Pharmaceutical Formulations

[0263] Pharmaceutical formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as acetate, Tris, phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol, and mcresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyllolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; tonicifiers such as trehalose and sodium chloride; sugars such as sucrose, mannitol, trehalose or sorbitol; surfactant such as polysorbate; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). The antibody preferably comprises the antibody at a concentration of between 5-200 mg/ml, preferably between 10-100 mg/ml.

[0264] The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, in addition to the anti-ECM-complex antibody which internalizes, it may be desirable to include in the one formulation, an additional antibody, e.g. a second anti-ECM-complex antibody which binds a different epitope on ECM-complex, or an antibody to some other target such as a growth factor that affects the growth of the particular cancer. Alternatively, or additionally, the composition may further comprise a chemotherapeutic agent, cytotoxic agent, cytokine, growth inhibitory agent, anti-hormonal agent, and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

[0265] The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

[0266] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−) hydroxybutyric acid.

[0267] The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Methods and Treatment Using Anti-ECM-complex Antibodies

[0268] According to the present invention, the anti-ECM-complex antibody that binds to ECM-complex or internalizes upon binding to ECM-complex on a cell surface is used to treat a subject in need thereof having a cancer characterized by ECM-complex-expressing cancer cells, in particular, breast or ovarian cancer, and associated metastases.

[0269] The cancer will generally comprise ECM-complex-expressing cells, such that the anti-ECM-complex antibody is able to bind thereto. While the cancer may be characterized by overexpression of the ECM-complex molecule, the present

application further provides a method for treating cancer which is not considered to be an ECM-complex-overexpressing cancer.

[0270] This invention also relates to methods for detecting cells or tissues which overexpress ECM-complex and to diagnostic kits useful in detecting cells or tissues expressing ECM-complex or in detecting ECM-complex in bodily fluids from a patient. Bodily fluids include blood, serum, plasma, urine, ascites, peritoneal wash, saliva, sputum, seminal fluids, tears, mucous membrane secretions, and other bodily excretions such as stool. The methods may comprise combining a cell-containing test sample with an antibody of this invention, assaying the test sample for antibody binding to cells in the test sample and comparing the level of antibody binding in the test sample to the level of antibody binding in a control sample of cells. A suitable control is, e.g., a sample of normal cells of the same type as the test sample or a cell sample known to be free of ECM-complex overexpressing cells. A level of ECM-complex binding higher than that of such a control sample would be indicative of the test sample containing cells that overexpress ECM-complex. Alternatively the control may be a sample of cells known to contain cells that overexpress ECM-complex. In such a case, a level of ECM-complex antibody binding in the test sample that is similar to, or in excess of, that of the control sample would be indicative of the test sample containing cells that overexpress ECM-complex. Additionally, the methods may comprise combining a test sample with an antibody of this invention, assaying the test sample for antibody binding to ECM-complex in the test sample and comparing the level of antibody binding in the test sample to the level of antibody binding in a control sample. A suitable control is, e.g., a non-diseased sample of the same type as the test sample, sample known to be free of ECM-complex or a sample of known quantity of ECM-complex. A level of ECM-complex binding higher than that of such a control sample would be indicative of the test sample containing overexpression of ECM-complex. Alternatively the control may be a sample known to overexpress ECM-complex. In such a case, a level of ECM-complex antibody binding in the test sample that is similar to, or in excess of, that of the control sample would be indicative of the test sample overexpressing ECM-complex.

[0271] ECM-complex overexpression may be detected with a various diagnostic assays. For example, over expression of ECM-complex may be assayed by immunohistochemistry (IHC). Paraffin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded an ECM-complex protein staining intensity criteria as follows.

[0272] Score 0 no staining is observed or membrane staining is observed in less than 10% of tumor cells.

[0273] Score 1+ a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.

[0274] Score 2+ a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

[0275] Score 3+ a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

[0276] Those tumors with 0 or 1+ scores for ECM-complex expression may be characterized as not overexpressing ECM-complex, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing ECM-complex.

[0277] Alternatively, or additionally, FISH assays such as the INFORM™ (sold by Ventana, Ariz.) or PATHVISION™ (VySiS, Ill.) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of ECM-complex overexpression in the tumor. ECM-complex overexpression or amplification may be evaluated using an in vivo diagnostic assay, e.g. by administering a molecule (such as an antibody of this invention) which binds ECM-complex and which is labeled with a detectable label (e.g. a radioactive isotope or a fluorescent label) and externally scanning the patient for localization of the label.

[0278] A sample suspected of containing cells expressing or overexpressing ECM-complex is combined with the antibodies of this invention under conditions suitable for the specific binding of the antibodies to ECM-complex. Binding and/or internalizing the ECM-complex antibodies of this invention is indicative of the cells expressing ECM-complex. The level of binding may be determined and compared to a suitable control, wherein an elevated level of bound ECM-complex as compared to the control is indicative of ECM-complex overexpression. The sample suspected of containing cells overexpressing ECM-complex may be a cancer cell sample, particularly a sample of breast or ovarian cancer. A serum sample from a subject may also be assayed for levels of ECM-complex by combining a serum sample from a subject with an ECM-complex antibody of this invention, determining the level of ECM-complex bound to the antibody and comparing the level to a control, wherein an elevated level of ECM-complex in the serum of the patient as compared to a control is indicative of overexpression of ECM-complex by cells in the patient. The subject may have a cancer such as breast or ovarian cancer.

[0279] Currently, depending on the stage of the cancer, breast or ovarian cancer treatment involves one or a combination of the following therapies: surgery to remove the cancerous tissue, radiation therapy, androgen deprivation (e.g., hormonal therapy), and chemotherapy. Anti-ECM-complex antibody therapy may be especially desirable in elderly patients who do not tolerate the toxicity and side effects of chemotherapy well, in metastatic disease where radiation therapy has limited usefulness, and for the management of prostatic carcinoma that is resistant to androgen deprivation treatment. The tumor targeting and internalizing anti-ECM-complex antibodies of the invention are useful to alleviate ECM-complex-expressing cancers, e.g., breast or ovarian cancers upon initial diagnosis of the disease or during relapse. For therapeutic applications, the anti-ECM-complex antibody can be used alone, or in combination therapy with, e.g., hormones, antiangiogens, or radiolabelled compounds, or with surgery, cryotherapy, and/or radiotherapy, notably for breast or ovarian cancers, also particularly where shed cells cannot be reached. Anti-ECM-complex antibody treatment can be administered in conjunction with other forms of conventional therapy, either consecutively with, pre- or post-conventional therapy, Chemotherapeutic drugs such as Taxotere® (docetaxel), Taxol® (paclitaxel), estramustine and mitoxantrone are used in treating metastatic and hormone refractory breast or ovarian cancer, in particular, in good risk patients. In the present method of the invention for treating or alleviating cancer, in particular, androgen independent and/or metastatic breast or ovarian cancer, the cancer patient can be administered anti-ECM-complex antibody in conjunction with treatment with the one or more of the preceding chemotherapeutic agents. In particular, combination therapy with paclitaxel and modified derivatives (see, e.g., EP0600517) is contemplated. The anti-ECM-complex antibody will be

administered with a therapeutically effective dose of the chemotherapeutic agent. The anti-ECM-complex antibody may also be administered in conjunction with chemotherapy to enhance the activity and efficacy of the chemotherapeutic agent, e.g., paclitaxel. The Physicians' Desk Reference (PDR) discloses dosages of these agents that have been used in treatment of various cancers. The dosing regimen and dosages of these aforementioned chemotherapeutic drugs that are therapeutically effective will depend on the particular cancer being treated, the extent of the disease and other factors familiar to the physician of skill in the art and can be determined by the physician.

[0280] Particularly, an immunoconjugate comprising the anti-ECM-complex antibody conjugated with a cytotoxic agent may be administered to the patient. Preferably, the immunoconjugate bound to the ECM-complex protein is internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate in killing the cancer cell to which it binds. Preferably, the cytotoxic agent targets or interferes with the nucleic acid in the cancer cell. Examples of such cytotoxic agents are described above and include maytansin, maytansinoids, saporin, gelonin, ricin, calicheamicin, ribonucleases and DNA endonucleases.

[0281] The anti-ECM-complex antibodies or immunoconjugates are administered to a human patient, in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The antibodies or immunoconjugates may be injected directly into the tumor mass. Intravenous or subcutaneous administration of the antibody is preferred. Other therapeutic regimens may be combined with the administration of the anti-ECM-complex antibody.

[0282] The combined administration includes co-administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preferably such combined therapy results in a synergistic therapeutic effect.

[0283] It may also be desirable to combine administration of the anti-ECM-complex antibody or antibodies, with administration of an antibody directed against another tumor antigen associated with the particular cancer. As such, this invention is also directed to an antibody "cocktail" comprising one or more antibodies of this invention and at least one other antibody which binds another tumor antigen associated with the ECM-complex-expressing tumor cells. The cocktail may also comprise antibodies that are directed to other epitopes of ECM-complex. Preferably the other antibodies do not interfere with the binding and or internalization of the antibodies of this invention.

[0284] The antibody therapeutic treatment method of the present invention may involve the combined administration of an anti-ECM-complex antibody (or antibodies) and one or more chemotherapeutic agents or growth inhibitory agents, including co-administration of cocktails of different chemotherapeutic agents. Chemotherapeutic agents include, e.g., estramustine phosphate, prednimustine, cisplatin, 5-fluorouracil, melphalan, cyclophosphamide, hydroxyurea and hydroxyureataxanes (such as paclitaxel and doxetaxel) and/or anthracycline antibiotics. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992).

[0285] The antibody may be combined with an anti-hormonal compound; e.g., an anti-estrogen compound such as tamoxifen; an anti-progesterone such as onapristone (see, EP 616 812); or an anti-androgen such as flutamide, in dosages known for such molecules. Where the cancer to be treated is androgen independent cancer, the patient may previously have been subjected to anti-androgen therapy and, after the cancer becomes androgen independent, the anti-ECM-complex antibody (and optionally other agents as described herein) may be administered to the patient.

[0286] Sometimes, it may be beneficial to also co-administer a cardioprotectant (to prevent or reduce myocardial dysfunction associated with the therapy) or one or more cytokines to the patient. In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy, before, simultaneously with, or post antibody therapy. Suitable dosages for any of the above co-administered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and anti-ECM-complex antibody.

[0287] For the prevention or treatment of disease, the dosage and mode of administration will be chosen by the physician according to known criteria. The appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Preferably, the antibody is administered by intravenous infusion or by subcutaneous injections. Depending on the type and severity of the disease, about 1 pg/kg to about 50 mg/kg body weight (e.g. about 0.1-15 mg/kg/dose) of antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A dosing regimen can comprise administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the anti-ECM-complex antibody. However, other dosage regimens may be useful. A typical daily dosage might range from about 1 pg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy can be readily monitored by conventional methods and assays and based on criteria known to the physician or other persons of skill in the art.

[0288] Aside from administration of the antibody protein to the patient, the present application contemplates administration of the antibody by gene therapy. Such administration of a nucleic acid molecule encoding the antibody is encompassed by the expression "administering a therapeutically effective amount of an antibody". See, for example, WO 96/07321 published Mar. 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

[0289] There are two major approaches to introducing the nucleic acid molecule (optionally contained in a vector) into the patient's cells; in vivo and ex vivo. For in vivo delivery the

nucleic acid molecule is injected directly into the patient, usually at the site where the antibody is required. For ex vivo treatment, the patient's cells are removed, the nucleic acid molecule is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, e.g. U.S. Pat. Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acid molecules into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for ex vivo delivery of the gene is a retroviral vector.

[0290] The currently preferred in vivo nucleic acid molecule transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). For review of the currently known gene marking and gene therapy protocols see Anderson et at., Science 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

Articles of Manufacture and Kits

[0291] The invention also relates to an article of manufacture containing materials useful for the detection of ECM-complex levels in samples, ECM-complex overexpressing cells and/or the treatment of ECM-complex expressing cancer, in particular breast or ovarian cancer. The article of manufacture comprises a container and a composition contained therein comprising an antibody of this invention. The composition may further comprise a carrier. The article of manufacture may also comprise a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for detecting ECM-complex expressing cells and/or treating a cancer condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ECM-complex antibody of the invention. The label or package insert indicates that the composition is used for detecting ECM-complex levels, ECM-complex expressing cells and/or for treating breast or ovarian cancer, in a patient in need thereof. The label or package insert may further comprise instructions for administering the antibody composition to a cancer patient. Additionally, the article of manufacture may further comprise a second container comprising a substance which detects the antibody of this invention, e.g., a second antibody which binds to the antibodies of this invention. The substance may be labeled with a detectable label such as those disclosed herein. The second container may contain e.g., a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

[0292] Kits are also provided that are useful for various purposes, e.g., for ECM-complex cell killing assays, for purification or immunoprecipitation of ECM-complex from cells or for detecting the presence of ECM-complex in a bodily fluid sample or detecting the presence of ECM-complex-expressing cells in a cell sample. For isolation and purification of ECM-complex, the kit can contain an anti-ECM-complex antibody coupled to a solid support, e.g., a tissue culture plate or beads (e.g., sepharose beads). Kits can be provided which contain the antibodies for detection and quantitation of ECM-complex in vitro, e.g. in an ELISA or a Western blot. As with the article of manufacture, the kit comprises a container and a composition contained therein comprising an antibody of this invention. The kit may further comprise a label or package insert on or associated with the container. The kits may comprise additional components, e.g., diluents and buffers, substances which bind to the antibodies of this invention, e.g., a second antibody which may comprise a label such as those disclosed herein, e.g., a radiolabel, fluorescent label, or enzyme, or the kit may also comprise control antibodies. The additional components may be within separate containers within the kit. The label or package insert may provide a description of the composition as well as instructions for the intended in vitro or diagnostic use.

EXAMPLES

Example 1

Production and Isolation of Monoclonal Antibody Producing Hybridomas

[0293] The following MAb/hybridomas of the present invention are described below:

[0294] ECM.D1, ECM.D2, ECM.D3, ECM.D4, ECM.D5, ECM.D6, ECM.D7, ECM.D8, ECM.D9, ECM.D10, ECM.D11, ECM.D12, ECM.D13, ECM.D14, ECM.D15, ECM.D16, ECM.D17, ECM.D18, ECM.D19, ECM.D20, ECM.D21, ECM.D22, ECM.D23, ECM.D24, ECM.D25, ECM.D26, ECM.D27, ECM.D28, ECM.D29, ECM.D30, ECM.D31, ECM.D32, ECM.D33, ECM.D34, ECM.D35, ECM.D36, ECM.D37, ECM.D38, ECM.D39, ECM.D40, ECM.D41, ECM.D42, ECM3.G1, ECM3.G3, ECM3.G4, ECM3.G5, ECM3.G6, ECM3.G8, ECM3.G9, ECM3.G11, ECM3.G12, ECM3.G13, ECM3.G14, ECM3.G15, ECM3.G16, ECM3.G17, MamA.H1, MamA.H2, MamA.H3, MamA.H4, MamA.H5, MamA.H6, MamA.H7, MamA.H8, MamA.H9, MamA.H10, MamA.H11, MamA.H12, MamA.H13, ECM3.J1, ECM3.J3, ECM3.J4, ECM3.J5, ECM3.J6, ECM3.J7, ECM3.J8, ECM3.J9, ECM3.J10, ECM3.J11, ECM3.J12, ECM3.J13, ECM3.J14, ECM3.J15, ECM3.J17, ECM3.J18, ECM3.J19, ECM3.J21, ECM3.J23, ECM3.J24, ECM3.J25, ECM3.J26, LipA.J1, LipA.J2, LipA.J3, and LipA.J4.

[0295] If the MAb producing hybridoma has been cloned, it is assigned the nomenclature

[0296] "X#.1," e.g., the first clone of ECM.D10 will be referred to as D10.1, the second clone of D10 will be referred to as D10.2, etc. Sub-clones are designated by a subsequent ".#", e.g. the first sub-clone of ECM.D10.1 is referred to as D10.1.1, the second sub-clone of D10.1 is D10.1.2, etc. Further generations of sub-clones are annotated in the same format. For the purposes of this invention, a reference to an anti-PCan065 antibody producing hybridoma, e.g. ECM.D10 or D10, will include all clones and sub-clones of the antibody, e.g., D10.1, D10.2, D10.1.1, etc. Furthermore, the nomencla-

ture ECM.D10.3, for example, may reference the antibody producing hybridoma, or the antibody itself.

Immunogens and Antigens (Recombinant Proteins, His Tags)

[0297] For the ECM complex constructs described herein, nucleic acid molecules encoding ECM family member proteins or fusion proteins of ECM family members were inserted into various expression vectors to produce recombinant proteins. These nucleic acid sequences were isolated by PCR using the primers which are routine to design.

[0298] For purposes of illustration, the predicted amino acid sequence encoded by each construct is also included. However, the constructs may include naturally occurring variants (e.g. allelic variants, SNPs) within the ECM family members as isolated by the primers. These variant sequences, and antibodies which bind to them are considered part of the invention as described herein.

[0299] ECM2/ECM3 Complex (Construct 1) Sequence and Protein Production

[0300] An ECM-complex was created by co-expressing recombinant ECM2 and ECM3. ECM2 was expressed using a modified pTT3 His-tagged vector. A representative amino acid sequence of ECM2 expressed by this vector is presented in SEQ ID NO:1.

ECM2 Amino Acid Sequence (SEQ ID NO: 1)

MKLSVCLLLVTLALCCYQANAEFCPALVSELLDFFFISEPLFKLSLAKFD

APPEAVAAKLGVKRCTDQMSLQKRSLIAEVLVKILKKCSVASHHHHHHHH

HH

ECM3 was expressed using a modified pTT3 vector. A representative amino acid sequence of ECM3 expressed by this vector is presented in SEQ ID NO:2.

ECM3 Amino Acid Sequence (SEQ ID NO: 2)

MKLLMVLMLAALLLHCYADSGCKLLEDMVEKTINSDISIPEYKELLQEFI

DSDAAAEAMGKFKQCFLNQSHRTLKNFGLMMHTVYDSIWCNMKSN

ECM2 and ECM3 were co-expressed to form ECM2 and ECM3 tertramers comprised of ECM2/ECM3 heterodimers. This ECM2/ECM3 complex is herein referred to as "Construct 1" or ECM2/ECM3. The complex was purified using standard protein techniques.

ECM3/LipA Complex (Construct 2) Sequence and Protein Production

[0301] An ECM-complex was created by co-expressing recombinant ECM3 and LipA. LipA was expressed using a modified pTT3 His-tagged vector. A representative amino acid sequence of ECM3 expressed by this vector is presented in SEQ ID NO:3.

LipA Amino Acid Sequence (SEQ ID NO: 3)

MRLSVCLLLLTLALCCYRANAVVCQALGSEITGFLLAGKPVFKFQLAKFK

APLEAVAAKMEVKKCVDTMAYEKRVLITKTLGKIAEKCDRASHHHHHHHH

HH

ECM3 was expressed using a modified pTT3 vector as described above. A representative amino acid sequence of ECM3 expressed by this vector is presented in SEQ ID NO:2.

ECM3 Amino Acid Sequence (SEQ ID NO: 2)

MKLLMVLMLAALLLHCYADSGCKLLEDMVEKTINSDISIPEYKELLQEFI

DSDAAAEAMGKFKQCFLNQSHRTLKNFGLMMHTVYDSIWCNMKSN

LipA and ECM3 were co-expressed to form LipA and ECM3 tertramers comprised of LipA/ECM3 heterodimers. This LipA/ECM3 complex is herein referred to as "Construct 2" or LipA/ECM3. The complex was purified using standard protein techniques.

ECM2/MamA Complex (Construct 3) Sequence and Protein Production

[0302] An ECM-complex was created by co-expressing recombinant ECM2 and MamA.

[0303] ECM2 was expressed using a modified pTT3 His-tagged vector as described above. A representative amino acid sequence of ECM2 expressed by this vector is presented in SEQ ID NO:1.

ECM2 Amino Acid Sequence (SEQ ID NO: 1)

MKLSVCLLLVTLALCCYQANAEFCPALVSELLDFFFISEPLFKLSLAKFD

APPEAVAAKLGVKRCTDQMSLQKRSLIAEVLVKILKKCSVASHHHHHHHH

HH

MamA was expressed using a modified pTT3 vector. A representative amino acid sequence of MamA expressed by this vector is presented in SEQ ID NO:4.

MamA Amino Acid Sequence (SEQ ID NO: 4)

MKLLMVLMLAALSQHCYAGSGCPLLENVISKTINPQVSKTEYKELLQEFI

DDNATTNAIDELKECFLNQTDETLSNVEVFMQLIYDSSLCDLF

ECM2 and LipA were co-expressed to form ECM2 and LipA tertramers comprised of ECM2/LipA heterodimers. This ECM2/ECM3 complex is herein referred to as "Construct 3" or ECM2/LipA. The complex was purified using standard protein techniques.

Immunization

[0304] Four immunization series were performed. Immunogens were the ECM2/ECM3 complex (Construct 1) for the ECM.D-series mAbs, the ECM2/MamA complex (Construct 2) for the MamA.H-series mAbs, and the ECM3/LipA complex (Construct 3) for the ECM3.G and ECM3.J-series mAbs.

[0305] For each series, nine mice (Balb/c, FVB or C3H) were immunized intradermally in both rear footpads. All injections were 25 uL per foot. The first injection of 10 ug of antigen per mouse was in Dulbecco's phosphate buffered saline (DPBS) mixed in equal volume to volume ratio with Titermax gold adjuvant (Sigma, Saint Louis, Miss.). Subsequently, mice were immunized twice weekly for 5 weeks. For the 2nd through 10th injection, mice were immunized with 10 ug of antigen in 20 uL of DPBS plus 5 uL of Adju-phos adjuvant (Accurate Chemical & Scientific Corp., Westbury,

N.Y.) per mouse. The final immunization consisted of 10 ug antigen diluted in DPBS alone.

Hybridoma Fusion

[0306] Four days after the final immunization, mice were sacrificed and draining lymph node (popliteal) tissue was collected by sterile dissection. Lymph node cells were dispersed using a Tenbroeck tissue grinder (Wheaton #347426, VWR, Brisbane, Calif.) followed by pressing through a sterile sieve (VWR) into DMEM and removing T-cells via anti-CD90 (Thy1.2) coated magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany).

[0307] These primary B-cell enriched lymph node cells were then immortalized by electro-cell fusion (BTX, San Diego, Calif.) with the continuous myeloma cell line P3x63Ag8.653 (Kearney, J. F. et al., J. Immunology 123: 1548-1550, 1979). The myeloma and B-cells were pooled at a 1:1 ratio for the fusion. These fusion cultures were distributed at 2 million cells per plate into wells of 96 well culture plates (Costar #3585, VWR). The remainder of the cells was cultured in bulk in HAT selection medium for 10 days and cryopreserved for future screens. Successfully fused cells were selected by culturing in selection medium (DMEM/15% FBS) containing 2.85 μM Azaserine, 50 μM Hypoxanthine (HA) (Sigma) or 50 μM Hypoxanthine, 0.2 μM Aminopterin, 8 μM Thymidine (HAT) (Sigma) supplemented with recombinant human IL-6 (Sigma) at 0.5 ng/mL. Cultures were transitioned into medium (DMEM/10% FBS) without selection and IL-6 supplements for continued expansion and antibody production.

[0308] Supernatants from wells were screened by enzyme linked solid phase immunoassay (ELISA). Monoclonal cultures, consisting of the genetically uniform progeny from single cells, were established after the screening procedure, by sorting of single viable cells into wells of two 96 well plates, using flow cytometry (Coulter Elite; Beckman-Coulter, Miami, Fla.). The resulting murine B-cell hybridoma cultures were expanded using standard tissue culture techniques. Selected hybridomas were cryopreserved in fetal bovine serum (FBS) with 10% DMSO and stored in Liquid Nitrogen at −196° C. to assure maintenance of viable clone cultures.

Direct ELISA Screening & Selection of Hybridomas Producing ECM Specific Antibodies

ECM.D-Series MAbs

[0309] Hybridoma cell lines were selected for production of ECM2/ECM3 specific antibodies by direct ELISA. Separate wells were coated with ECM2/ECM3 Protein Construct 1, ECM2/MamA Protein Construct 3, ECM3/LipA Protein Construct 2 and a negative control protein. One ug/mL protein in PBS (100 uL/well) was incubated overnight in 96 well polystyrene EIA plates (Costar #9018, VWR) at 4° C. The plate wells were washed twice with Tris buffered saline with 0.05% Tween20, pH 7.4 (TBST). Nonspecific binding capacity was blocked by filling the wells (300 ul/well) with TBST/0.5% bovine serum albumin (TBST/BSA) and incubating for >30 minutes at room temperature (RT). The wells were emptied and filled with 50 uL/well TBST/BSA to prevent them from drying out during the sample collection process. Hybridoma culture medium sample (50 uL) was added to the wells and incubated for 1 hour at RT. The wells were washed 3 times with TBST. One hundred uL of alkaline phosphatase conjugated goat anti-mouse IgG (Fc) with minimal cross-reactivity to human Fc (P/N115-055-071, Jackson Immunoresearch), diluted 1:5000 in TBST/BSA, was added to each well and incubated for >1 hour at RT. The wells were washed 3 times with TBST. One hundred uL of alkaline phosphatase substrate para-nitrophenylphosphate (pNPP) (Sigma) at 1 mg/mL in 1 M Diethanolamine buffer pH 8.9 (Pierce) was added to each well and incubated for 20 min at RT. The enzymatic reaction was quantified by measuring the solution's absorbance at 405 nm wavelength. Results are depicted in Table 1.

TABLE 1

ELISA signals of D series hybridomas

	Protein coated to wells			
Hybridoma	ECM2/ECM3	ECM2/MamA	ECM3/LipA	negative control
ECM.D1	1.665	2.467	0.160	0.175
ECM.D2	0.998	3.150	0.125	0.127
ECM.D3	2.705	3.315	0.107	0.104
ECM.D4	2.452	3.006	0.181	0.119
ECM.D5	3.262	3.305	0.111	0.111
ECM.D6	3.304	3.052	0.106	0.115
ECM.D7	1.331	2.798	0.129	0.138
ECM.D8	2.832	3.452	0.104	0.118
ECM.D9	1.064	2.800	0.240	0.118
ECM.D10	1.560	3.081	0.458	0.136
ECM.D11	3.263	4.000	0.142	0.132
ECM.D12	1.464	0.981	0.209	0.136
ECM.D13	3.620	4.000	0.173	0.140
ECM.D14	3.423	2.828	0.122	0.120
ECM.D15	2.517	3.110	0.143	0.136
ECM.D16	0.774	3.220	0.138	0.142
ECM.D17	3.016	3.260	0.127	0.127
ECM.D18	1.300	4.000	0.215	0.159
ECM.D19	2.597	2.962	0.113	0.121
ECM.D20	1.759	2.409	0.124	0.140
ECM.D21	0.587	1.239	0.129	0.121
ECM.D22	1.062	1.602	0.202	0.133
ECM.D23	1.671	4.000	0.440	0.132
ECM.D24	3.066	2.681	0.122	0.118
ECM.D25	2.796	3.282	0.132	0.116
ECM.D26	1.172	1.028	0.147	0.187
ECM.D27	2.101	3.161	0.114	0.122
ECM.D28	1.298	2.531	0.158	0.172
ECM.D29	1.609	0.102	4.000	0.104
ECM.D30	3.491	0.112	4.000	0.117
ECM.D31	3.230	0.117	3.319	0.119
ECM.D32	3.422	0.124	2.259	0.135
ECM.D33	3.539	0.112	3.525	0.116
ECM.D34	3.063	0.117	3.593	0.114
ECM.D35	3.293	0.115	1.010	0.112
ECM.D36	3.338	0.121	3.181	0.124
ECM.D37	2.204	0.124	2.520	0.121
ECM.D38	3.172	0.123	3.277	0.136
ECM.D39	2.961	0.111	3.312	0.112
ECM.D40	2.448	2.283	2.447	0.150
ECM.D41	3.586	3.377	4.000	0.108
ECM.D42	2.165	1.213	3.148	0.203

[0310] Supernatants from forty-two hybridomas, named ECM.D1 to ECM.D42, reacted specifically with the ECM2/ECM3 complex. Twenty-eight supernatants (ECM.D1 to ECM.D28) also reacted with ECM2/MamA, but not with ECM3/LipA indicating ECM2 specificity. Supernatants from eleven hybridomas (ECM.D29 to ECM.D39) also reacted with ECM3/LipA but not with ECM2/MamA indicating ECM3 specificity. Supernatants from three hybridomas (ECM.D40 to ECM.D42) reacted with all three ECM protein complexes, ECM2/ECM3, ECM2/MamA and ECM3/LipA,

but not with the negative control protein indicating that these antibodies recognize a common epitope shared between the three ECM complexes. Hybridoma D1 to D42 were expanded and cryopreserved.

ECM3. G-Series MAbs

[0311] ELISA screens of hybridoma supernatants were performed as described for the ECM.D-series. Results are depicted in Table 2.

TABLE 2

	ELISA signals of G series hybridomas			
	Protein coated to wells			
Hybridoma	ECM3/LipA	ECM2/ECM3	ECM2/MamA	negative control
ECM3.G1	3.0319	2.5327	0.0929	0.0936
ECM3.G3	3.6707	3.4132	0.0979	0.0883
ECM3.G4	1.2538	2.7194	0.0988	0.1100
ECM3.G5	2.9902	3.2247	0.1121	0.1195
ECM3.G6	3.0542	2.8546	0.1624	0.1595
ECM3.G8	3.1919	3.1973	0.1183	0.1121
ECM3.G9	3.2120	3.0028	0.0985	0.0924
ECM3.G11	3.3120	3.0576	0.0970	0.0884
ECM3.G12	3.0489	3.0562	0.1016	0.1060
ECM3.G13	2.2281	2.1773	0.1108	0.1068
ECM3.G14	1.2710	1.4675	0.1081	0.1092
ECM3.G15	1.7999	2.0085	0.1157	0.1040
ECM3.G16	1.5368	1.4968	0.1057	0.1018
ECM3.G17	3.1662	3.2568	0.0909	0.0926

Supernatants from fourteen hybridomas (ECM3.G1, ECM3.G3 to ECM3.G6, ECM3.G8, ECM3.G9, and ECM3.G11 to ECM3.G17) reacted with ECM3/LipA and ECM2/ECM3 proteins, but not with ECM2/MamA protein indicating specificity for ECM3. These hybridomas were expanded and cryopreserved.

MamA.H-Series MAbs

[0312] ELISA screens of hybridoma supernatants were performed as described for the ECM.D-series. Results are depicted in Table 3.

TABLE 3

	ELISA signals of H series hybridomas			
	Protein coated to wells			
Hybridoma	MamA/ECM2	ECM3/ECM2	LipA/ECM3	negative control
MamA.H1	1.545	0.116	0.100	0.111
MamA.H2	2.271	0.300	0.108	0.099
MamA.H3	1.478	0.120	0.141	0.111
MamA.H4	2.894	0.095	0.104	0.103
MamA.H5	2.883	0.106	0.109	0.092
MamA.H6	2.050	0.245	0.149	0.128
MamA.H7	2.589	0.118	0.119	0.124
MamA.H8	1.992	0.098	0.083	0.088
MamA.H9	0.660	0.106	0.087	0.102
MamA.H10	2.514	1.510	0.087	0.100
MamA.H11	1.783	0.856	0.124	0.211
MamA.H12	2.207	0.460	0.117	0.108
MamA.H13	2.178	0.847	0.131	0.148
MamA.H14	2.508	0.785	0.127	0.131

[0313] Supernatants from fourteen hybridomas (MamA.H1 to MamA.H14) reacted specifically with ECM2/MamA protein. Nine of these supernatants (MamA.H1 to MamA.H9) only reacted with ECM2/MamA protein, but did not bind to ECM2/ECM3 and ECM3/LipA proteins, indicating specificity for MamA. Five supernatants (MamA.H10 to MamA.H14) reacted with ECM2/MamA and ECM2/ECM3, indicating ECM2 specificity. Hybridoma H1 to H14 were expanded and cryopreserved.

ECM3.J-Series and LipA.J-Series MAbs

[0314] ELISA screens of hybridoma supernatants were performed as described for the ECM.D-series. Results are depicted in Table 4.

TABLE 4

	ELISA signals of J series hybridomas			
	Protein coated to wells			
	ECM2/ECM3	ECM2/MamA	ECM3/LipA	negative control
ECM3.J 1	0.2175	0.0789	0.3735	0.0803
ECM3.J 3	0.7652	0.0849	1.0247	0.0963
ECM3.J 4	2.2109	0.1032	3.8972	0.0982
ECM3.J 5	0.2834	0.1215	0.3298	0.1173
ECM3.J 6	0.8080	0.1118	1.0580	0.1063
ECM3.J 7	0.2091	0.1131	0.2332	0.0892
ECM3.J 8	1.1426	0.1003	1.7912	0.0959
ECM3.J 9	1.3929	0.0829	2.0594	0.0927
ECM3.J 10	0.1199	0.0851	0.2300	0.1523
ECM3.J 11	0.1140	0.0895	0.2375	0.0874
ECM3.J 12	0.1287	0.0993	0.3337	0.1146
ECM3.J 13	2.0456	0.1077	3.1970	0.1289
ECM3.J 14	0.2012	0.1141	0.2341	0.1160
ECM3.J 15	0.8115	0.1031	0.8162	0.1036
ECM3.J 17	0.1141	0.0789	0.1251	0.0830
ECM3.J 18	1.2943	0.0959	1.8191	0.0907
ECM3.J 19	0.5457	0.0829	1.3310	0.0843
ECM3.J 21	1.3646	0.1098	0.1752	0.1075
ECM3.J 24	0.3994	0.0950	0.3268	0.0947
ECM3.J 25	1.3994	0.0848	1.8599	0.0840
ECM3.J 26	2.8494	0.3075	3.0127	0.0943
LipA.J1	0.1318	0.0941	1.5871	0.1194
LipA.J2	0.1648	0.1028	0.6944	0.1071
LipA.J3	0.1605	0.1018	1.6954	0.1029
LipA.J4	0.0963	0.0861	1.6788	0.0893

[0315] Supernatants from twenty-five hybridomas reacted specifically with ECM3/LipA protein as indicated by higher absorbance values in wells coated with ECM3/LipA than in wells coated with negative control protein. Twenty-one of these supernatants (ECM3.J1 to ECM3.J5 to ECM3.J15, ECM3.J17 to ECM3.J19, ECM3.J21 and ECM3.J24 to ECM3.J26) also reacted with ECM2/ECM3 protein, but did not bind to ECM2/MamA proteins, indicating specificity for ECM3. Four supernatants (LipA.J1 to LipA.J4) only reacted with ECM3/LipA protein, but not with ECM2/MamA and ECM2/ECM3, indicating LipA specificity. Hybridoma ECM3.J1 to ECM3.J26 and LipA.J1 to LipA.J4 were expanded and cryopreserved.

Off-Ranking Analysis of ECM Hybridoma Supernatants

[0316] Dissociation constants (kd) were calculated from surface plasmon resonance measurements using a BIACORE 3000 instrument (BiaCore, Piscataway, N.J.). A RAM-Fc

surface was used to capture each antibody supernatant, followed by an injection of the indicated ECM protein over the captured antibody.

[0317] Flow cell 1 of a CM5 sensor chip (BiaCore) was used as a blank surface for reference subtractions, and was activated and then inactivated with ethanolamine per standard BiaCore protocols. Flow cell 2 was used to immobilize RAM Fc using an injection time of 12 minutes and a flow of 5 ul/min. The RAM-Fc (BiaCore) was diluted to 35 ug/mL in 10 mM acetate as suggested. Standard amine coupling (BiaCore) was used. Hybridoma supernatants were diluted 1:2 in HBS-EP running buffer (BiaCore) and passed over flow cells 1 and 2. Antibodies were captured at 5 ul/min flow rate, 3 minute injection, and an ECM protein was injected at 5 ug/mL for 2 minutes. The dissociation time was 3 minutes. The regeneration of the chip surface, or removal of captured hybridoma supernatants binding to the antigen between cycles, was performed by injecting 10 mM glycine pH 1.75 for 30 seconds at 100 uL/minute.

[0318] The above procedure was performed by using the BiaCore's surface preparation and binding wizard included in the BiaCore control software. Results were automatically fitted using the separate ka/kd function included in the BiaCore analysis software, assuming a 1:1 Langmuir binding model. Results in Table 5 include the antibody producing hybridoma and the dissociation constant (kd).

TABLE 5

Dissociation rate constants kd of ECM-specific antibodies

mAb	ECM2/ECM3 kd [1/s]	ECM2/MamA kd [1/s]	ECM3/LipA kd [1/s]
D1	4.64E-04	6.64E-04	
D19	1.18E-03	8.97E-04	
D22	1.01E-03	7.86E-04	
D28	5.09E-04	6.30E-04	
D32	2.30E-04		1.44E-04
D34	7.82E-04		
D37	3.87E-04		5.55E-04
G1	1.99E-03		
G12	1.01E-03		5.61E-04
G17	1.60E-04		6.05E-05
G3	2.23E-04		9.86E-05
G4	6.73E-04		3.52E-04
G5	3.44E-04		4.60E-04
G6	1.17E-03		1.14E-03
G7	6.70E-04		2.98E-04
G8	1.61E-04		7.88E-05
G9	1.83E-04		1.17E-04
H10	9.10E-05	4.55E-05	
H11	1.08E-03	1.60E-03	
H12	3.54E-04	1.62E-04	
H13	1.65E-04	2.76E-04	
H14	3.57E-04	2.06E-04	
H2	3.56E-04	4.68E-05	
H7		1.69E-04	

Epitope Mapping of ECM Complex Specific MAbs

[0319] Overlapping peptides covering the sequences of ECM2, ECM3, MamA and LipA were used to analyze the epitopes of all ECM complex-specific antibodies from the D, G, H and J series. Peptides were fifteen amino acids long with five amino acid overlap between adjacent peptides, e.g. the first peptides covered amino acids 1 to 15, the second peptide covered amino acids 10 to 25. Peptides were coated to wells and antibody reactivity towards the peptides was determined by direct ELISA as described above. Antibodies ECM2.D3, ECM2.D7, ECM2.D12, ECM2.D13, ECM2.D24 and ECM2.D42 reacted with peptide ECM2-5 (peptide sequence VKRCTDQMSLQKRSL (SEQ ID NO:5)). Antibody ECM3.J6 reacted with peptide ECM3-4 (peptide sequence: FIDSDAAAEAMGKFK (SEQ ID NO:6)). Antibody ECM3.J19 reacted with peptide ECM3-1 (peptide sequence: DSGCKLLEDMVEKTI (SEQ ID N:7)). All other antibodies did not react with any peptide, indicating that these antibodies bind conformational epitopes.

Cloning of Hybridomas Producing ECM Complex Specific MAbs

[0320] Based on the data above, the following hybridomas were selected for single cell cloning into 96 well culture plates by cell sorting (Coulter Elite): ECM2.D1, ECM2.D8, ECM2.D10, ECM2.D19, ECM2.D22, ECM2.D24, ECM2.D25, ECM2.D28, ECM2.H2, ECM2.H10, ECM2.H11, ECM2.H12, ECM3.D32, ECM3.D34, ECM3.D36, ECM3.D37, ECM3.G4, ECM3.G5, ECM3.G6, ECM3.G8, ECM3.G12, ECM3.G17, ECM3.J11, ECM3.J12, ECM3.J24, LipA.J1, LipA.J2, LipA.J3, LipA.J4, MamA.H5 and MamA.H7. After 2 weeks of culture, supernatants of each subclone were tested by direct ELISA. Three ELISA-positive subclones per parent hybridoma were expanded and cryopreserved.

ECM-D, -G, -H, -J Series MAb Checkerboard ELISA

[0321] A checkerboard ELISA analysis was carried out utilizing the different antigen complexes described above: ECM2/MamA (Construct 3), ECM2/ECM3 (Construct 1), and ECM3/LipA (Construct 2). For each antigen complex, each antibody was tested as both coating and detection antibody in combination with every other antibody, including itself, so that every possible pair was examined for its binding ability. Appropriate positive and negative antibody controls were included to establish the validity of each experiment.

[0322] High binding half-area-well polystyrene plates (Corning Life Sciences) were coated overnight at 4° C. with 25 μL/well of anti-ECM-complex mAb at 10 μg/mL in phosphate buffered saline (PBS). The coating solution was aspirated off and the plates were washed four times in Tris-buffered saline (TBS)/0.5% Tween-20 (TBS/T). The wells were blocked with 150 μL/well of Superblock-TBS (Pierce Biotechnology, Illinois) for 1 hour at room temperature (RT). The wells were washed four times with TBS/T and 25 μL/well of antigen in assay buffer (TBS, 1% BSA, 1% Mouse Serum, 1% Calf Serum, and 0.1% ProClin™) was added so that for each well containing antigen, there was a corresponding (in terms of coating and detecting antibody) well containing no antigen. In any given experiment, antigen was added at a fixed concentration, which varied with each antigen. The range of antigen concentrations was 3-50 ng/mL. The plate was incubated for 1 hour at RT followed by washing in TBS/T. Biotinylated detecting antibody was added at 25 μL/well and a concentration of 5 μg/mL in assay buffer and the plate was incubated for 1 hour at RT. In some experiments, prior to the addition of the detecting antibody, competitor antibody (i.e., the same antibody as the coating antibody) was added at a volume of 25 μL/well and final concentration of 10 μg/mL in assay buffer. The plate was then incubated for approximately 30 min at RT. Without washing the wells, the biotinylated detecting antibody was added to a final volume of 30 μL/well and a concentration of 5 μg/mL in assay buffer and the plate

was incubated for 1 hour at RT. After incubation in the presence of detecting antibody, the wells were washed four times with TBS/T and 25 μL/well streptavidin-HRP conjugate (Jackson Lab) was added at a 1:20,000 dilution in PBS. The plate was incubated for 30 min at RT and then washed four times in TBS/T. HRP substrate TMB (Stable Stop, Moss, Inc.) was added at 25 μL/well and the plate was incubated for up to 30 min at RT. The reaction was stopped by the addition of 25 μL/well 1N HCl, and the optical density (OD) at 450 nm was obtained on a Spectramax 190 plate reader (Molecular Devices).

[0323] A total of 29 antibodies were tested by checkerboard analysis, i.e., as both coating and detecting antibodies, for a total of 841 mAb pairs tested. 11 pairs were tested with the antigen complexes MamA/ECM2, ECM2/ECM3 and ECM3/LipA. or each pair and each antigen, a specific activity was calculated and expressed in terms of the signal-to-noise (s/n) ratio. The signal was the OD value obtained in the presence of antigen and the noise was the OD value obtained in the absence of antigen. The s/n ratio was obtained by dividing the signal by the noise.

[0324] The resulting profile of reactivities was evaluated with a view towards selecting candidate pairs for further analysis. Such factors as the s/n ratio, the magnitude of the noise for a given pair and antigen, and the identity or similarity in pattern of reactivity among two or more mAb pairs were considered in identifying mAbs able to detect antigen.

[0325] The pattern of reactivities demonstrated that for each antigen, there were several distinct epitopes. Antibody pairs with the highest signal/noise ratio were selected to test sensitivity for recombinant protein, and reactivity towards native protein in serum samples.

Hemi-Sandwich ELISA with Anti-ECM mAbs

[0326] Purified antibodies from cloned hybridomas were tested for binding efficacy by a hemi-sandwich ELISA to confirm the specificities assigned in the direct ELISA format. The protocol used was that for a standard ELISA (above). For the hemi-sandwich ELISA, the coating antibody was either an anti-HIS antibody or an anti-ECM-complex antibody. Antigen captured in the well was detected with either an anti-ECM-complex antibody (for antigen captured by the anti-HIS antibody) or an anti-HIS antibody (for antigen captured by the anti-ECM-complex antibody).

[0327] All antibodies demonstrating activity in the direct ELISA were tested in the hemi-ELISA. The specificities determined in the hemi-sandwich ELISA format agreed with the specificities assigned based on the direct ELISAs with the following exceptions: 1) the "pan-ECM-reactive" mAbs D42 and D43 had no activity as either coating or detecting antibodies towards the antigen MamA/ECM2 in the hemi-sandwich ELISA. The mAb D42 furthermore did not have activity towards this antigen in the checkerboard analysis, indicating that D42 may have a very low affinity for this antigen; 2) the "ECM2-reactive" mAb D24 had no activity as a detecting antibody towards the antigen MamA/ECM2 in the hemi-sandwich ELISA. The mAb D24 also did not have activity (as a coating or a detection antibody) towards this antigen in the checkerboard analysis. As D24 did have activity (in the hemi-sandwich ELISA and in the checkerboard analysis) towards the antigen ECM2/ECM3, the specificity assignment made in the direct ELISA is most likely correct. The discrepancy between the two assay formats might be due to a high degree of context dependence in the recognition by D24 of its epitope.

ECM -D, -G, -H, -J Series mAbs ELISA

[0328] High binding half-area-well polystyrene plates (Corning Life Sciences) were coated overnight at 4° C. with 25 μL/well of anti-ECM complex mAb at 10 μg/mL in phosphate buffered saline (PBS). The coating solution was aspirated off and the plates were washed four times in Tris-buffered saline (TBS)/0.5% Tween-20 (TBS/T). The wells were blocked with 150 μL/well of Superblock-TBS (Pierce Biotechnology, Illinois) for 1 hour at room temperature (RT). The wells were washed four times with TBS/T and 25 μL/well of antigen (recombinant antigen or sera) in assay buffer (TBS, 1% BSA, 1% Mouse Serum, 1% Calf Serum, and 0.1% ProClin™) was added at a dilution (for the recombinant antigen: in a titration) specific to each ECM antigen complex and to each mAb pair. The plate was incubated for 1 hour at RT. The wells were washed four times with TBS/T and the biotinylated detecting antibody was added at a final concentration of 5 μg/mL in assay buffer. The plate was incubated for 1 hour at RT. The wells were washed four times with TBS/T and 25 μL/well of streptavidin-HRP conjugate (Jackson Lab) was added at a 1:20,000 dilution in PBS. The plate was incubated for 30 min at RT and then washed four times in TBS/T. HRP substrate TMB (Stable Stop, Moss, Inc.) was added at 25 μL/well and the plate was incubated for 30 min at RT. The reaction was stopped by the addition of 25 μL/well 1N HCl, and the optical density (OD) at 450 nm was obtained on a Spectramax 190 plate reader (Molecular Devices). For determination of levels of the marker CA15.3 in serum, a commercially available EIA kit was used according to the manufacturer's instructions (BioCheck, Inc., Foster City, Calif.).

[0329] The concentration of analyte was calculated based on the standard curve for a given mAb pair and antigen complex. Samples with a signal below detectability were assigned a signal of the minimal detectable concentration (MDC) as follows: The MDC is defined as two standard deviations above the background value (value in the absence of antigen).

Example 2

Sandwich ELISA Detection of ECM Complexes in Human Serum

Human Serum Samples

[0330] Human cancer serum samples were obtained from IMPATH-BCP, Inc. (Franklin, Mass.), Clinical Research Center of Cape Cod (CRCCC), Inc. (West Yarmouth, Mass.) and ProteoGenex (Culver City, Calif.). The serum samples from healthy men and women were obtained from ProMedDx LLC (Norton, Mass.). All samples were aliquoted upon arrival and stored at minus 80° C. until use.

[0331] In the tables demonstrating detection of ECM complexes, MIC-1 (PCan065), B7-H4 (O110) and CA15.3 in serum, samples are grouped by type and identified by tissue and disease state of the tissue. Tissue annotation includes: BR=Breast, CN=Colon, LN=Lung, OV=Ovarian, and PR=Prostate. Disease states may be specifically indicated or abbreviated into groups as: CAN=Cancer. Samples from non-diseased men and women are annotated as NRM Male (NRM M) and NRM Female (NRM F), respectively.

Detection of ECM-complex mAbs in Screening Panels of Normal and Cancer Sera

[0332] Anti-ECM-complex mAb pairs reactive with any of the three recombinant antigen complexes in the checkerboard analysis were subjected to further screening through analysis

in several serum panels comprising pooled or individual sera from patients with various cancers, and normal healthy subjects. The first step of this screening procedure was analysis of mAb pair reactivity in a panel ("training panel") of pooled sera from cancer patients and individual sera from male and female controls, as described in Table xxx below. A total of 132 pairs of antibodies were tested with the "training panel". Those pairs reactive with any of the cancer pools in the "training panel" were further tested in a "primary panel" comprising sera from patients with various cancers and from normal healthy individuals as outlined in Tables 6a and 6b.

TABLE 6a

Composition of cancers in "training panel"

	NML F	NML M	BR CAN	CN CAN	LN CAN	OV CAN	PR CAN
Number of serum pools	0	0	2	3	2	1	2
Number of individual sera	3	3	0	0	0	0	0

TABLE 6b

Composition of cancers in "primary panel"

	NML F	NML M	BR CAN	CN CAN	LN CAN	OV CAN	PR CAN
Number of samples	21	19	24	24	24	24	24

[0333] A total of 30 pairs of antibodies were tested with the "primary panel". The mAb pairs were scored in terms of their ability to detect cancer. Table 7 provides a summary of the scoring which is expressed in terms of the identification (indicated by "+"), based on OD value, or non-identification (indicated by "−") of one or more individual samples in a given set as having an elevated signal relative to the average signal in the set of normals. Based on this scoring, the pairs were selected for further analysis in higher-resolution serum panels.

TABLE 7

Summary of performance of 30 mAb pairs in "primary panel"

Antigen Detected (complex)	mAb Pairs (Coat Ab/ Det'n Ab)	OD value		Relative Reactivity (compared to NMLs)				
ECM2/ECM3	D1/G5	Low	Low	−	−	−	+/−	−
ECM2/ECM2	D1/H2	Low	Low	−	−	−	+	+
ECM2/MamA	D19/H7	Med	Med	−	+/−	−	+	−
ECM2/ECM2	D22/H10	High	High	+/−	+/−	−	+	+/−
ECM2/ECM3	D28/G12	Low	Low	−	−	−	+/−	−
ECM2/ECM2	D28/H10	High	High	−	−	+/−	+	+/−
ECM3/ECM2	D34/D24	Low	Low	−	+/−	+/−	−	−
ECM3/ECM3	D37/D34	Low	Low	−	−	−	+/−	−
ECM2&3/ECM3	D42/D32	Low	Low	−	+/−	+/−	+/−	−
ECM3/ECM2	G4/H10	Low	Low	−	−	−	+/−	−
ECM3/ECM2	G5/D24	Low	Low	−	−	−	+/−	−
ECM3/ECM3	G5/G8	High	High	+/−	+/−	+/−	+	+/−

TABLE 7-continued

Summary of performance of 30 mAb pairs in "primary panel"

Antigen Detected (complex)	mAb Pairs (Coat Ab/ Det'n Ab)	OD value		Relative Reactivity (compared to NMLs)				
ECM3/ECM3	G5/G12	Low	Low	−	+/−	−	+/−	−
ECM3/ECM2	G8/D1	Low	Low	−	−	−	+/−	−
ECM3/ECM3	G8/D34	Low	Low	−	−	−	+/−	−
ECM3/ECM2	G8/H2	Low	Low	−	−	−	+/−	−
ECM3/ECM2	G17/D28	Low	Low	−	−	−	+/−	−
ECM3/ECM3	G17/G4	Low	Low	−	−	−	+/−	+/−
MamA/ECM2	H5/D1	High	High	−	−	−	+	+/−
MamA/ECM2	H5/H2	Med	High	−	−	−	+	+
ECM2/ECM2	H10/D1	High	High	−	−	−	+	+/−
ECM2/ECM3	H10/D32	Low	Low	−	−	−	+/−	−
ECM2/ECM3	H10/D34	Med	High	−	−	−	+	+/−
ECM2/ECM3	H10/D37	Low	Low	−	−	−	+/−	−
ECM2/ECM3	H10/G5	Low	Low	−	−	−	+/−	−
ECM2/ECM2	H12/D28	Med	Med	−	−	−	+	+/−
LipA/ECM3	LipA.J3/G12	Low	Low	−	+/−	−	+	+/−
LipA/ECM3	LipA.J4/D37	Med	Med	+/−	+	+/−	+	+
LipA/ECM2&3	LipA.J4/D43	Low	Low	−	+	−	−	−
LipA/ECM3	LipA.J4/G5	Low	Low	+/−	+	+/−	+	+

Antibody pairs D22/H10, H5/D1, H5/H2, and H10/D34 were selected to evaluate ECM-complex levels in breast cancer samples. Antibody pairs D28/H10, and H10/D1 were selected to evaluate ECM-complex levels in ovarian cancer samples.

Detection of ECM-complex mAbs in Breast Cancer Samples

[0334] The concentration of ECM complexes was measured by four anti-ECM-complex mAb pairs in 400 serum samples from normal, healthy females and individuals with breast cancer. PCan065 (MIC-1) and CA15.3 were evaluated as controls. Tables 8 provides an overview of all samples tested and Table 9 shows the OD values obtained for the standard curves for each mAb pair and antigen complex.

TABLE 8

Summary of breast cancer serum samples

Sample Type	No. of Samples Tested	No. of Samples in Analysis
Normal Controls (female)	150	150
All Breast Cancer stages	250	250
Early Stage (stg I and II) cancer	170	170
Late Stage (stg III and IV) cancer	76	76

*Four breast cancer samples were Tumor In Situ and not assigned a stage.

TABLE 9

Standard curves for six assays utilized to test breast cancer sera

MamA/ECM2 (H5/D1)		MamA/ECM2 (H5/H2)		ECM2/ECM3 (H10/D34)		ECM2/ECM2 (D22/H10)		Pcan065 (A10/B2)		CA15.3 (commercial kit)	
ng/mL	OD	ng/mL	OD	ng/mL	OD	ng/mL	OD	ng/mL	OD	U/mL	OD
1	3.012	1	0.739	2	2.638	1	2.52				
0.5	2.229	0.5	0.52	1	1.78	0.5	1.671				
0.25	1.385	0.25	0.329	0.5	1.022	0.25	0.931	6	1.334	240	0.923
0.125	0.85	0.125	0.211	0.25	0.621	0.125	0.568	2	0.51	120	0.55
0.0625	0.527	0.0625	0.141	0.125	0.383	0.0625	0.351	0.75	0.228	60	0.365
0.0313	0.344	0.0313	0.105	0.0625	0.248	0.0313	0.243	0.3	0.121	30	0.227
0.0156	0.268	0.0156	0.086	0.0313	0.195	0.0156	0.196	0.05	0.0692	15	0.159
0	0.15	0	0.066	0	0.129	0	0.151	0	0.0574	0	0.0952

[0335] Tables 10a and 10b provide a summary of the quantiles established for the measured levels of ECM complex in the serum of patients and control subjects.

TABLE 10a

Levels (ng/mL) of ECM-complexes in normal and breast cancer samples

	Antigen/Complex (mAb pair)							
	MamA/ECM2 (H5/D1)		MamA/ECM2 (H5/H2)		ECM2/ECM3 (H10/D34)		ECM2/ECM2 (D22/H10)	
	Samples							
	NML F	BR CAN	NML F	BR CAN	NML F	BR CAN	NML F	BR CAN
	Quantiles							
	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)
Minimum	0	0.002	0.002	0.006	0.001	0	0.004	0.011
25th Percentile	0.047	0.084	0.084	0.084	0.081	0.141	0.1	0.204
Median	0.117	0.198	0.092	0.1285	0.204	0.371	0.262	0.672
75th Percentile	0.233	0.434	0.118	0.276	0.418	0.772	0.699	1.205
Maximum	2.82	4.66	1.54	3.04	4.38	12.88	7.43	16.64

TABLE 10b

Levels of Pcan065 and CA15.3 in normal and breast cancer samples

	Antigen/Complex (mAb pair)			
	Pcan065 (A10/B2)		CA15.3 (commercial kit)	
	Samples			
	NML F	BR CAN	NML F	BR CAN
	Quantiles			
	(ng/mL)	(ng/mL)	(U/mL)	(U/mL)
Minimum	0.01	0.01	447	355
25th Percentile	0.360	0.453	1322	1167
Median	0.794	0.899	1987	1745
75th Percentile	1.10	2.17	3741	3437
Maximum	4.27	10.29	26531	33451

[0336] Tables 10a and 10b demonstrate that MamA/ECM2, ECM2/ECM3, and ECM2/ECM2 complexes are detected at a higher level in sera from individuals with breast cancer than in individuals without breast cancer.

Detection of ECM-complex mAbs in Ovarian Cancer Samples

[0337] The concentration of ECM complexes was measured in 119 serum samples from normal/healthy females and individuals with ovarian cancer. Table 11 provides an overview of all samples tested and Table 12 shows the OD values obtained for the standard curves for each mAb pair and antigen complex.

TABLE 11

Summary of ovarian cancer serum samples

Sample Type	No. of Samples Tested	No. of Samples in Analysis
Normal Controls (female)	40	40
Ovarian Cancer	79	79

TABLE 12

Standard curves for three assays utilized to test ovarian cancer sera

ECM2/ECM2 (D28/H10)		ECM2/ECM2 (H10/D1)		O110 (A57/A7)	
ng/mL	OD	ng/mL	OD	pg/mL	OD
1	2.664	1	2.53		
0.5	1.998	0.5	1.58		
0.25	1.204	0.25	0.986	6	1.544
0.125	0.707	0.125	0.569	2	0.904
0.0625	0.42	0.0625	0.377	0.75	0.573
0.0313	0.274	0.0313	0.268	0.3	0.328
0.0156	0.199	0.0156	0.201	0.05	0.259
0	0.094	0	0.141	0	0.213

[0338] Table 13 provides a summary of the quantiles established for the measured levels of ECM complex in the serum of patients and control subjects.

TABLE 13

Levels of ECM-complexes and O110 in normal and ovarian cancer samples

	Antigen/Complex (mAb pair)					
	ECM2/ECM2 (D28/H10)		ECM2/ECM2 (H10/D1)		O110 (A57/A7)	
	Samples					
	NML F	OV CAN	NML F	OV CAN	NML F	OV CAN
	Quantiles					
	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	(pg/mL)	(pg/mL)
Minimum	0.078	0.081	0.098	0.111	8.32	8.32
25th Percentile	0.1420	0.1760	0.120	0.136	108	177
Median	0.205	0.2750	0.130	0.162	152	361
75th Percentile	0.248	0.5720	0.153	0.244	220	987
Maximum	0.744	1.82	0.356	0.750	2207	6356

Table 13 demonstrates that the ECM2/ECM2 complex is detected at a higher level in sera from individuals with ovarian cancer than in individuals without ovarian cancer.

Results

[0339] Elevated levels of ECM-complexes were observed in individuals with breast and ovarian cancer. Specifically, MamA/ECM2, ECM2/ECM3 and ECM2/ECM2 complexes were elevated in individuals with breast cancer and ECM2/ECM2 complexes were elevated in individuals with ovarian cancer. These results demonstrate that elevated levels of ECM-complexes is indicative of an individual having breast or ovarian cancer. Further, elevated levels of PCan065 are indicative early stage (stage 1/2) mucinous or serous ovarian cancer. Additionally, ECM-complex ELISAs are able to determine levels of ECM-complexes and discriminate individuals with breast or ovarian cancers from individuals without disease.

Example 3

ROC Analysis of ECM-Complex Levels in Serum

[0340] The ability of a test or assay to discriminate diseased cases from normal cases is evaluated using Receiver Operating Characteristic (ROC) curve analysis (Metz, 1978; Zweig & Campbell, 1993). ROC curves can also be used to compare the diagnostic performance of two or more laboratory or diagnostic tests (Griner et al., 1981).

[0341] An ROC curve is generated by plotting the sensitivity against the specificity for each value. From the plot, the area under the curve (AUC) can be determined. The value for the area under the ROC curve (AUC) can be interpreted as follows: an area of 0.84, for example, means that a randomly selected positive result has a test value larger than that for a randomly chosen negative result 84% of the time (Zweig & Campbell, 1993). When the variable under study can not distinguish between two result groups, i.e. where there is no difference between the two distributions, the area will be equal to 0.5 (the ROC curve will coincide with the diagonal). When there is a perfect separation of the values of the two groups, i.e. there no overlapping of the distributions, the area under the ROC curve equals 1 (the ROC curve will reach the upper left corner of the plot).

[0342] The 95% confidence interval for the area can be used to test the hypothesis that the theoretical area is 0.5. If the confidence interval does not include the 0.5 value, then there is evidence that the laboratory test has the ability to distinguish between the two groups (Hanley & McNeil, 1982; Zweig & Campbell, 1993).

ROC Analysis of ECM-Complexes in Breast Cancer

[0343] Univariate ROC Analysis of ECM-complexes, Pcan065, and CA15.3 in Breast Cancer The sensitivity and specificity for MamA/ECM2, ECM2/ECM3, ECM2/ECM2, PCan065 and CA15.3 alone or in combination to distinguish breast cancers from non-cancers was calculated through receiver operating characteristic (ROC) analysis. Table 14a shows the AUC values from the ROC analysis in case (all cancer samples) versus controls (normal healthy samples). Table 14b shows the AUC values from the ROC analysis in cases (early stage or late stage cancer samples) versus controls (normal healthy samples). AUC values were calculated based on measurements of the serum levels of MamA/ECM2, ECM2/ECM3, ECM2/ECM2, PCan065 and CA15.3 as described in the above standard ELISA protocol.

TABLE 14a

AUC values of ECM-complexes, Pcan065 and CA15.3 in breast cancer

Antigen (mAb pair)	AUC	Std Error	95% Confidence Interval	p-value
MamA/ECM2 (H5/D1)	0.638	0.028	0.588-0.685	0.0001
MamA/ECM2 (H5/H2)	0.619	0.028	0.570-0.667	0.0001
ECM2/ECM3 (H10/D34)	0.621	0.028	0.571-0.669	0.0001
ECM2/ECM2 (D22/H10)	0.641	0.028	0.592-0.688	0.0001
Pcan065	0.606	0.029	0.556-0.654	0.0002
CA15.3	0.541	0.030	0.491-0.590	0.1727

TABLE 14b

AUC values of ECM-complexes, Pcan065, and CA15.3 in breast cancer by stage

Antigen (mAb pair)	Cancer Stage	AUC	95% Confidence Interval
MamA/ECM2 (H5/D1)	Early	0.647	0.571-0.680
	Late	0.708	0.634-0.758

TABLE 14b-continued

AUC values of ECM-complexes, Pcan065, and CA15.3 in breast cancer by stage

Antigen (mAb pair)	Cancer Stage	AUC	95% Confidence Interval
ECM2/ECM3 (H10/D34)	Early	0.584	0.528-0.639
	Late	0.692	0.628-0.752
ECM2/ECM2 (D22/H10)	Early	0.622	0.571-0.680
	Late	0.709	0.645-0.767
Pcan065	Early	0.605	0.550-0.659
	Late	0.612	0.545-0.676
CA15.3	Early	0.533	0.476-0.588
	Late	0.552	0.485-0.618

Bivariate ROC Analysis of ECM-complexes, Pcan065 and CA 15.3 in Breast Cancer

[0344] The sensitivity and specificity in the detection of breast cancer for the ECM-complexes, PCan065, and CA15.3 in combination was calculated through ROC analysis as described above. Table 15 shows the AUC from the ROC analysis of MamA/ECM2, ECM2/ECM3, ECM2/ECM2, PCan065, and CA15.3 levels in cases (cancer samples) versus controls (normal healthy samples) as combination markers. AUC values were calculated based on measurements of the serum levels of the markers as described above.

TABLE 15

AUC values of ECM-complexes, Pcan065 and CA15.3 in breast cancer

Antigen (mAb pair)	MamA/ ECM2 (H5/D1)	MamA/ ECM2 (H5/H2)	ECM2/ ECM3 (H10/D34)	ECM2/ ECM2 (D22/H10)	Pcan065
MamA/ECM2 (H5/D1)	0.638				
MamA/ECM2 (H5/H2)	0.638	0.619			
ECM2/ECM3 (H10/D34)	0.642	0.635	0.621		
ECM2/ECM2 (D22/H10)	0.658	0.645	0.648	0.641	
Pcan065	0.662	0.663	0.674	0.681	0.606

[0345] Summary of ROC Analysis in Breast Cancer with the ECM-Complexes

[0346] The ROC analysis of the ECM-complex ELISAs using four anti-ECM-complex mAb pairs demonstrates that ECM-complexes alone are useful for detecting breast cancer. The ECM-complexes perform better than the established marker CA15.3 for detection of breast cancer. Additionally, the ROC analysis demonstrates that ECM-complexes are unregulated in early stage breast cancer. Furthermore, the ECMs in combination with Pcan065 have a higher AUC for detecting breast cancer than either marker alone. These results demonstrate that MamA/ECM2, ECM2/ECM3, and ECM2/ECM2 alone or in combination with other markers, are useful for detecting cancer, in particular breast cancer.

ROC Analysis of ECM-Complexes in Ovarian Cancer

Univariate ROC Analysis of ECM-Complexes, Pcan065, and CA15.3 in Ovarian Cancer

[0347] The sensitivity and specificity for ECM2/ECM2, and O110 alone or in combination to distinguish ovarian cancers from non-cancers was calculated through receiver operating characteristic (ROC) analysis. Table 16 shows the AUC values from the ROC analysis in case (cancer samples) versus controls (normal healthy samples). AUC values were calculated based on measurements of the serum levels of ECM2/ECM2m and O110 as described in the above standard ELISA protocol.

TABLE 16

AUC values of ECM-complexes, and O110 in ovarian cancer

Antigen (mAb pair)	AUC
ECM2/ECM2 (D28/H10)	0.665
ECM2/ECM2 (H10/D1)	0.729
O110	0.760

Bivariate ROC Analysis of ECM-complexes, and O110 in Ovarian Cancer

[0348] The sensitivity and specificity in the detection of ovarian cancer for the ECM-complexes, and O110 in combination was calculated through ROC analysis as described above. Table 17 shows the AUC from the ROC analysis of ECM2/ECM2, and O110 levels in cases (cancer samples) versus controls (normal healthy samples) as combination markers. AUC values were calculated based on measurements of the serum levels of the markers as described above.

TABLE 17

AUC values of ECM-complexes and O110 in ovarian cancer

Antigen (mAb pair)	ECM2/ECM2 (D28/H10)	ECM2/ECM2 (H10/D1)	O110	All three markers
ECM2/ECM2 (D28/H10)	0.665			
ECM2/ECM2 (H10/D1)	0.727	0.729		
O110	0.799	0.806	0.760	
All three markers				0.810

Summary of ROC Analysis in Ovarian Cancer with the ECM-complexes

[0349] The ROC analysis of the ECM-complex ELISAs using two anti-ECM-complex mAb pairs demonstrates that ECM-complexes alone are useful for detecting ovarian cancer. The ECMs perform equally as well as the marker O110 for detection of ovarian cancer. Furthermore, the ECMs in combination with O110 have a higher AUC for detecting ovarian cancer than either marker alone. These results demonstrate that ECM2/ECM2, as detected by different assays, alone or in combination with other markers, are useful for detecting cancer, in particular ovarian cancer.

Example 4

Deposits

Deposit of Cell Lines and DNA

[0350] Hybridoma cell lines were deposited with the American Type Culture Collection (ATCC) located at 10801 University Boulevard, Manassas, Va. 20110-2209, U.S.A., and accorded accession numbers.

TABLE 18

ATCC deposits		
Hybridoma	ATCC Accession No.	Deposit Date

[0351] The names of the deposited hybridoma cell lines may be shortened for convenience of reference. E.g. D10.3 corresponds to ECM.D10.3. These hybridomas correspond to the clones (with their full names) listed in Table 18. Subclones of hybridomas are listed which have the same characteristics and properties of parental clones. Reference to a parent clone or hybridoma producing an anti-ECM complex antibody, such as ECM.D10 or ECM.D2, includes all subclones such as those listed in Table 1.

[0352] These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations there under (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between diaDexus, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 3 7 CFR §1.14 with particular reference to 886 OG 638).

[0353] The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strains are not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws. The making of these deposits is by no means an admission that deposits are required to enable the invention.

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Phe Asp Ala Pro Pro Glu Ala Val Ala Ala Lys Leu Gly Val Lys Arg
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Cys Thr Asp Gln Met Ser Leu Gln Lys Arg Ser Leu Ile Ala Glu Val
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Phe Ile Asp Ser Asp Ala Ala Ala Glu Ala Met Gly Lys Phe Lys Gln
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Gly Phe Leu Leu Ala Gly Lys Pro Val Phe Lys Phe Gln Leu Ala Lys
35 40 45

Phe Lys Ala Pro Leu Glu Ala Val Ala Ala Lys Met Glu Val Lys Lys
50 55 60

Cys Val Asp Thr Met Ala Tyr Glu Lys Arg Val Leu Ile Thr Lys Thr
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His His His His His His
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<210> SEQ ID NO 4
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Ile Asn Pro Gln Val Ser Lys Thr Glu Tyr Lys Glu Leu Leu Gln Glu
35 40 45

Phe Ile Asp Asp Asn Ala Thr Thr Asn Ala Ile Asp Glu Leu Lys Glu
50 55 60

Cys Phe Leu Asn Gln Thr Asp Glu Thr Leu Ser Asn Val Glu Val Phe
65 70 75 80

Met Gln Leu Ile Tyr Asp Ser Ser Leu Cys Asp Leu Phe
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-continued

<210> SEQ ID NO 5
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 5

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1 5 10 15

<210> SEQ ID NO 6
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<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
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1 5 10 15

What is claimed is:

1. An isolated antibody which competes for binding to the same epitope as the epitope bound by an antibody which binds an ECM-complex.

2. The antibody of claim **1** which competes for binding to the same epitope as the epitope bound by the monoclonal antibody produced by a hybridoma selected from the group comprising ECM.D1, ECM.D22, ECM.D28, ECM.D34, MamA.H2, MamA.H5 and MamA.H10.

3. The antibody of claim **1** which is a monoclonal antibody, a chimeric antibody, a human or humanized antibody, or an antibody fragment.

4. The antibody of claim **1** which binds an ECM-complex wherein such ECM-complex is a heterodimer or homodimer comprising secretogloblin family members.

5. The antibody of claim **4** wherein the heterodimer or homodimer is selected from the group of dimers comprising MamA and ECM2, ECM2 and ECM3, and ECM2 and ECM2.

6. The antibody of claim **5** wherein the heterodimer or homodimer is bound to an identical heterodimer or homodimer to form a tetramer.

7. The antibody of claim **1** where the antibody competes for binding with a molecule that binds a secretoglobin domain.

8. The antibody of claim **3** which is detectably labeled or which is conjugated to a growth inhibitory agent or a cytotoxic agent.

9. The antibody of claim **1** where the antibody inhibits the growth of ECM-complex-expressing cancer cells.

10. The antibody of claim **9**, wherein the cancer cells are from a cancer selected from the group consisting of breast and ovarian cancer.

11. A cell that produces the antibody of claim **3**.

12. The cell of claim **11**, wherein the cell is selected from the group consisting of a hybridoma selected from the group comprising ECM.D1, ECM.D22, ECM.D28, ECM.D34, MamA.H2, MamA.H5 and MamA.H10.

13. A method of producing the antibody of claim **3** comprising culturing an appropriate cell and recovering the antibody from the cell culture.

14. A composition comprising the antibody of claim **3** and a carrier.

15. The composition of claim **14**, wherein the antibody is a humanized form of an anti-ECM-complex antibody produced by hybridoma selected from the group comprising ECM.D1, ECM.D22, ECM.D28, ECM.D34, MamA.H2, MamA.H5 and MamA.H10.

16. A method of inhibiting growth of an ECM-complex-expressing tumor, comprising binding an ECM-complex with the antibody of claim **1**, thereby inhibition ECM-complex activity and inhibiting growth of the tumor.

17. The method of claim **16**, wherein the tumor is selected from the group consisting of breast, ovarian, metastatic breast and metastatic ovarian tumors.

18. The method of claim **16**, wherein the antibody is a humanized form of the antibody produced by hybridoma selected from the group comprising ECM.D1, ECM.D22, ECM.D28, ECM.D34, MamA.H2, MamA.H5 and MamA.H10.

19. The method of claim **16** wherein the ECM-complex-expressing tumor is in a mammal.

20. The method of claim **19** wherein the mammal is administered at least one chemotherapeutic agent in conjunction with the antibody.

21. The method of claim **20** wherein the chemotherapeutic agent is paclitaxel or derivatives thereof.

22. An article of manufacture comprising a container and a composition contained therein, wherein the composition comprises an antibody of claim **3**.

23. The article of manufacture of claim **22** further comprising a package insert indicating that the composition can be used to detect or treat breast or ovarian cancer.

24. A method for determining the presence of an ECM-complex in a sample comprising:

(a) contacting a sample with an ECM-complex antibody of claim **3** under conditions suitable for specific binding of the ECM-complex antibody to ECM-complex, and

(b) determining the level of binding of the antibody to ECM-complex in the sample,

wherein ECM-complex antibody binding to ECM-complex in the sample indicates the presence of an ECM-complex in the sample.

25. The method of claim **24** wherein said sample is from a subject who has a cancer, is suspected of having a cancer or who may have a predisposition for developing cancer.

26. A method for detecting ECM-complex overexpression in a subject in need thereof comprising,

(a) determining the level of ECM-complex in the sample by combining a sample of a subject with an ECM-complex antibody of claim **3** under conditions suitable for specific binding of the ECM-complex antibody to ECM-complex in said sample, and

(b) comparing the level of ECM-complex determined in step (a) to the level of ECM-complex in a control,

wherein an increase in the level of ECM-complex in the sample from the subject as compared to the control is indicative of ECM-complex overexpression in the subject.

27. The method of claim **26** wherein the subject has cancer, is suspected of having a cancer or who may have a predisposition for developing cancer.

28. The method of claim **27** wherein the cancer is breast cancer, ovarian cancer, metastatic breast cancer or metastatic ovarian cancer.

29. The method of claim **26**, wherein the sample is selected from the group consisting of tissues, cells, blood, serum, plasma, urine, ascites, peritoneal wash, saliva, sputum, seminal fluids, tears, mucous membrane secretions, and other bodily excretions such as stool.

30. The method of claim **26** wherein the control is selected from the group comprising a sample from a subject without a cancer overexpressing ECM-complex, a sample of known concentration of ECM-complex and a sample of normal tissue adjacent to cancerous tissue.

31. A method for detecting the presence of breast or ovarian cancer in a subject comprising:

(a) determining the level of ECM-complex in a sample from the subject, and

(b) comparing the level of ECM-complex determined in step (a) to the level of ECM-complex in a control,

wherein an increase in the level of ECM-complex in the sample from the subject as compared to the control is indicative of the presence of breast or ovarian cancer.

32. The method of claim **31** wherein the sample is selected from the group consisting of cells, tissues, bodily fluids, blood, serum, plasma, urine, ascites, peritoneal wash, saliva, sputum, seminal fluids, tears, mucous membrane secretions, and other bodily excretions such as stool.

33. The method of claim **31** wherein the control sample is selected from the group comprising a sample from a subject without a cancer overexpressing an ECM-complex, a sample of known concentration of ECM-complex and a sample of normal tissue adjacent to cancerous tissue.

34. A screening method for antibodies that bind to an epitope which is bound by an antibody of claim **3** comprising,

(a) combining an ECM-complex-containing sample with a test antibody and an antibody of claim **3** to form a mixture,

(b) determining the level of ECM-complex antibody bound to ECM-complex in the mixture, and

(c) comparing the level of ECM-complex antibody bound in the mixture of step (a) to a control mixture,

wherein the level of ECM-complex antibody binding to ECM-complex in the mixture as compared to the control is indicative of the test antibody's binding to an epitope that is bound by the anti-ECM-complex antibody of claim **3**.

* * * * *

专利名称(译)	ECM-复合抗体组合物和使用方法		
公开(公告)号	US20100074893A1	公开(公告)日	2010-03-25
申请号	US12/421836	申请日	2009-04-10
[标]申请(专利权)人(译)	BISTRUP ANNETTE DIEDRICH GUNDO MILLER保尔·勒维		
申请(专利权)人(译)	BISTRUP ANNETTE DIEDRICH GUNDO MILLER保尔·勒维		
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IPC分类号	A61K39/395 C07K16/00 C12N5/16 C12P21/06 G01N33/53 G01N33/566		
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优先权	61/123689 2008-04-10 US		
外部链接	Espacenet USPTO		

摘要(译)

本发明提供了与ECM-复合物结合的分离的抗ECM复合物抗体。本发明还包括含有抗ECM复合物抗体和载体的组合物。这些组合物可以在制品或试剂盒中提供。本发明的另一方面是编码抗ECM复合物抗体的分离的核酸，以及包含分离的核酸的表达载体。还提供了产生抗ECM复合物抗体的细胞。本发明包括产生抗ECM复合物抗体的方法。本发明的其他方面是检测表达癌症的ECM复合物的方法，抑制表达ECM复合物的肿瘤生长的方法，以及减轻或治疗哺乳动物中表达ECM复合物的癌症的方法，包括：向哺乳动物施用治疗有效量的抗ECM复合物抗体。

TABLE 1

ELISA signals of D series hybridomas

	Protein coated to wells			
Hybridoma	ECM2/ECM3	ECM2/MamA	ECM3/LipA	negative control
ECM.D1	1.665	2.467	0.160	0.175
ECM.D2	0.998	3.150	0.125	0.127
ECM.D3	2.705	3.315	0.107	0.104
ECM.D4	2.452	3.006	0.181	0.119
ECM.D5	3.262	3.305	0.111	0.111
ECM.D6	3.304	3.052	0.106	0.115
ECM.D7	1.331	2.798	0.129	0.138
ECM.D8	2.832	3.452	0.104	0.118
ECM.D9	1.064	2.800	0.240	0.118
ECM.D10	1.560	3.081	0.458	0.136
ECM.D11	3.263	4.000	0.142	0.132
ECM.D12	1.464	0.981	0.209	0.136
ECM.D13	3.620	4.000	0.173	0.140
ECM.D14	3.423	2.828	0.122	0.120
ECM.D15	2.517	3.110	0.143	0.136
ECM.D16	0.774	3.220	0.138	0.142
ECM.D17	3.016	3.260	0.127	0.127
ECM.D18	1.300	4.000	0.215	0.159
ECM.D19	2.597	2.962	0.113	0.121
ECM.D20	1.759	2.409	0.124	0.140
ECM.D21	0.587	1.239	0.129	0.121
ECM.D22	1.062	1.602	0.202	0.133
ECM.D23	1.671	4.000	0.440	0.132
ECM.D24	3.066	2.681	0.122	0.118
ECM.D25	2.796	3.282	0.132	0.116
ECM.D26	1.172	1.028	0.147	0.187
ECM.D27	2.101	3.161	0.114	0.122
ECM.D28	1.298	2.531	0.158	0.172
ECM.D29	1.609	0.102	4.000	0.104
ECM.D30	3.491	0.112	4.000	0.117
ECM.D31	3.230	0.117	3.319	0.119
ECM.D32	3.422	0.124	2.259	0.135
ECM.D33	3.539	0.112	3.525	0.116
ECM.D34	3.063	0.117	3.593	0.114
ECM.D35	3.293	0.115	1.010	0.112
ECM.D36	3.338	0.121	3.181	0.124
ECM.D37	2.204	0.124	2.520	0.121
ECM.D38	3.172	0.123	3.277	0.136
ECM.D39	2.961	0.111	3.312	0.112
ECM.D40	2.448	2.283	2.447	0.150
ECM.D41	3.586	3.377	4.000	0.108
ECM.D42	2.165	1.213	3.148	0.203