Title: ANTI-IDIOTYPE ANTIBODY OF DISINTEGRIN AND USES THEREOF

Abstract: Anti-idiotypic antibody of a disintegrin, preferably cantortrostatin, mimics the structure and function of the disintegrin, allowing its use as an integrin antagonist. Thus, monoclonal or polyclonal anti-idiotypic antibody of disintegrin can be administered to a patient in need of anti-angiogenic or anti-metastatic therapy. The anti-idiotypic antibody blocks integrin function on newly growing vessels, endothelial cells, or tumor cells. Such antibodies can be used for diagnosis and therapy of diseases in which integrin receptors play a role.
ANTI-IDIOTYPE ANTIBODY OF DISINTEGRIN
AND USES THEREOF

STATEMENT OF GOVERNMENT SUPPORT

This invention was supported in whole or in part with funding from the United States National Institutes of Health. The United States government may have certain rights in the invention.

FIELD OF THE INVENTION

[0001] This invention relates to inhibitors of integrin function and to anti-idiotypic antibodies of disintegrins such as contortrostatin.

BACKGROUND OF THE INVENTION

[0002] Disintegrins are the most potent known inhibitors of integrin function and include a class of cystine-rich peptides isolated from the venom of the Viperidae and Crotalidae families of snakes (1, 2). The sequence K/RGD (Lys/Arg-Gly-Asp) is conserved in all of these peptides (1, 2). Disintegrins bind to the fibrinogen receptor αllβ3, which results in the inhibition of fibrinogen-dependent platelet aggregation (3). Except for barbourin, a KGD-containing disintegrin which is a relatively specific antagonist for αllβ3 (4), other disintegrins are rather nonspecific and can block function of other β3 integrins, as well as β1 integrins (3, 5).

[0003] Contortrostatin is a disintegrin isolated from Agkistrodon contortrix contortrix (southern copperhead) venom (6). Unlike other monomeric disintegrins, contortrostatin is a homodimer with a mass of 13,505 for the intact molecule and 6,750 for the reduced peptide as shown by mass spectrometry.
Receptors of contortrostatin identified so far include integrins αIIbβ3, αvβ3, αvβ5, and α5β1 (6-10). The interactions between contortrostatin and the integrins are all RGD-dependent.

(0004) In addition to its platelet aggregation inhibitory activity through binding to αIIbβ3, contortrostatin inhibits adhesion of many human cancer cell lines to vitronectin by binding to vitronectin receptor(s). These cell lines including melanoma cells (7), breast cancer cells (9), Kaposi’s sarcoma cells, glioblastoma cells, ovarian cancer cells, bladder cancer and prostate cancer cells. Contortrostatin is also a potent inhibitor of vascular endothelial cell (8) and osteoclast adhesion (11). In addition, contortrostatin inhibits invasion of the above cells through Matrigel basement membrane, which is an artificial membrane composed of predominantly laminin and type IV collagen, independent of its anti-adhesion effect. In the chick chorioallantoic membrane angiogenesis model, contortrostatin inhibits neovascularization induced by bFGF, VEGF, and various tumors (8). In an orthotopic xenograft nude mouse model of breast cancer established by transplantation of human metastatic breast cancer cells (MDA-MB-435) into the mammary fat pad, daily intratumoral injection of contortrostatin significantly inhibits growth and metastasis of the tumor (9). Furthermore, immunohistochemical study of the tumor tissue sections indicates that the microvascular density is significantly reduced in contortrostatin treated tumors (8).

Receptors of contortrostatin identified so far include integrins αIIbβ3, αvβ3, αvβ5, and α5β1. It has been demonstrated that αvβ3 undergoes upregulation in endothelial cells during vasculogenesis (12), wound healing (13), and angiogenesis (14, 15). A monoclonal antibody (mAb) to integrin αvβ3, as well as a cyclic Arg-Gly-Asp (RGD)-containing peptide, perturbed angiogenesis and produced regression of human breast cancer growing on the chick embryo chorioallantoic membrane (CAM) (14, 15). Antagonists of αvβ3 apparently
cause apoptosis of vascular endothelial cells, which results from the activation of p53 and increase of \textit{bcl-2/bax} ratio (16-18). Pathological studies strongly suggest that the invasiveness of cancer cells is proportional to the expression level of \(\alpha\nu\beta3\) (19-21). Recently, Wong \textit{et al.} (22) compared the expression level of \(\alpha\nu\beta3\) in different breast cancer cell lines, and found that the highly metastatic MDA-MB-435 cells express substantial levels of the integrin, whereas MDA-MB-231 and MCF-7, which are less metastatic, do not express \(\alpha\nu\beta3\). Apart from mediating adhesion to vitronectin, \(\alpha\nu\beta3\) transmits signals from the matrix to regulate cellular locomotion functions (23).

\textbf{[0006]} Integrin \(\alpha\nu\beta5\) has also been found to play a role in angiogenesis and cancer metastasis (14, 15, 24-26). It has been reported that an anti-\(\alpha\nu\beta5\) antibody blocks vascular endothelial growth factor (VEGF) induced angiogenesis (27). There is evidence that \(\alpha\nu\beta5\) is involved in cell adhesion (28). Recent studies have shown that \(\alpha\nu\beta5\) requires activation of insulin-like growth factor-1 (IGF-1) to mediate cancer cell migration (25, 26). OVCAR-5 is a human ovarian carcinoma cell line, which expresses \(\alpha\nu\beta5\) but not \(\alpha\nu\beta3\) (10). In our invasion assay, HT-1080 conditioned medium was used as chemoattractant. The specific cytokines triggering \(\alpha\nu\beta5\)-mediated signal transduction in this medium have not been identified. However, OVCAR-5 cells invade through the Matrigel-coated membrane toward the gradient of HT-1080 conditioned medium. It is likely that invasion of this cell line is mediated by \(\alpha\nu\beta5\), since the cells are \(\alpha\nu\beta3\) negative and anti-\(\alpha\nu\beta3\) (7E3) failed to inhibit invasion. Both contortrostatin and anti-\(\alpha\nu\beta5\) (P1F6) partially prevent invasion of these cells, suggesting that antagonism of \(\alpha\nu\beta5\) is an effective way to block invasion of OVCAR-5 cells (10).

\textbf{[0007]} The function of integrin \(\alpha5\beta1\) in angiogenesis has been neglected until recently. Varner's group (29) reported that \(\alpha5\beta1\) and its ligand fibronectin are coordinately up-regulated on blood vessels in human tumor biopsies. The
interaction of the ligand and receptor plays critical roles in angiogenesis, and results in tumor growth in vivo. It has been shown that the central cell-binding domain of fibronectin induces angiogenesis in an α5β1 dependent manner. An antagonist of α5β1 blocks angiogenesis induced by several growth factors but has little effect on angiogenesis induced by VEGF. α5β1 antagonists inhibit tumor angiogenesis, and cause regression of human tumors xenograft in animal models. Investigation reveals that α5β1 and αvβ3 participate in the same pathways of angiogenesis. This conclusion is in agreement with the study by Bayles et al. (30) that αvβ3 and α5β1 are both involved in vacuolation and lumen formation in three-dimensional fibrin matrices, and complete inhibition of lumen formation can only be achieved by combined blockage of both αvβ3 and α5β1.

[0008] Contortrostatin is an antagonist of platelet fibrinogen receptor, integrin αIIbβ3, and inhibits platelet aggregation (6). The role of platelets in cancer metastasis (31-35) (36, 37) has long been established. There are reports that thrombocytopenia induced by anti-αIIbβ3 antibody significantly reduces metastasis (38). This antibody prevents cancer/platelet interaction and activation of platelets by factors released by cancer cells (e.g. tissue factor). Other reports suggest that blockage of αIIbβ3 in platelets disrupts cancer/platelet interaction, which is critical for metastasis (31). On the other hand, tumor-released VEGF is believed to change the anti-thrombotic surface of the vasculature into a prothrombotic one, causing fibrin formation and platelet adhesion and activation (39). Recently, the function of platelets in angiogenesis has attracted the attention of investigators (40). Platelets are rich resources of angiogenic factors such as PDGF and VEGF (41). There is recent evidence suggesting that megakaryocytes are able to synthesize VEGF via de novo route (42, 43). It is well known that upon activation, platelets release their contents including angiogenic factors, like VEGF (44). In an in vitro assay, platelets at physiological concentrations were found to stimulate proliferation of HUVECs,
indicative of proangiogenic activity in vivo (39). It has been demonstrated that an anti-\(\alpha llb\beta 3\) antibody inhibits release of angiogenic factor VEGF from the platelets by blocking aggregation (45).

SUMMARY OF THE INVENTION

[0009] In accordance with one aspect of the present invention, there is provided an anti-idiotypic antibody ("Ald") of disintegrin, the antibody being characterized in binding to one or more integrins, the integrins being a receptor for said disintegrin. In a preferred embodiment, the disintegrin is contortrostatin ("CN"). The anti-idiotypic antibody can be monoclonal antibody or polyclonal antibody. In a further embodiment, the monoclonal antibody is a human antibody or a non-human antibody, or a chimeric or humanized antibody. In yet a further embodiment, anti-idiotypic antibody of disintegrin binds to integrins selected from the group consisting of \(\alpha llb\beta 3\), \(\alpha v\beta 3\), \(\alpha v\beta 5\) and \(\alpha 5\beta 1\).

[0010] A pharmaceutical composition comprising the anti-idiotypic antibodies of disintegrin and optionally a pharmaceutically acceptable carrier is provided for the treatment of angiogenesis, cancer, bone metabolism, or inflammation. Further provided are cells that express the antibodies such as hybridoma cells.

[0011] In accordance with a further aspect of the present invention, there is provided a method of making an anti-idiotypic monoclonal antibody of disintegrin, the method comprising immunizing an animal with an anti-disintegrin antibody, immortalizing lymphocytes from the animal, and selecting immortalized lymphocytes producing anti-idiotypic antibody of the invention. In a preferred embodiment, the disintegrin is contortrostatin.

[0012] Also provided is a method of making an anti-idiotypic monoclonal antibody of a disintegrin, the method comprising immunizing an animal with an anti-disintegrin antibody, preparing a phage display antibody library using nucleic
acid from the animal, and selecting phage which display anti-idiotype antibody of the invention. In a preferred embodiment, the disintegrin is contortrostatin.

[0013] In accordance with a further aspect of the present invention, there is provided a method of antagonizing the activity of disintegrin receptors in an individual, the method comprising administering an effective amount of a composition comprising the anti-idiotype antibodies of disintegrin.

[0014] In accordance with a yet another aspect of the present invention, there is provided a use of an anti-idiotypic antibody of disintegrin for the preparation of a medicament for the treatment of angiogenesis, cancer, bone metabolism, or inflammation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1: α-CN10 binds to contortrostatin in a concentration-dependent manner. Native contortrostatin (0.1 μg/well) was immobilized in the wells of ELISA plate. After blockage with BSA, α-CN10 hybridoma cell culture supernatant at various dilutions was applied into the well. Bound antibody was detected using goat anti-mouse IgG conjugated with horseradish peroxidase. Result shows that α-CN10 binds to immobilized contortrostatin (squares) in concentration-dependent manner, whereas it does not bind to immobilized BSA (triangles).

[0016] Figure 2: SDS-PAGE of purified α-CN10 immunoglobulin. 10 μg of purified α-CN10 and F(ab)2′ fragment of α-CN10 were dissolved in loading buffer containing 2% β-mercaptoethanol and 10 mM DTT, and boiled for 10 min before analysis with SDS-PAGE on a 7.5% acrylamide gel. Sample in lane A is the reduced whole molecule of IgG, which shows a heavy chain greater than 50 kDa and a light chain at about 30 kDa. Sample in lane B is the reduced Fab'
fragment which contains a light chain and truncated heavy chain of about 30 kDa.

[0017] Figure 3: α-CN10 recognizes contortrostatin in Western Blot. Contortrostatin (5 μg) was reduced and resolved by SDS-PAGE using 4 - 20% gradient acrylamide gel in duplicate. A Western blot was prepared from one gel and developed with α-CN10 hybridoma supernatant (1:200 dilution). Lane A is the molecular weight standards, lane B is a Coomassie blue stained SDS-PAGE gel showing contortrostatin as 6.8 kDa subunit, and lane C is the Western blot (Lane C).

[0018] Figure 4: α-CN10 neutralizes anti-platelet aggregation activity of contortrostatin. Platelet aggregation was induced by ADP after one minute incubation with different agents at 37°C. Panel A: complete aggregation was induced by 10 mM ADP (a), this aggregation is completely inhibited by one minute pre-incubation with 100 nM contortrostatin (b). Panel B: anti-aggregation activity of contortrostatin is partially neutralized by α-CN10 at 200 nM (c), and 1000 nM (d), and completely neutralized at 5000 nM (e), whereas PBS (f) or an irrelevant IgG at 200, 1000 and 5000 nM (represented by g, h, i, respectively) does not reverse anti-aggregation effect of contortrostatin.

[0019] Figure 5: α-CN10 inhibits anti-adhesion activity of contortrostatin. Human breast cancer cells (MDA-MB-435) were treated with contortrostatin at various concentrations with and without the presence of 10 μM of α-CN10. The treated cells were allowed to bind to a microtiter plate pre-coated with human vitronectin (0.5 μg/well). Contortrostatin inhibits adhesion of the cells to vitronectin dose-dependently (squares). α-CN10 shifts the dose-response relationship curve to the right (triangles).

[0020] Figure 6: α-CN10 neutralizes anti-adhesion effect of contortrostatin in a dose-dependent manner. Human breast cancer cells (MDA-MB-435) were treated with contortrostatin at 10 nM with increasing concentration of α-CN10
before the cells were allowed to bind to a vitronectin-coated plate. Anti-adhesion activity of contortrostatin was dose-dependently neutralized by α-CN10, as shown by a dose dependent increase in adhering cells with increasing concentrations of the antibody.

Figure 7: α-CN10 inhibits binding of contortrostatin to immobilized integrin αvβ5. Purified integrins αvβ3 and αvβ5 were immobilized in the wells of ELISA plate at 0.1 μg/well. Contortrostatin pre-incubated with various concentrations of α-CN10 were allowed to bind to the plate and probed with rabbit antiserum to contortrostatin followed by goat anti-rabbit IgG conjugated with alkaline phosphatase. α-CN10 inhibits binding of contortrostatin to αvβ5 (triangles), but not to αvβ3 (squares).

Figure 8: Immune response in a mouse immunized with α-CN10. A mouse was immunized with α-CN10 as described herein. Sera obtained was three days after the final boost injection was tested in an ELISA for antibody binding to an Fab' fragment of α-CN10 (coated at 0.1 μg/well). The α-CN10 immunized serum reacted with the Fab' fragment in a concentration dependent manner (filled squares), whereas serum immunized by an irrelevant antigen was unreactive in the assay (filled triangles).

Figure 9: SDS-PAGE of purified Ald40-8 immunoglobulin. 10 μg of purified Ald40-8 was dissolved in loading buffer containing 2% β-Mercaptoethanol and 10 mM DTT, and boiled for 10 min before analyzing by SDS-PAGE on a 7.5% Acrylamide gel. Sample in the left lane is reduced whole molecule of IgG, which shows a heavy chain at about 50 kDa and a light chain at about 30 kDa.

Figure 10. Saturation binding curves of Ald40-8 to integrins αvβ3, αllβ3 and αvβ5. ELISA plates were coated with purified integrins αvβ3, αllβ3 and αvβ5 (0.1 μg/ml). Ald40-8 at various concentrations was allowed to bind to the pre-coated plates, and the bound antibody was detected with goat anti-
mouse IgG. The antibody binds to αvβ3 (triangles) and α1β1β3 (squares) in a similar manner, whereas the maximum magnitude of binding to αvβ5 (up-side-down triangles) is lower than to β3 integrins.

[0025] Figure 11: Contortrostatin competes with Ald40-8 to bind to αvβ5. ELISA plates were coated with purified αvβ5 (0.1 μg/ml). Ald40-8 at 1 μM was pre-incubated with contortrostatin at various concentrations, and was allowed to bind to the pre-coated plates. The bound antibody was detected with goat anti-mouse IgG. Contortrostatin inhibits binding of Ald40-8 in a dose-dependent manner (triangles).

[0026] Figure 12: Ald40-8 binds to cells expressing integrins αvβ3 and αvβ5. Human embryonic kidney carcinoma 293 cells expressing exogenous αvβ3 (293-β3) and αvβ5 (293-β5) cells were incubated with Ald40-8 at 100 μg/ml. The bound Ald40-8 was detected with goat anti-mouse IgG conjugated with FITC. The fluorescence of the stained cells are determined by FACScan flow cytometry. The background was defined using the FITC-labeled secondary antibody alone. The result demonstrated that Ald40-8 (100 μg/ml) binds to both cell lines which express αvβ3 (panel A) and αvβ5 (panel B), respectively.

[0027] Figure 13. Both contortrostatin and Ald40-8 inhibits adhesion of MDA-MB-435 cells to immobilized vitronectin. Human breast cancer cells (MDA-MB-435) were treated with contortrostatin, Ald40-8, and an irrelevant IgG, respectively, at various concentrations. The treated cells were allowed to bind to a microtiter plate pre-coated with human vitronectin (0.5 μg/well). Both contortrostatin (up-side-down triangles) and Ald40-8 (squares) inhibit adhesion of the cells to vitronectin in a dose-dependent manner. The concentration of Ald40-8 to achieve the maximum inhibition obtained by contortrostatin is about 20 times higher than for contortrostatin. Irrelevant IgG did not affect adhesion of the cells (solid triangles).
Figure 14: An agarose gel showing synthesis of an scFv antibody library by overlap PCR. The library was obtained by extracting nucleic acid from mice immunized with anti-contortrostatin antibody α-CN10.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides anti-idiotype antibodies of disintegrin which have an antigen binding site that shares functional and, in some cases, structural characteristics of the disintegrin. In some embodiments, anti-idiotype antibodies of disintegrin are characterized in having functional properties that mimic the binding between the disintegrin and its integrin receptor. Structural characteristics that may be shared between the anti-idiotype antibody and its disintegrin can be an RGD or related motif amino acid sequence. In the case of the antibody, the RGD or related sequence is usually located in the antibody combining site and generally in one of the complementary determining regions or CDRs.

Anti-idiotype antibodies (anti-idiotypic or "anti-ids") are antibodies directed against epitopes (called idiotypes) located in the antigen binding region or variable region of another antibody molecule. Idiotypes (Ab1) and anti-idiotypes (Ab2) are believed to play a role in immune homeostasis. An "internal image" anti-idiotypic antibody is one that mimics the three-dimensional structure of the antigen recognized the Ab1. Thus, anti-disintegrin antibodies are Ab1 type antibodies and anti-idiotype antibodies of disintegrin are Ab2 type antibodies.

Anti-idiotype antibodies of disintegrin are characterized in having some or all of the integrin binding characteristics of the disintegrin. In this regard, the anti-idiotype antibody of disintegrin is characterized in binding to integrins that are receptors for the disintegrin. In some embodiments, the anti-idiotype antibody will bind to the same integrins that are receptors for the
particular disintegrin. In other embodiments, the anti-idiotype antibody will bind to a subset of integrins that are receptors for the particular disintegrin. In still other embodiments, the anti-idiotype antibody will bind to more integrins than are receptors for the particular disintegrin.

[0032] Anti-idiotype antibodies of disintegrin also may be characterized in inhibiting binding between integrins and the particular disintegrin that pertains to that antibody. For example, if the disintegrin is contortrostatin, then the anti-idiotype antibody of contortrostatin may inhibit binding between contortrostatin and one or more of its integrin receptors, e.g., αιιββ3, ανββ3 ανββ5 and αιββ1. Inhibition can be evaluated in liquid or solid phase assays (e.g. ELISA) as are well known in the art.

[0033] The anti-idiotype antibodies of the invention also can be characterized in inhibiting a functional activity of the disintegrin to which the anti-idiotype antibody pertains. The activity to be inhibited may be inhibition of cell adhesion, inhibition of metastases, inhibition of angiogenesis, and the like. Assays to measure cell adhesion, metastasis and angiogenesis are well known in art. Exemplary such assays are described in the examples.

[0034] Preferred anti-idiotype antibodies of the invention are anti-idiotype antibodies of contortrostatin. Such anti-idiotype antibodies share functional characteristics of this disintegrin. As already described, contortrostatin is a 13.5 kDa homodimeric structure (Figure 3), which contains RGD sequence in each of the chains. Contortrostatin specifically binds to integrin ανββ3, ανββ5, and αιββ1. Through antagonism of these integrins, the disintegrin (contortrostatin) blocks adhesion, migration and invasion of cancer and vascular endothelial cells, and thus exerts anti-metastatic and anti-angiogenic activities in vitro and in vivo. Contortrostatin also binds to integrin αιιββ3, a fibrinogen receptor on the surface of blood platelets. This antagonism not only allows the disintegrin to inhibit platelet aggregation as an anti-thrombotic agent, but also
disrupts platelet-mediated interaction between metastatic cancer cells and vascular endothelium, and inhibits release of angiogenic factors such as VEGF from platelets.

[0035] In a preferred embodiment, the anti-idiotypic antibodies of disintegrin are monoclonal antibodies. In a preferred embodiment, the anti-idiotypic antibody of contortrostatin is Ald 40-8 or an antibody with higher binding affinity to integrin αIIbβ3, αvβ3, and αvβ5 than Ald 40-8.

[0036] The specificity of an antibody for a particular antigen is determined by its antigen-binding site, a distinct region of the antibody molecule that makes contact with an antigen. This site is found within the variable regions of immunoglobulin heavy and light chains. An idiotypic, or surface marker, is the unique variable region of a monospecific population of antibody molecules. Idiotypes are also unique determinants in the immunoglobulin that can stimulate production of anti-idiotypic antibodies.

[0037] Historically speaking, the first studies to characterize idiotypic determinants on antibody molecules were performed by Oudin and Michel (47) and by Kunkel et al. (48) in the 1960s. These studies examined antisera generated against homogeneous immunoglobulins (myelomas and Bence Jones proteins) and revealed the antigenic individuality of immunoglobulin molecules. In 1974, Jerne (49) proposed a biological significance for idiotypic determinants in his network theory of immune regulation. Jerne hypothesized that an immune response to a given antigen is controlled by a series of idiotypic/anti-idiotypic reactions that may either enhance or suppress an immune response. The antibody made in response to the original antigen becomes itself an antigen and elicits the synthesis of a second, anti-idiotypic antibody. Thus, a given idiotypic is under control of an anti-idiotypic, and the anti-idiotypic may be regulated by another set of antibodies referred to as anti-anti-idiotypic antibody.
Internal imagery of anti-idiotypes was first reported when anti-idiotypes raised against antibodies to insulin reproduced certain physiologic actions of the hormone itself when bound to insulin receptors (50). Subsequently, anti-idiotype mimicry of bacterial, viral, parasitic, and tumor antigens proved successful in generating experimental anti-idiotype vaccines (51, 52). All evidence to date indicates that anti-idiotype mimicry of antigen is due to similarities in three-dimensional structural conformations between antigen and anti-idiotype (53, 54).

Anti-disintegrin antibodies suitable for eliciting the anti-idiotype antibodies of the invention may be obtained by various approaches. In one approach, animals are immunized with the disintegrin or portions thereof. Disintegrins may be obtained from natural sources or may be synthesized chemically by methods well known in the art. The disintegrin may be administered by any of a variety of routes and optionally in combination with adjuvants as is well known in the art.

For example, antibodies to the disintegrin, contortrostatin, may be prepared by immunizing animals with contortrostatin purified from the venom of southern copperhead snake. Pharmacokinetic studies in mice using $^{125}$I-contortrostatin suggested a short half life, as short as one hour. Bolus injection of one effective dosage of the $^{125}$I-contortrostatin can be completely excreted after 12 hours. Radiolabeled contortrostatin quickly accumulates in the spleen, presumably due to its affinity to platelets. These results, which are consistent with the small size of the contortrostatin subunit (13.5 kDa), suggest conjugating small disintegrins to larger molecules to enhance the immunogenicity.

In another embodiment, anti-idiotype antibody producing lymphocytes from immunized animals may be removed and propagated in vitro providing a
source of the antibody. The hybridoma method, is a well known method for preparing immortalized antibody producing cells, can be used for this purpose.

[0042] Anti-disintegrin antibodies useful for eliciting anti-idiotype antibodies of the invention may be polyclonal or monoclonal. Polyclonal antibodies may be obtained from animals by obtaining serum or plasma using well known methods. The antibody may be further purified by biochemical means for affinity chromatography using well known methods.

[0043] Monoclonal anti-disintegrin antibodies may obtained using the cell fusion/hybridoma method and related methods, well known in the art. For example, as described herein, the hybridoma approach was used to prepare a monoclonal antibody (mAb) designated α-CN10 against the disintegrin, contortrostatin. The mouse from which the hybridoma was obtained was immunized with purified native contortrostatin conjugated to agarose 4B. The resulting α-CN10 antibody binds to contortrostatin (Figure 1) and neutralizes its anti-aggregation and anti-adhesion functions in in vitro assays (Figure 4-6). The hybridoma which produces α-CN10 has been deposited with the American Type Culture Collection under accession No. ___.

[0044] Anti-disintegrin antibodies also may be prepared without immunization by using recombinant DNA technologies. For example, an antibody library may be prepared using nucleic acid from non-immunized animals in accordance with methods known in the art (see, e.g., U.S. patent no. 6,291,650; 6,222,447; 6,140,471; 5,962,255; 6,248,516; 6,291,161; 6,291,160; 6,291,159; 6,291,158). An unimmunized antibody library may be selected for binding to disintegrins by displaying the library on phage as is well known in the art (see, e.g., U.S. Patent No. 5,969,108; 6,172,197; 5,969,108; 5,773,743). Site specific mutation of amino acid residues critical for binding to the disintegrin may be used to improve antibody affinity and specificity for the disintegrin.
Various methods are available for preparing anti-idiotype antibodies of disintegrin using the anti-disintegrin antibodies prepared as described above. In one approach, animals are immunized with polyclonal anti-disintegrin antibodies or with one or more monoclonal anti-disintegrin antibodies. Immunization may be made with the complete antibody or with fragments of the antibody that contain the idiotype. Such fragments include fragments prepared by proteolysis such as Fab, Fab’, and non-natural antibody fragments such as Fv prepared by recombinant DNA techniques. Synthetic antibody fragments also may be used.

Animals may be immunized with antibodies or fragments by administration using well known routes and optionally with any of various adjuvants. Plasma, serum or other fluids from animals immunized with anti-disintegrin may be used a source of polyclonal anti-idiotype antibodies of the disintegrin. In another approach, monoclonal anti-idiotype antibody may be obtained by immortalizing lymphocytes from the immunized animals using the hybridoma or related methods. Immortalized cells producing anti-idiotype antibodies may be screened to identify those with desirable properties as described herein. Polyclonal antibodies also can be similarly screened.

For example, as described herein, the anti-contortrostatin monoclonal antibody, α-CN10, was used to immunize a mouse to generate anti-idiotypic antibody of contortrostatin. Hybridomas prepared using spleen cells of the immunized mouse resulted in the identification of anti-idiotype monoclonal antibodies against α-CN10, one of which was designated Ald 40-8 (Figure 9). Mab Ald 40-8 binds to contortrostatin receptors including integrins αvβ3, αvβ5, and αIIbβ3 in a concentration-dependent manner in ELISA-based assays (Figure 10). Contortrostatin competes with the binding between Ald40-8 and αvβ5 integrin in a solid phase binding assay (Figure 11) and also binds to cell expressed integrins (Figure 12). Ald40-8 is a partial internal image of native contortrostatin, and is a functional surrogate with respect to binding the
integrins bound by contortrostatin. The hybridoma which produces Aid40-8 has been deposited with the American Type Culture Collection under accession No.

[0048] Anti-idiotypic antibodies of disintegrin may also be prepared from immunized animals by cloning antibodies using recombinant DNA technologies. Thus, antibody libraries from immunized lymphocytes of the animal may be prepared using methods discussed above. Phage display of heavy and light chains also may be used to select those anti-idiotypic antibodies with desirable properties.

[0049] For example, pCANTAB 5E™ (a phagemid vector) provided by Amersham Biosciences (Recombinant Phage Antibody System/Expression Module) can be used for the construction of the library. Alternatively, T7Select® (a bacteriophage derivative) from Novagen T7Select® can be employed to generate more diverse libraries. The high ligation and packaging efficiencies of T7Select® vector may enhance the size and diversity of the candidate pool. The phagemid pCANTAB 5E™ allows the scFv fragment to be displayed on the phage capsid protein III for further selection by biopanning. pCANTAB 5E™ vector can generate immune libraries containing over 10^7 clones (library diversity) constructed from 250 ng of vector DNA.

[0050] An scFv cDNA repertoire can be synthesized from total RNA isolated from activated-B cells of the disintegrin-immunized mice. The scFv mAb cDNA library may be generated by PCR amplification and overlap extension assembly of variable light- and heavy-chain coding sequences using a complete set of degenerate primers. The scFv repertoire may be digested and ligated in pCANTAB 5E™, which is used to transform TG1 E. coli strain with M13K07 helper phage co-transformation.

[0051] The scFv displaying phage pool can be selected by any of various techniques described herein including biopanning on immobilized CN. This
selected primary library may then be used to further immunize mice to elicit a second generation anti-idiotypic antibody. In this regard, total RNA from immunized mice may be isolated and a second cDNA library in scFv format constructed. The second generation scFv cDNA repertoire may be displayed on pCANTAB 5ETM, and further selected by biopanning on immobilized integrins (αIIbβ3, αvβ3, αvβ5, and α5β1), to obtain candidate anti-idiotypic antibodies worthy of further testing.

[0052] For uses in human, it is preferred that the anti-disintegrin antibody be human in origin or have been genetically modified to make it human-like (e.g., humanized) such as by CDR grafting and exchanging the non-human heavy or light chain constant regions with those from human. A variety of antibody humanization methods are known in the art (see e.g., U.S. patent no. 5,225,539; 5,530,101; 5,585,009; 5,693,761; 5,693,762; 6,180,370; 5,639,641; 5,871,907; 5,888,657; and 5,565,332). Humanization and related techniques reduces the immunogenicity of foreign antibodies in humans as was observed in the case of several humanized monoclonal antibodies that have undergone clinical trials as anticancer (55), or anti-angiogenic drugs (56-59). Second, the half-life of a humanized monoclonal antibody in vivo is as long as 22 to 30 days, similar to that of native IgG (60-63). In contrast disintegrins such as contortrostatin have a half-life of 1 to 2 hours. This pharmacodynamic feature of IgG favors anti-metastatic and anti-angiogenic therapy, where long-term sustained drug concentration at the active site is required.

[0053] Antibodies raised in animals can be administered to humans but in many cases, a human anti-immunoglobulin antibody immune response will inactivate the antibody (e.g., a mouse antibody as described in the examples). The immunogenicity may be addressed by humanizing the antibody and/or by attaching polymers that reduce immunogenicity such as long chain polyethylene glycols (PEG). Long chain PEG and other polymers are known for their ability to mask foreign epitopes, resulting in the reduced immunogenicity of therapeutic
proteins that display foreign epitopes (64,65). Immunosuppressant drugs such as cyclosporin A, anti-CD3 antibody, and the like, may also be used to reduce the likelihood that an immune response to the anti-idiotypic antibody will develop.

[0054] The anti-idiotypic antibodies of the present invention may comprise a full length natural antibody or may be an antibody fragment such as F(ab)2 or Fab' fragment, or a recombinant form of an antibody such as a single chain variable fragment (scFv). The anti-idiotypic antibodies of contortrostatin or of any other disintegrins may comprise any known type of immunoglobulin, including IgM, IgG, IgE, IgA and IgD. The antibody may be raised in any animal host including any mammal or avian.

[0055] In another embodiment, there is provided anti-idiotypic antibody of contortrostatin that is preferentially reactive with one or several integrins. For example, anti-idiotypic antibody with selective affinity to αvβ3 and αvβ5, but little or no affinity for αIIbβ3 is useful for anti-angiogenic/anti-metastatic therapy. In addition, anti-idiotypic antibody that has selective affinity to αIIbβ3 is useful for antiaggregation therapy. Such antibodies can be obtained by screening a phage display antibody library prepared from an animal immunized with contortrostatin.

[0056] Antagonism of integrins is an important approach for anti-angiogenic and anti-metastatic therapy (46). Contortrostatin blocks the function of multiple integrins, which play critical roles in cancer cell adhesion, migration, invasion, and angiogenesis. It thus has broad spectrum of inhibitory effects towards these pathophysiological procedures, and has potent antineoplastic activity.

[0057] Although not wishing to be bound by any theory, it is believed that the anti-idiotypic antibody to contortrostatin acts as a surrogate antigen because it mimics the structure and function of contortrostatin. The resulting human anti-idiotypic antibodies bind to integrins αIIbβ3, αvβ3 αvβ5, to α5β1. The present
approach can be used to block multiple critical integrins in angiogenic vascular endothelial cells and metastatic cancer cells. In addition, it can block the release of angiogenic factors, such as PDGF and VEGF from aggregating platelets, and disrupt platelet-mediated interactions between metastatic cancer cells and vascular endothelium. Because of the wide spectrum of integrin antagonism, the possibility for the angiogenic vascular endothelial and metastatic cancer cells to circumvent the integrin blockage is minimized.

[0058] The anti-idiotypic antibodies of the present invention find use in treating angiogenic disorders. Such disorders involve abnormal neovascularization where growth of new blood vessels is the cause of, or contributes to, the pathology associated with a disease. In these situations, inhibition of angiogenesis will reduce the deleterious effects of the disease. Other therapeutic targets for the compounds of the instant invention are eye diseases characterized by neovascularization. Such eye diseases include corneal neovascular disorders, such as corneal transplantation, herpetic keratitis, luetic keratitis, pterygium and neovascular pannus associated with contact lens use. Additional eye diseases include age-related macular degeneration, presumed ocular histoplasmosis, retinopathy of prematurity, neovascular glaucoma, and the like.

[0059] Where the growth of new blood vessels is required to support growth of a deleterious tissue, inhibition of angiogenesis will reduce the blood supply to the tissue and thereby contribute to reduction in tissue mass based on blood supply requirements. Cancer is an example where neovascularization is a continual requirement in order for the tumor to grow and establish tumor metastases. Thus, the ability of anti-idiotypic antibodies of disintegrin to target integrins can be used to inhibit tumor tissue angiogenesis, thereby preventing tumor metastasis and tumor growth.
Accordingly the invention provides methods of treating angiogenesis, cancer, bone metabolism, or inflammation by administering an effective amount of anti-idiotypic antibodies of the invention. The antibodies also may be used in the preparation of a medicament for treatment of these diseases or conditions. In addition to their use as inhibitors of integrin binding, anti-idiotypic antibodies of the invention may be conjugated with a drug or therapeutic agent.

In addition to therapeutic applications, anti-idiotypic antibodies of disintegrin (e.g., of contortrostatin) may be used for imaging cells or tissues such as tumor cells as is well known in the art. Accordingly, provided is a method of imaging cells or tissue in an individual wherein said cells or tissue expresses an integrin which is the target of a disintegrin such as contortrostatin, the method comprising administering to the individual an effective amount of an anti-idiotypic antibody of disintegrin linked to a suitable radioisotope or detectable label.

An anti-idiotypic antibody of the present invention can be administered as a pharmaceutical composition wherein the invention compound is optionally formulated with a pharmaceutically acceptable carrier. Accordingly, the invention compounds may be used in the manufacture of a medicament. Pharmaceutical compositions of the invention compounds may be formulated as solutions or lyophilized powders for parenteral administration. Powders may be reconstituted by addition of a suitable diluent or other pharmaceutically acceptable carrier prior to use. Powders also may be sprayed in dry form. The liquid formulation may be a buffered, isotonic, aqueous solution. Examples of suitable diluents are normal isotonic saline solution, standard 5% dextrose in water or buffered sodium or ammonium acetate solution. Such formulation is especially suitable for parenteral administration, but may also be used for oral administration or contained in a metered dose inhaler or nebulizer for insufflation. It may be desirable to add excipients such as polyvinylpyrrolidone,
gelatin, hydroxy cellulose, acacia, polyethylene glycol, mannitol, sodium chloride or sodium citrate.

[0063] Alternately, anti-idiotypic antibodies may be encapsulated, tableted or prepared as an emulsion or syrup for oral administration. Pharmaceutically acceptable solid or liquid carriers may be added to enhance or stabilize the composition, or to facilitate preparation of the composition. Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, magnesium stearate or stearic acid, talc, pectin, acacia, agar or gelatin. Liquid carriers include syrup, peanut oil, olive oil, saline and water. The carrier may also include a sustained release material such as glycercyl monostearate or glycercyl distearate, alone or with a wax. The amount of solid carrier varies but, preferably, will be between about 20 mg to about 1 g per dosage unit. The pharmaceutical preparations are made following the conventional techniques of pharmacy involving milling, mixing, granulating, and compressing, when necessary, for tablet forms; or milling, mixing and filling for hard gelatin capsule forms. When a liquid carrier is used, the preparation will be in the form of syrup, elixir, emulsion or an aqueous or non-aqueous suspension. For rectal administration, the invention compounds may be combined with excipients such as cocoa butter, glycerin, gelatin or polyethylene glycols and molded into a suppository.

[0064] Anti-idiotypic antibodies of the invention may be formulated to include other medically useful drugs or biological agents. The compounds also may be administered in conjunction with the administration of other drugs or biological agents useful for the disease or condition that the invention compounds are directed (see e.g., U.S. Pat. No. 6,413,955 for active ingredients useful for osteoporosis).

[0065] As employed herein, the phrase “an effective amount,” refers to a dose sufficient to provide concentrations high enough to impart a beneficial
effect on the recipient thereof. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated, the severity of the disorder, the activity of the specific compound, the route of administration, the rate of clearance of the compound, the duration of treatment, the drugs used in combination or coincident with the compound, the age, body weight, sex, diet and general health of the subject, and like factors well known in the medical arts and sciences. Various general considerations taken into account in determining the “therapeutically effective amount” are known to those of skill in the art and are described, e.g., in Gilman et al., eds., Goodman And Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1990. Dosage levels typically fall in the range of about 0.001 up to 100 mg/kg/day; with levels in the range of about 0.05 up to 10 mg/kg/day are generally applicable. A compound can be administered parenterally, such as intravascularly, intravenously, intrarterially, intramuscularly, subcutaneously, or the like. Administration can also be orally, nasally, rectally, transdermally or inhalationally via an aerosol. The composition may be administered as a bolus, or slowly infused.

[0066] The invention will be described in greater detail by reference to the following non-limiting examples.
EXAMPLES

[0067] Example 1. Generation and characterization of monoclonal antibody against contortrostatin:

[0068] Preparation of antigen—coupling contortrostatin with Agarose 4B: 0.2 grams of cyanogen-bromide activated agarose 4B (purchased from Sigma) was swollen and washed with cold HCl (1mM) and distilled water. After washing, the beads were mixed immediately with 0.6 mg of HPLC purified contortrostatin dissolved in 0.5 ml of 0.1 M sodium carbonate buffer (pH 8.5), and incubated in room temperature for 2 hours. The free primary amine groups were then blocked with 0.2 M glycine (pH 8.0). The contortrostatin-agarose conjugates were then washed with phosphate buffer saline (PBS, pH 7.2) and stored at -20°C until use.

[0069] Immunization of mice with contortrostatin-agarose conjugates: Balb/c mice six-week-old, were injected intraperitoneally with 50 μg of contortrostatin coupled to Agarose 4B beads after emulsification in equal volume of complete Freund’s adjuvant. The mice were boosted by intraperitoneal injection of the same amount of antigen (emulsified in incomplete Freund’s adjuvant) 14 days later. Two more boost injections were given once every two weeks thereafter. The final boost was performed via i.v. route with the same amount of antigen but not coupled to the beads. The immunized animals were sacrificed, and the fusion of hybridoma cells was conducted 3 days after the final boost.

[0070] Cell fusion and generation of hybridomas: The cell fusion was performed on the 4th day after the final immunization. The immunized animals were euthanized and the spleens were removed and washed with phosphate buffer saline (PBS) which contained 200 U/ml of Penicillin and 200 μg/ml of Streptomycin. The spleen cells were removed by slow injection of sterile PBS in
multiple sites, while squeezing the spleen with forceps. Both the spleen and the myeloma cells were washed and centrifuged at 400 g for 10 min at 4°C, resuspended with 50 ml of washing media, and mixed at the ratio of 1:1. For cell fusion, one milliliter of sterile polyethylene glycol -1500 (PEG-1500) pre-warmed in 37°C was added drop-wise within 45 seconds, followed by constant swirling over the next minute. Fifteen milliliters of serum-free Dulbecco’s Modified Eagle Medium (DMEM) was added drop-wise for 15 min, pausing for 10 sec every 2 min, while the tube was swirled gently. After centrifugation at 140 g for 10 min at 4°C, the pellet was resuspended in 50 ml of 20% fetal bovine serum (FBS) complete media, with 50 units/ml of mouse recombinant interleukin-6 (IL-6). The fused cells were plated into 96-well plates with 0.1 ml per well, and incubated at 37°C with 5% CO2. After an overnight incubation, the medium was replaced with hypoxanthine, aminopterin, and thymidine (HAT) medium, and changed every two days. Hybridoma cells became visible in about 10 days after fusion. The acid hybridoma supernatants were collected, and screened.

[0071] Selection of hybridomas producing antibodies reacting with contortrostatin using Enzyme Linked Immunoassay (ELISA): Contortrostatin (0.1 μg in 100 μl of PBS) was immobilized in wells of a 96-well ELISA plate. The plate was incubated at 4°C overnight. The plate was then rinsed three times with PBS to wash away the unbound protein. Nonspecific binding was blocked by treating wells with 1% bovine serum albumin (BSA) in PBS at room temperature for an hour. One hundred microliters of the hybridoma cell culture supernatants at 1:1 dilution was added into the wells, and incubated in room temperature for one hour. After aspiration of the supernatants, the wells were rinsed three times with PBS with 0.2% of Tween-20 (washing buffer). One hundred microliters of goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson Immunoresearch laboratory) at 1:1,000 dilution was added into the wells, and incubated in room temperature for an hour. The bound
antibody was detected by using o-phenylenediamine dihydrochloride (OPD) substrate and determined with plate reader at 492 nm wavelength. Four hybridomas, designated α-CN 2, 8, 10 and 11, were found to react with contortrostatin. Among them α-CN10 gave the highest signal, and was selected for further study. A titer curve of α-CN10 supernatant determined by ELISA is demonstrated in Figure 1. The positive clones then were colonized twice using the limiting dilution methods in 96-well plates.

[0072] **Antibody purification:** Large scale preparation of the antibody was performed by proliferating the hybridoma cells in a bio-reactor followed by purification with protein A - affinity chromatography and ion exchange chromatography. Two liters of α-CN10 hybridoma supernatant were harvested following cell culture and loaded on a protein A affinity column at a flow rate of 2 ml/minute using Bio-Rad Bio-Logic low pressure chromatography system. Un-bound protein was removed by washing the column with 150 ml of PBS (pH 7.4) at a flow rate of 4 ml/minute. The antibody was eluted by 0.1 M citrate buffer (pH 3.0). The elute collected into a sterile bottle was diluted in 20 mM sodium acetate buffer (1:8 v/v) and adjusted to pH 5.5 ± 0.2. The partially purified antibody was subsequently loaded onto a SP-Sepharose column at a flow rate of 4 ml/minute, followed by washing with 20 mM sodium phosphate buffer (pH 6.0). The antibody was eluted with PBS (20 mM sodium phosphate and 150 mM sodium chloride, pH 7.4). The antibody was quantitated by OD at 280 nm, and the purity of the antibody was confirmed by SDS-PAGE (Figure 2).

[0073] **Western Blot of α-CN10:** Ten micrograms of purified native contortrostatin was dissolved in 20 μl of PBS. Five microliters of 5X sample loading buffer containing 10 mM Dithiothreitol (DTT), and 2% b-Mercaptoethanol was added into each sample. The mixture was heated in boiling water bath for 5 min, and loaded in a 4-20% gradient Acrylamide mini gel. The electrophoresis was performed at 110 V for 1 hour. The resolved protein was transferred to a nitrocellulose membrane according to standard
procedure. The membrane was blocked with 1% BSA at room temperature for an hour. The membrane was then incubated in 10 µg/ml of α-CN10 dissolved in 1% BSA/PBS in room temperature for one hour. The membrane was washed three times in Tris-buffered saline (TBS) buffer. Each washing took 15 min. Then the membrane was incubated with goat anti-mouse IgG conjugated with alkaline phosphatase (Jackson Immunoresearch laboratory) using 1:1,000 dilution at room temperature for one hour. Eventually, the bound antibodies were detected by using nitroblue tetrazolium chloride (NTB) and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) according to standard protocol. A band about 6.8 kDa, which represents a subunit of contortrostatin was detected (Figure 3).

α-CN10 neutralizes anti-aggregation activity of contortrostatin in a dose-dependent manner: Contortrostatin is an antagonist of the platelet fibrinogen receptor (GP IIb/IIIa or αIIbβ3), and thus inhibits fibrinogen-dependent aggregation (6). In this assay, it was demonstrated that the anti-aggregation activity of contortrostatin can be neutralized by α-CN10.

Human blood was obtained from healthy volunteers who had not taken any drugs or alcoholic beverages during the previous week. The blood was anticoagulated with 1/10 volume of 3.8% trisodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 180 g for 20 min at room temperature. Platelet aggregation was studied photometrically with 0.5 ml citrated PRP using an aggregometer and siliconized test tubes. During the test, the tube was kept at 37°C with stirring at 900 rpm. The light transmission was continuously recorded. The tentative inhibitory components were added to PRP in the aggregometer 1 min before the addition of 10 µM ADP. After addition of ADP, the incubation was continued for 3 min. Contortrostatin completely inhibits ADP-induced platelet aggregation at 100 nM. α-CN10 is able to neutralize the inhibitory activity of contortrostatin in a dose-dependent manner (Figure 4).
result suggests that α-CN10 is a functional inhibitor of contortrostatin. It disrupts interaction between contortrostatin and αIIbβ3.

α-CN10 neutralizes anti-adhesion activity of contortrostatin: MDA-MB-435, an estrogen-receptor negative cell line established from cancer cells isolated from the pleural effusion of a woman with metastatic, ductal adenocarcinoma of the breast (67), was obtained from Dr. J. Price (MD Anderson Cancer Center, University of Texas, Houston, TX). This cell line expressed integrin αvβ3 as its major vitronectin receptor (22). Low level of αvβ5 is also expressed. Both αv integrins are involved in adhesion of the cells to vitronectin (28). Contortrostatin inhibits adhesion of the cells to plates coated with vitronectin (9). Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum was employed for culture of MDA-MB-435 cells. Cells were incubated at 37°C with 5% CO2. Vitronectin purchased from Becton Dickinson (Bedford, MA) was dissolved in PBS at concentration of 10 μg/ml, and was immobilized on Immulon-II 96-well microtiter plate (Dynex Technologies, Inc., Chantilly, VA) by incubating 100 μl of vitronectin solution in the wells overnight at 4°C. The amount of vitronectin was shown to be able to support more than 90% cell adhesion. Excess proteins were washed away with PBS. Unbound sites were blocked with 1% bovine serum albumin (BSA) in PBS, and the plates were washed with PBS. One hundred μl of MDA-MB-435 cells (7.5 x 105/ml) were seeded in the coated microtiter plate wells. The cells were treated with various reagents by incubation at 25°C for 20 minutes prior to seeding. Seeded cells were allowed to adhere for one hour at 37°C. After unbound cells were washed away, the extent of cells adhesion was determined by CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). The tests were performed in triplicate. It has been demonstrated previously (9) that contortrostatin inhibits adhesion of MDA-MB-435 cells to immobilized vitronectin in a dose-dependent manner. Maximum inhibition was achieved at about 10 nM. With the presence of 10 μM α-CN10, the dose-
response relationship curve was shifted rightward. The concentration of contortrostatin to achieve complete inhibition of adhesion increases from 10 nM to 1000 nM (Figure 5). This is a strong indication that α-CN10 inhibits contortrostatin by blocking its functional domain mediating disintegrin/integrin interactions. The anti-adhesion activity of contortrostatin is reversed by α-CN10. Ten nM of contortrostatin was found to completely inhibit adhesion of MDA-MB-435 cells to vitronectin. The inhibitory effect of contortrostatin was reduced with increasing concentrations of α-CN10, as shown by the increased number of adhered cells (Figure 6). It is concluded that α-CN10 inhibits the anti-adhesion activity of contortrostatin by disrupting interaction between contortrostatin and αvβ3, and perhaps αvβ5 well as.

[0077] α-CN10 inhibits binding of contortrostatin to immobilized αvβ5: This assay evaluated whether α-CN10 blocks the interaction between contortrostatin and αvβ5. Purified αvβ5 was purchased from Chemicon Corp. 100 ng of soluble αvβ5 was immobilized on wells of a 96-well plate at 4°C overnight. It is known that contortrostatin binds to the plate coated with αvβ5 dose-dependently (10). Contortrostatin at 0.5 nM was mixed with increasing concentration of α-CN10, and allowed to bind to the coated plate at room temperature for one hour. Bound contortrostatin was detected with a 1:1,000 dilution of rabbit antiserum against contortrostatin. Goat anti-rabbit antibody conjugated with alkaline phosphatase was used as a secondary antibody. The secondary antibody was absorbed with mouse serum, and was shown not to cross react with α-CN10. The bound antibodies were quantitated by adding disodium p-nitrophenyl phosphate (pNPP) and determining the absorbance at 405 nm. The result demonstrated that with the increasing concentration of α-CN10, the bound contortrostatin to αvβ5 is reduced dose-dependently, whereas there is no significant changes in the level of contortrostatin bound to αvβ3 (Figure 7), suggesting that α-CN10 blocks interaction of contortrostatin with αvβ5.
Example 2. Generation of anti-idiotype antibody against contortrostatin

Mice were immunized with anti-α-CN10 antibody using a similar approach to immunizing with contortrostatin. Mice were injected intraperitoneally with 50 µg of α-CN10, which had been emulsified in equal volume of complete Freund’s adjuvant. The mice were boosted by an intraperitoneal injection of the same amount of antigen emulsified in incomplete Freund’s adjuvant 14 days later. Two additional boost injections were performed at 2-week intervals. Fusion was conducted as described above.

Generation of F(ab)2 fragment of α-CN10: F(ab)’2 fragments was generated using an immobilized ficin column (Pierce) according to the protocol provided by the manufacturer. Briefly, 5 mg of α-CN10 was diluted in digestion buffer containing 0.2 mg/ml of Cysteine and applied to an immobilized ficin column for 20 hrs at 37°C. The solution that containing F(ab)’2 fragment was then eluted from ficin column and passed through a protein A column to remove Fc fragment and undigested antibody. The unbound F(ab)’2 fraction was collected and concentrated to 1 mg/ml. The antibody recovery was determined using absorbance at 280 nm and the F(ab)’2 was shown to be homogeneous as determined by gel electrophoresis (Figure 2).

Generation and screening of anti-α-CN10 idiotype hybridoma: Three days after the final immunization, the immunoreactivity of the mouse serum to α-CN10 was tested by means of ELISA. The ELISA protocol described above was followed. The F(ab)’2 fragment of α-CN10 was immobilized onto 96-well plate at 0.1 µg/well. Serum at various dilutions, ranging from 1:100 to 1:1,000,000 were tested. After incubation and washing, the bound antibodies were detected with goat-antimouse IgG Fc’ conjugated with alkaline

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phosphatase. It was found that the IgG in the serum binds to the plate coated
with F(ab')2 fragment of α-CN10 in a concentration-dependent manner, whereas
serum from a mouse immunized with irrelevant antigen did not show such a
binding pattern. This result strongly indicated that antibodies to α-CN10 had
been generated by the immunized animal (Figure 8).

[0082] The hybridomas were screened by their immunoreactivity to α-CN10
by ELISA. The protocol followed was identical with that previously described.
The F(ab')2 fragment of α-CN10 was immobilized onto 96-well plate at 0.1
μg/well overnight at 4°C. Supernatants from the hybridomas at 1:1 dilution
were added to the wells. After incubation and washing, the bound antibodies
were detected with goat-antimouse IgG Fc' conjugated with alkaline
phosphatase. A total of 72 colonies were screened. Samples with absorbance
(405 nm) higher than the mean absorbance plus 3 times that of the standard
deviation (Mean + 3 X SD) were regarded as positive. Based on these criteria,
25 out of 72 colonies were deemed positive and subjected to further testing.

[0083] Selection of hybridoma against purified integrin αvβ3, and αvβ5. The
vitronectin receptors αvβ3 and αvβ5 were employed for this assay. Purified αvβ3
and αvβ5 (both were purchased from Chemicon, Crop.) were immobilized in a
96-well plate with 0.1 μg/well in coating buffer (20 mM Tris, pH 7.4, 150 mM
NaCl, 1 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2). ELISA protocols identical
with the above assay were followed. Supernatants which showed reactivity to
α-CN10 were diluted 1:1 with PBS, and added into wells coated with αvβ3 and
αvβ5. Successful immobilization of the integrins was confirmed by a parallel
ELISA probed with anti-αvβ3 (7E3) and anti-αvβ5 (P1F6) mAbs. Bound
antibodies to the integrin-coated plate were detected with goat anti-mouse IgG
conjugated with alkaline phosphatase. The criteria for positive function were set
by absorbance (405 nm) higher than the mean absorbance plus 3 times the
standard deviation. Three out of 25 hybridomas screened demonstrated
reactivity with both integrins. Among them, clone number 40 produced the
highest level of signal. This hybridoma was selected for subcloning and was
designated Ald40-8. The procedures for hybridoma proliferation, and
immunoglobulin purification are identical with those used for α-CN10. Purified
Ald40-8 was analyzed with SDS-PAGE (Figure 9).

[0084] **Isotype determination.** Antibody isotype determination was performed
using IsoStrip kit (purchased from Boehringer). Briefly, 150 µl of freshly diluted
(1:100) supernatant of hybridoma was added into development tube, incubated
at room temperature for 30 seconds and agitated briefly so that the color latex
is completed resuspended. Insert IsoStrip into the development tube for 10 min.
The class or subclass of heavy chain and light chain of monoclonal antibody was
determined by the position of color bands on the IsoStrip. It is identified that
immunoglobulin Ald40-8 is a mouse IgG1.

[0085] **Example 3: Characterization of the anti-idiotypic antibody of contortrostatin, Ald40-8**

[0086] **Specificity and apparent affinity of Ald40-8 to integrins:** Saturation
binding curve of Ald40-8 on αvβ3, αvβ5, and αllbβ3 was determined by a
modified ELISA method. 100 ng of soluble αvβ3, αvβ5 (both purchased from
Chemicon) and αllbβ3 (kindly provided by Dr. Marian Nakada, Centecor) was
immobilized on a 96-well plate at 4°C overnight in coating buffer. Ald40-8 at
various concentrations was allowed to bind to the coated plate at room
temperature for one hour in the binding buffer containing 50 mM Tris, pH8.0,
200 mM NaCl, 0.05 mM CaCl2, 0.2 mM MnCl2, 0.05% Tween 20 and 1%
BSA. Goat anti-mouse antibody conjugated with alkaline phosphatase was used
as a secondary antibody. The bound antibodies were quantitated by adding
pNPP, and determining the absorbance at 405 nm. Background was determined
by the binding of an irrelevant IgG, at the same concentrations as Ald40-8, to
the integrins. Specific binding is obtained by subtracting the background from the total binding. Analysis of each concentration of ligand was performed in duplicate.

[0087] The result illustrates that Ald40-8 binds to all three integrins in a concentration-dependent and saturable manner (Figure 10). The magnitude of plateau phase to αvβ3 and αllbβ3 is higher than that to αvβ5. This is similar to the apparent binding pattern of native contortrostatin to the integrins (68). However, the concentrations required to reach the plateau for Ald40-8 is much higher than that of native contortrostatin. Quantitatively, the concentration of contortrostatin to achieve 50% of the total binding to αvβ3 is 0.5 nM, and to αvβ5 is 0.8 nM. In contrast, about 2000 nM of Ald40-8 is required to reach 50% of the maximum binding to αvβ3, whereas 1500 nM is required to achieve 50% of binding to αvβ5. Although the bound disintegrin and IgGs were probed with different secondary antibodies, which in turn introduced different kinetics of reaction, nonetheless, it is concluded that the affinity of the anti-idiotype antibody to the integrins is an order of magnitude lower than that of contortrostatin.

[0088] Contortrostatin competes with Ald40-8 on binding to αvβ5, but not to αvβ3. To confirm that Ald40-8 binds to the same epitope on integrins as contortrostatin, a competition binding assay with the antibody and contortrostatin was performed using modified ELISA method on immobilized integrins αvβ5 and αvβ3. Similarly to the previous experiment, 100 ng of soluble αvβ5, αvβ3 and αllbβ3 (purchased from Chemicon) were immobilized on a 96-well plate at 4°C overnight in the coating buffer. Ald40-8 at fixed concentration of 1 μM was mixed with contortrostatin at various concentrations in the binding buffer, and was allowed to bind to the coated plate at room temperature for one hour. Goat anti-rabbit antibody conjugated with alkaline phosphatase was used as a secondary antibody to detect the bound Ald40-8. Analysis of each concentration of contortrostatin was performed in duplicate.
The results demonstrated that contortrostatin inhibits binding of Ald40-8 to αβ5 in a dose-dependent manner, whereas there is no significant inhibition of the binding of Ald40-8 to αβ3 or α1β3. The result suggests that Ald40-8 competes with contortrostatin to bind to the same epitope on integrin αβ5, but may not bind to the same site on αβ3 and α1β3 (Figure 11). The fact that Ald40-8 does not compete with contortrostatin to bind to αβ3 is consistent with the observation that α-CN10 only blocks binding of contortrostatin to αβ5, but not to αβ3 (section 8, Figure 7). Although the interaction of contortrostatin to both αv and β3 integrins is mediated by RGD domain, the functional blocking sequence in contortrostatin for each integrin may be slightly different. It is possible that the idiotypic domain of α-CN10 is sufficient to block interaction of contortrostatin to αβ5, but is not optimized for functional blockage of αβ3 binding of the disintegrin. It is also possible that neutralization of contortrostatin antiaggregation activity by α-CN10 is due to steric hindrance. As a faithful internal image of α-CN10, Ald40-8 precisely competes with contortrostatin for the identical binding site on αβ5, but fails to do so in αβ3 and α1β3, although structural similarity of the anti-idiotype allows it to bind to the latter integrins.

Ald40-8 binds to αβ3 and αβ5 integrins expressed by cells: Human embryonic kidney carcinoma 293 cells expressing exogenous αβ3 (293-β3) and αβ5 (293-β5) cells (69) (kindly provided by Dr. Jeffrey Smith, the Burnham Institute, La Jolla, CA) were harvested from subconfluent flasks, and were resuspended in 1% BSA/PBS at density of 1 x10^7 /ml and aliquoted 100 μl per tube. Cells were incubated at room temperature with antibodies at the concentration of 100 μg/ml for 30 minutes. The cells were washed twice and resuspended in 1% BSA/PBS. Goat anti-mouse IgG conjugated with FITC (Jackson ImmunoResearch, West Grove, PA) was added to the suspension at a final dilution of 1:200. After 30 minutes incubation at room temperature in darkness, unbound FITC-conjugated IgG was washed off, and the fluorescent
intensity of the cells was analyzed using flow cytometry (FACScan, Becton Dickinson, Bedford, MA). The background was defined using the FITC-labeled secondary antibody alone. The result demonstrated that Ald40-8 (100 \mu g/ml) binds to both cell lines which express \alpha \nu \beta 3 and \alpha \nu \beta 5, respectively (Figure 12).

Ald40-8 inhibits adhesion of cells MDA-MB-435 to vitronectin:
Vitronectin (Becton Dickinson) at concentration of 10 \mu g/ml was immobilized on Immulon-II 96-well microtiter plate by incubating 100 \mu l of vitronectin solution in the wells overnight at 4\degree C. Excess proteins were washed away with PBS. Unbound sites were blocked with 1% BSA in PBS, and the plates were washed with PBS. One hundred \mu l of MDA-MB-435 cells (7.5 x 105/ml) were seeded in the coated microtiter plate wells. The cells were pre-treated with various concentrations of Ald40-8, or contortrostatin, by incubation at 25\degree C for 20 minutes prior to seeding. Seeded cells were allowed to adhere for one hour at 37\degree C. After unbound cells were washed away, the extent of cells adhesion was determined by CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). The tests were performed in triplicate. It is found that both contortrostatin and Ald40-8 inhibit adhesion of MDA-MB-435 cells to vitronectin in a dose-dependent manner. However, the concentration of Ald40-8 to achieve the maximum inhibition is, many times higher than that of native contortrostatin (Figure 13).

With the above example, it was demonstrated that it is feasible to generate an anti-idiotype antibody to disintegrin, contortrostatin in this case, as its functional surrogate. The anti-idiotype antibody described herein not only binds to both the purified and cell expressed receptors of contortrostatin (including \alpha \nu \beta 3, \alpha \nu \beta 5, and \alpha l l \beta 3), but also competes with the antigen to bind to one of its receptors \alpha \nu \beta 5. The mimicry of function through the anti-idiotype antibody is demonstrated by the anti-adhesion activity of Ald40-8 on adhesion
of MDA-MB-435 cells to vitronectin. This is one of the major mechanisms of action for contortrostatin.

[0093] Ald40-8 is a partial image of contortrostatin, as shown by the fact that it only competes with the disintegrin on αvβ5, but not the rest of the integrins to which both Ald40-8 and contortrostatin bind. This results from the selectivity of the first generation antibody, α-CN10. It was found that α-CN10 blocks some of contortrostatin’s function, such as its antiaggregation and anti-adhesion effect. α-CN10 partially blocks the function of contortrostatin, with αvβ5, but not to αvβ3. This subtle difference in binding specificity is reflected in the second generation antibody, Ald40-8, as it solely competes with contortrostatin to bind to αvβ5.

[0094] It is noticed that the affinity of the antibody to the integrins is much lower than that of the native contortrostatin, as illustrated by the saturation binding curve and the potency in anti-adhesion assay. However, affinity maturization can be achieved with several methods, such as altering the immunization protocol and mutation of CDR sequences through antibody engineering technology. Given the right antagonist spectrum, a high affinity clone should be able to be selected from a large candidate pool of hybridomas or phage display library.

[0095] Example 4: Preparation and selection of anti-idiotypic antibodies to contortrostatin using an antibody phage display library

[0096] Immunization of Balb/c mice with native CN and α-CN 10 monoclonal antibody and generation of cDNA from spleen cells: Five Balb/c immunocompetent mice (6 to 8 week old, male or female) were immunized with native purified CN, and another five with α-CN10. Both antigens (CN and α-CN 10) were first conjugated to cyanogen-bromide activated Sepharose 4B beads
(preparation of antigen described above). The subcutaneous route was used for the first two round of immunization (days 1 and 14). The antigen dosage for the subcutaneous administrations was 200 μg/mouse of conjugated CN, and 400 μg/mouse of conjugated α-CN10. Freund’s complete adjuvant was used in the first subcutaneous administration followed by Freund’s incomplete adjuvant in the second subcutaneous administration. A final boost by intravenous (tail vein) injection was administered seven days following the second subcutaneous administration. The intravenous boost for CN and CN10 immunized animals was free native CN and α-CN10 monoclonal antibody, respectively, without adjuvants, at a dosage of 10 μg/mouse CN, and 400 μg/mouse α-CN10. This dosage avoids toxicity, especially in the case of CN.

[0097] The serum Ab titer was monitored by ELISA against native CN and against integrins. When the serum titers of the immunized animals reached a plateau (usually 4 days after the intravenous boost), the spleens were harvested, immediately sliced and dipped in an RNA later a Quigen solution RNAlater Stabilization Reagent®, which contains powerful RNA inhibitors (Qiagen RNeasy® Protect Kit). The slices were then homogenized using a hand-held homogenizer and total RNA extracted using a Qiagen RNA extraction protocol. The total quantity of pure RNA extracted from spleens of mice immunized with CN was 340 μg (with a purity of 2.0 based of OD260/OD280 ratio) and 540 μg for the α-CN 10 monoclonal antibody immunized mice. (with a purity of 1.77 based of OD260/OD280 ratio). Finally, first-strand cDNA was synthesized from total the RNA pool using an Invitrogen SuperScript™ first-strand synthesis system for RT-PCR kit (an oligo (dT) primer and reverse transcriptase).

[0098] **Generation of Mouse scFv cDNA library:** The individual rearranged heavy- and light-chain variable regions of cDNA repertoire from the mice immunized with CN (or with the anti-CN antibody αCN10) were amplified separately and further assembled through an overlap extension PCR step in the final scFv library that was used for cloning into pCANTAB 5E™ phagemid
vector. The design of the scFv from the amino terminus was \( V_H \)-
linker(Gly\(_4\)Ser\(_3\)) - \( V_L \). Briefly, in the 1\(^{st} \) round of PCR, the rearranged light- and
heavy-chain variable regions were selected using a set degenerate primers
described in the literature (70) and modified as noted herein. The \( V_H \) forward
primers were modified to include a Not I cloning site at the 5' end of the primer
(table 1; bold lettering) while the \( V_H \) back primers include at the 5' end a portion
of the sequence encoding the (Gly\(_4\)Ser\(_3\)) linker (table 2; small case lettering). \( V_L \)
back primers (sense) were designed to encode at the 3' end a FLAG which is a
tag sequence that renders the displayed scFv fragment immunologically
recognizable by an anti-FLAG monoclonal antibody (table 3; small case lettering
underlined) while the \( V_L \) forward primers were modified to include at the 5' end
the sequence encoding the (Gly\(_4\)Ser\(_3\)) linker (table 4; small case lettering).

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Table 2. V<sub>H</sub> back primers

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**Table 3.** V\_\textsubscript{L} back primers

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Table 4. V_{L} forward primers

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<td>ggagccgcgcgcgcgcc(agaaccaccaccacc)\textsubscript{2}ACCT AGGACAGTCAGTTTG</td>
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</table>

PCR conditions for the first round of amplifications: 3 min denaturation at 92°C; add 2 U of Taq DNA polymerase; seven cycles of 1 min at 92°C, 30 s at 63°C, 50 s at 58°C, 1 min at 72°C; 23 cycles of 1 min at 92°C, 30 s at 63°C, 1 min at 72°C; and final elongation for 10 min at 72°C. The amplified VL and VH products were purified and further concentrated in Microcon DNA columns produced by Amicon. The amplified VL and VH products were fused by overlap extension PCR using the sense and reverse extension primers, sback and nfor (table 5).
Table 5.  sFv Overlap extension primers

<table>
<thead>
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<th>Primer ID</th>
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The back overlap extension primer (sback) introduced a sequence tail containing an Sfi I restriction site (bold small case lettering). Overlap extension PCR conditions: denaturation for 3 min at 92°C; 2 cycles of 1 min at 92°C, 30 s at 63°C, 50 s at 58°C, 1 min at 72°C; add primers scback and nfor; 5 cycles of 1 min at 92°C, 30 s at 63°C, 50 s at 58°C, 1 min at 72°C; 23 cycles of 1 min at 92°C, 30 s at 63°C, 1 min at 72°C; and elongation for 10 min at 72°C. Both VL and VH repertoires and the overlap extension scFv library analyzed by agarose gel (1.5%) electrophoresis showed the expected size (approximately 750 bases) of the fused product (Figure 14). Further details for PCR amplification and amplified product purification were described previously (70, 71).

Ligation of library and transformation of TG1 E. coli cells: The scFv DNA fragments were ligated into the pCANTAB 5E™ phagemid vector after digesting the fragments with Sfi I (4 hour digest) followed by Not I (4 hour digest). Ligation was achieved (1 hour) following the instructions from the Recombinant Phage Antibody System/Expression Module, Amersham Biosciences. Analysis by agarose gel electrophoresis showed the expected size for the ligated vector with sFv insert (pCANTAB/scFv). Electrocompetent TG1 E. coli cells were transformed with pCANTAB/scFv library by electroporation with a BioRad electrorporation system. The transformed cells were grown for 1 hr at 37°C with shaking at 250 rpm, the cells were plated SOBAG plates and incubated at 37°C for 16-24 hr. The library was titrated by plating 1:10,000, 1:100,000 and 1:1,000,000 of the total volume of the library (10 ml) on different SOBAG plates, revealing a complexity of more than 10⁷ different
clones. This initial library was further grown in 2x YT medium to yield a final scFv library of \(10^{11}-10^{12}\) total clones. The final library was then co-transformed with helper phage M13K07 (Amersham Biosciences) and incubated overnight in 2x YT-AK medium (containing 100 µg/ml Ampicillin and 50 µg/ml Kanamycin) at 37°C with shaking at 250 rpm. The M13K07 helper phage was used to rescue the phagemid and its scFv insert gene from the transformed E. coli TG1 cells. The phage particles rescued were then concentrated from the bacterial media by precipitation with 4% PEG-8000 and 3% NaCl, titered and used for biopanning.

[00105] **Screening libraries by biopanning on CN-coated plate:** 50 µl of freshly prepared phage library (in the estimated range of 1012 phage) is added in the wells of ELISA plates pre-coated with native CN, and incubated at 37°C for 2 hr. The plates are washed three times with 0.5% Tween in PBS, and the bound phage is eluted with 50 µl of 100 nM glycine-HCl (pH 2.2), and immediately neutralized by 3µl of 2M Tris-base. The selected phage in the eluant is amplified by re-transformation of TG1 E.coli with co-transformation of the helper phage (71). Three to six rounds of panning are performed, and the enrichment of the specific binders in the selected phage pool is monitored by phage ELISA in a plate coated with CN, and detected with anti-M13 antiserum (Stratagene) after each round of panning. The eluant from the last round of panning is spread out in agarose plate, and single phage plaques are picked up and amplified in TG1 E.coli. Small-scale preparation of PEG-precipitated scFv-displaying phage is analyzed for specificity by phage ELISA, and subject to functional tests.

[00106] **Selection for scFv-displaying phage which are function blockers:** CN disrupts the function of integrins by inhibition of fibrinogen-dependent platelet aggregation, and cell adhesion to vitronectin (Vn) and fibronectin (Fn). A function blocker of CN presented by the phage can reverse the inhibitory effects of CN in the following assays.
(a) **Platelet aggregation assay:** αIIbβ3 functions as a fibrinogen receptor on the surface of blood platelets, and mediates aggregation in response to various stimulators, such as ADP. Human blood is obtained from healthy volunteers. Platelet-rich plasma (PRP) is obtained by centrifugation at 180 x g for 20 min at room temperature. Platelet aggregation is studied photometrically with PRP using an aggregometer (72). The agents to be tested are added to PRP 1 min before the addition of 10 μM ADP. The CN concentration for complete inhibition of ADP-induced platelet aggregation is determined experimentally. Various concentrations of the scFv-displaying phage obtained from Experiment 3 are mixed with CN to test their effect on aggregation. Blocking of the inhibitory action of CN on platelet aggregation in the presence of the phage implies that the mAb blocks the CN binding site for αIIbβ3.

(b) **Adhesion assay:** Integrins αvβ3 and αvβ5 are Vn receptors which mediate adhesion of cells to Vn; αvβ1 is a Fn receptor that mediates cell attachment to Fn. Human embryo kidney carcinoma 293 cells transfected either with exogenous αvβ3 or αvβ5 (293-β3 and 293-β5 cells, respectively, provided by Dr. Jeffery Smith, The Burnham Institute, La Jolla, CA, are used for cell adhesion on Vn, and human chronic erythroleukemia cell K562 (α5β1 positive, purchased from ATCC) is used for adhesion on Fn. CN inhibits adhesion of these cells to the immobilized ECM protein dose-dependently. Concentration of CN for complete inhibition of adhesion is defined experimentally. Vn and Fn (purchased from BD Bioscience, prepared at 10 μg/ml) are immobilized on Immulon-II 96-well microtiter plate by incubating 100 μl of the solution in the wells overnight at 4°C. A established protocol (73) is followed. Various concentrations of the scFv-displaying phage obtained from Experiment 3 are mixed with CN at fixed concentration, and tested for the effect on adhesion. The cells are treated with the selected phage in the presence of CN by incubation at 25°C for 20 minutes prior to seeding. Blockage of the inhibitory
effect of CN by the phage implies that the phage-displayed scFv blocks the
binding site in CN for the corresponding integrin.

(c) **Solid phase binding assay**: CN binds to integrins αvβ3, αvβ5, 
αIIbβ3, and α5β1 when they are immobilized on microtiter plates. A function
blocking scFv to CN can disrupt this binding. Purified αvβ3, αvβ5 and α5β1 are
purchased from Chemicon Corp. Purified αIIbβ3 is a generous gift from Dr.
Marian Nakada from Centocor, Inc., Malvern, PA. Soluble integrins are
immobilized on wells of 96-well plates at 4°C overnight. CN at an appropriate
concentration (~100 nM) is mixed with increasing concentrations of scFv-
displaying phage selected after aggregation and adhesion assays (described
above), and the mixture is applied to the coated plate at room temperature for
one hour. Bound CN is detected with a 1:1,000 dilution of rabbit antiserum
against CN previously generated. Goat anti-rabbit Ab conjugated with alkaline
phosphatase (Jackson ImmunoResearch) is used as a second Ab. The second
Ab is absorbed with mouse serum, and does not cross react with mouse Ab.
The bound antibody is quantitated by adding disodium p-nitrophenyl phosphate
(pNPP) and determining the absorbance at 405 nm. Those phage-displayed scFv
fragments that inhibit binding of CN to integrin(s) are selected. The inhibition
specificity of the different scFv may vary.

[00110] **Generation of Ald mAbs (2nd generation mAbs) by immunization of
animals with the scFv displaying phage, construction of the 2nd generation scFv
phage library, and selection for phage displayed scFv which bind to all CN
receptors (integrins αIIbβ3, αvβ3, αvβ5, and α5β1):** Balb/c mice (6 to 8 weeks,
males or females) are immunized with library of phage displaying sFv prepared as
described above. Primary and booster administration of phage is performed
intravenously. Antibody serum titer is monitored by ELISA using plates coated
with the corresponding soluble recombinant scFv, obtained using HB2151 E. coli
non-suppressor bacterial expression system.
A phage display library of the scFv is constructed from the spleen cells of these phage-immunized mice using methods identical to those described above. The splenocytes from the animals immunized with α-CN 10 monoclonal antibody were also used for building a 2nd generation scFv phage library. The 2nd generation libraries were subjected to a series of selection by in vitro panning on immobilized integrins αIβ3, αvβ3, αvβ5, and α5β1, by ex-vivo panning on cancer cell lines expressing the integrins and by in vivo panning in nude mice bearing orthotopic human tumors.

Screening the phage display library by panning on immobilized integrins (αIβ3, αvβ3, αvβ5, and α5β1): Integrins are immobilized on ELISA plates. The scFv displaying phage library generated from the experiment described above, is selected by panning on plates coated with αvβ3 and αvβ5 in series. Acceptable integrin specificity of the Abs is listed in Table I. The phage pool is then selected by panning on immobilized αIβ3 and α5β1 to obtain combination A (Table I), on immobilized αIβ3 to obtain C, on α5β1 to obtain D, and negative selection on both αIβ3 and α5β1 to obtain B. Three cycles of panning are performed with each integrin. Enrichment of bound phage is monitored by phage ELISA. The resultant phage pool is amplified.

Screening the phage display library by panning in vivo in nude mice grafted with MDA-MB-435 cancer cells, a human breast cancer cell line, displaying a broad spectrum integrin profile – orthotopic breast cancer model: Twelve nude mice (3 mice per round of panning) are engrafted with MDA-MB-435 human breast cancer cells surgically into the right mammary fat pad. The MDA-MB-435 cell express a broad spectrum of integrins on their surface including αvβ3, αvβ5, and α5β1, making them an attractive candidate to use for in vivo panning of the above phage library. The tumors are allowed to develop until they reached a volume of approximately 1 cm³. At this point, the purified phage (contained in 300 μl PBS) are injected into mice intravenously (tail vein) and allowed to circulate for 5-10 min before the animals were sacrificed and
tumors flushed (through intra-cardiac catheterization) and harvested (74-75). The tumors are further processed for phage recovery according to an established protocol (76). The recovered phage are further amplified in TG1 E. coli cells, and then used for subsequent rounds of in vivo panning (4 rounds in total).

[00114] **Cell-surface selection of the scFv displaying phage with CN-receptor integrins expressed on cells:** 293-β3, 293-β5, and K562 cells that express integrin αvβ3, αvβ5 and α5β1, respectively, are employed for this assay. Phage pool resulting from the experiment described above, is screened with combinations of the cell lines according to the specificity spectrum determined above. Cells are incubated with the phage at room temperature for 30 min. After washing, the bound phage is eluted with acidic buffer and separated from the cells by centrifugation. Three cycles of selection are performed with each cell line. Eventually, the ability of the selected phage pools to bind to the cell-expressed integrins is confirmed with flow cytometry. To do this, the above cell lines are incubated with phage at room temperature for 30 min, and bound phage is detected with mouse anti-M13 antiserum followed by goat anti-rabbit IgG-FITC (purchased from Jackson ImmunoResearch). The fluorescent density is measured by FACS analysis. Cells treated with goat anti-rabbit IgG-FITC alone serve as background. Rightward shift of the fluorescent density peak suggests binding of the phage to cell expressed integrins.

[00115] **Negative selection for the scFv displaying phage whose binding to cell-expressed integrins are inhibited by CN:** Binding to the identical epitopes that CN binds to is a prerequisite for Alds to mimic the function of CN. Binding of such a phage-displayed scFv to the integrins are competitively inhibited by CN. In the experiment, the selected phage pool from Experiment 3 with 293-β3, 293-β5, and K562 cells is incubated in the presence of 10 μM CN. Phages that do not bind the cells are recovered and amplified.
[00116] After selection on each cell line, the phage is colonized by spreading on agarose plates. Single colonies are picked up and amplified. A small-scale phage preparation is performed by PEG-precipitation. The integrin binding capacity of each phage clone is reconfirmed by FACS analysis.

[00117] Selection of scFv displaying phage for full spectrum functional surrogate of CN using in vitro assays, and expression of the final candidate in form of mouse-human chimeric IgG. It is provided that Al ds that block the epitope-binding site on the 1st generation mAb are internal images of the antigen, and work as functional surrogate. A series of functional tests, including solid phase competitive binding, adhesion, invasion, and CAM assays, are performed to select the functional surrogates of CN among the colonized phage-displayed scFv. The resultant mouse scFv is grafted into a human IgG frame for expression in a eukaryotic system.

[00118] Adhesion assay: Integrin  αvβ3 and αvβ5 are expressed in 293-β3 and -β5 cells, respectively, and mediate adhesion of the cells to Vn. These two cell lines are chosen as working models, and their adhesion to immobilized Vn are tested. Chronic erythroleukemia K562 is employed to test α5β1-mediated adhesion to Fn. Native CN serves as a positive control. The phage-displayed scFv that are capable of inhibiting adhesion of at least 293-β3 and -β5 cell lines is regarded as functional. Inhibition dose to block 50% adhesion (IC50) is estimated by determining the dose-response relationship curve, and compared to that of CN.

[00119] Invasion assay: The concentrations of the phage-displayed scFv are chosen at which complete inhibition of adhesion is achieved from the above experiment. Those scFv with anti-invasive activity (77) against both human breast cancer (MDA-MB-435) cells and human umbilical vein endothelial cells are selected. Native CN is used as a positive control.
CAM angiogenesis assay: The selected scFv has \textit{in vivo} antiangiogenic activity similar to CN. This activity is tested in chick embryo chorioallantoic membrane (CAM) angiogenesis assay, since native CN shows significant antiangiogenic activity in this assay. Ten-day old chick embryos are purchased from a local poultry farm (AA Labs, Westminster, CA). Preparation of the CAM is described in detail elsewhere (77). Angiogenesis is induced by filter discs impregnated with 200 ng bFGF or VEGF. Saline-soaked discs are used to determine the angiogenesis background. CN is used as positive control, and 0.9% NaCl solution is used as negative control. Ten embryos are included in each group. Appropriate dosage of the scFv displaying phage (pre-determined by pilot studies) is used for treatment 24 hours after implantation of the discs, and the embryos are incubated for another 48 hours. Quantitation of angiogenesis is carried out by determination of the number of blood vessel branch points within the confined region of the filter disc with a stereomicroscope. The number of branch points is related to the number of newly sprouting angiogenic vessels (77).

Production of recombinant 1st generation mAb: The NS0 murine myeloma cell line is employed with the Glutamine Synthetase Gene Amplification System (Lonza Biologics, UK) to produce recombinant IgG. The expression vectors are constructed using standard techniques (78). The expression vector pEE12 HC (79) is a plasmid containing the cDNA sequence for the human IgG heavy chain, under the control of the cytomegalovirus major immediate early promoter, and the cDNA sequence for a selective marker, glutamine synthetase, under the control of the SV40 early promoter. pEE6 LC is an expression vector for human IgG light chain with similar construction (79). The PCR products of the VH and VL coding sequences of the functional Abs are subcloned into the heavy- and light-chain expression vectors in place of the corresponding human-originated sequences. Appropriate restriction sites are introduced into the PCR products by the PCR primers. The NS0 murine
myeloma cells are grown in non-selective medium consisting of Hybridoma-SFM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum, L-glutamine and non-essential amino acids. The vectors are co-expressed in the host cells according to the method described previously (79). The recombinant IgG is purified stepwise from cell culture medium by protein A affinity and ion-exchange chromatography (79). The activity of the recombinant human/mouse chimeric IgG is confirmed by the assays described in this section.

CITED REFERENCES


[00205] The invention thus has been disclosed broadly and illustrated in reference to representative embodiments described above. Those skilled in the art will recognize that various modifications can be made to the present invention without departing from the spirit and scope thereof. All publications, patent applications, and issued patents, are herein incorporated by reference to the same extent as if each individual publication, patent application or issued patent were specifically and individually indicated to be incorporated by reference in its entirety. Provisional patent application serial no. 60/349,623 filed January 16, 2002, also incorporated by reference herein.
CLAIMS

1. An anti-idiotypic antibody of disintegrin, said antibody being characterized in binding to one or more integrins, said integrins being a receptor for said disintegrin.

2. The anti-idiotypic antibody of claim 1, wherein said antibody is characterized in inhibiting binding between said one or more integrins and said disintegrin.

3. The anti-idiotypic antibody of claim 2, wherein said antibody inhibits binding between some but not all integrins which are a receptor for said disintegrin.

4. The anti-idiotypic antibody of claim 1, wherein said antibody inhibits a functional activity of the disintegrin, said activity selected from the group consisting of inhibition of cell adhesion, inhibition of metastases, and inhibition of angiogenesis.

5. The anti-idiotypic antibody of claim 1, wherein said disintegrin is contortrostatin.

6. The anti-idiotypic antibody of claim 1, wherein said antibody is a monoclonal antibody.

7. The anti-idiotypic antibody of claim 1, wherein said antibody is polyclonal antibody.
8. The anti-idiotypic antibody of claim 1, wherein said antibody is a human antibody, a chimeric antibody or a humanized antibody.

9. The anti-idiotypic antibody of claim 1, wherein said disintegrin is contortrostatin.

10. The anti-idiotypic antibody of claim 9, wherein said antibody is Ald 40-8.

11. The anti-idiotypic antibody of claim 9, wherein said antibody exhibits higher binding affinity of integrins than Ald 40-8.

12. The anti-idiotypic antibody of claim 1, wherein said integrin is selected from the group consisting of integrin αllbβ3, αvβ3, αvβ5 and α5β1.

13. The anti-idiotypic antibody of claim 2, wherein said antibody and said disintegrin binds to integrin αllbβ3, αvβ3, αvβ5 and α5β1 but said antibody does not inhibit binding between at least one of the integrins and said disintegrin.

14. A pharmaceutical composition comprising an anti-idiotypic antibody as recited in any one of claims 1-13 and optionally a pharmaceutically acceptable carrier.

15. The pharmaceutical composition of claim 14 for the treatment of angiogenesis, cancer, bone metabolism, or inflammation.

16. A cell line producing the antibody of any of claims 1-13.

17. The cell line of 16, wherein said cell line is a hybridoma.
18. A method of making the anti-idiotypetype antibodies as recited in any of claims 1-13, said method comprising immunizing an animal with an anti-disintegrin antibody, immortalizing lymphocytes from the animal, and selecting immortalized lymphocytes producing said anti-idiotypetype antibodies.

19. The method of claim 18 wherein said disintegrin is contortrostatin.

20. A method of making the anti-idiotypetype antibodies as recited in any of claims 1-13, said method comprising preparing a phage display antibody library using nucleic acid from an animal, and selecting phage which display said anti-idiotypetype antibody.

21. The method of claim 20, wherein said nucleic acid is obtained from an animal immunized against the disintegrin.

22. The method of claim 20, wherein said disintegrin is contortrostatin.

23. A method of antagonizing the activity of disintegrin receptors, in an individual, said method comprising administering an effective amount of a composition comprising the antibody of any of claims 1-13.

24. The method of claim 23, wherein said disintegrin receptors are contortrostatin receptors.

25. The method of claim 23, wherein said contortrostatin receptors are selected from the group consisting of integrin αⅠβ3, αvβ3, αvβ5 and α5β1.
26. Use of an anti-idiotypic antibody as recited in any one of claims 1-13 for the preparation of a medicament for the treatment of angiogenesis, cancer, bone metabolism, or inflammation.
FIG. 1
Absorbance

Concentration of α-CN10 (nM)

FIG. 7
FIG. 8
FIG. 10
FIG. 14