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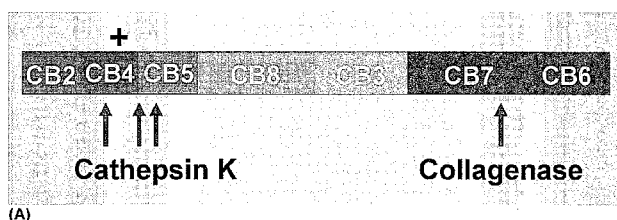
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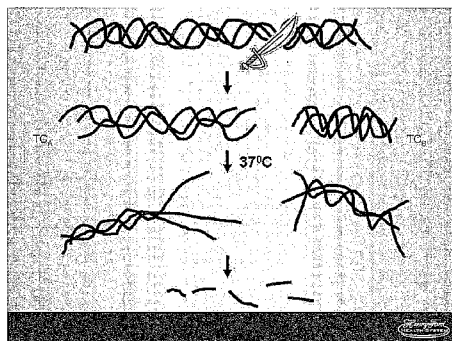
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(54) Title: COLLAGEN-DERIVED PEPTIDE AS BIOMARKER, THERAPEUTIC AGENT AND TARGET



(A)



(B)

FIGURES 1(A)-(B)

(57) Abstract: Some embodiments comprise a novel peptide derived from Type 1 collagen which is a potent activator of ECM synthesis and remodeling and which may facilitate the detection and/or assessment of the presence of tumor cells, or other cells of interest, by detecting or assessing the presence of the peptide and/or its subfragments, and which may stimulate the production of proteases in a subject.

COLLAGEN-DERIVED PEPTIDE AS BIOMARKER, THERAPEUTIC AGENT AND TARGET

RELATED APPLICATIONS

(0001) This application claims priority to U.S. Provisional Patent Application No. 61/033,248 filed March 3, 2008, which is hereby incorporated in full.

FIELD OF THE INVENTION

(0002) The invention relates generally to the fields of molecular and cellular biology.

BACKGROUND

(0003) Collagen remodeling and turnover are some of the most fundamental processes that occur in mammals, including humans, during growth and aging. Type I collagen is the most abundant protein found in humans. Proteolysis of the collagen matrix is mediated by proteolytic enzymes, mainly collagenases belonging to the matrix metalloproteinases ("MMP") family. Collagen turnover must be carefully regulated, as too little results in fibrosis and too much leads to matrix destruction and tissue damage.

(0004) The understanding of the regulation of physiological vs. pathological turnover of collagen is a challenging one. Many related proteolytic enzymes are made in "pro" forms and have complex activation cascades. Another approach to understanding collagen cleavage has been to remove the collagenase cleavage site and create a collagenase-resistant collagen mouse, which resulted in discovering another collagenase site. More recently, other researchers have shown that other MMPs that were thought not to be involved in collagen turnover can overcome these modifications and can remodel the collagen matrix. However, one common denominator in collagen turnover is the generation of collagen fragments during its proteolysis.

(0005) The activity of connective tissue cells is modulated by a number of factors present in their environment. In addition to the soluble factors such as hormones, cytokines or growth factors, cells also receive signals from the surrounding extracellular matrix ("ECM") macromolecules. Moreover, they may degrade ECM proteins and liberate peptides which may by themselves constitute new signals for the surrounding cells. Therefore, a regulation loop may exist in connective tissue, constituted by peptides generated by ECM degradation and connective tissue cells. The term of "matrikine" has been proposed to designate such ECM-derived peptides able to regulate cell activity.

(0006) The study of matrix degradation products that have novel biological properties is not new, but is a field still in its infancy. One of these matrikines is the C-terminal domains of type XVIII collagen, also known as endostatin. The study of endostatin has increased significantly since it was first proposed by Dr. Judah Folkman and has led to its use as an antitumor agent. The original hypothesis of this mechanism was rejected and opposed by the scientific community; without the perseverance of Dr. Folkman's group, this research would not have resulted in this innovation.

(0007) Yet the identity, function, and regulation of many of these so-called matrikines remain elusive, as does the determination of whether or not specific matrikines reside or are active in particular tissues during pathological processes and related disease states. Thus, an unmet need remains for novel means to detect the presence of pathological conditions where novel molecules of interest, including but not limited to matrikines, may be present, as well as to permit therapeutic modalities in light of same.

BRIEF DESCRIPTION OF THE DRAWINGS

(0008) Some embodiments of the invention will now be described, by way of example only, with reference to the accompanying drawings, in which:

(0009) FIGS. 1(A)-(B) are a schematic of protease cleavage sites of Cathepsin-K and collagenases that can generate CB4II from type I collagen and a schematic of cleavage of triple helical type I collagen that exposes and generates collagen fragments that contain CB4II sequence in physiological conditions.

(00010) FIG. 2 is a chart showing stimulation of proteases and bone associated genes by CB4II in human MDA-MB-231 breast tumor cells.

(00011) FIG. 3 shows the results of a Western Blot of MMP-1 and MMP-13 production from MDA-MB-231 cells that were stimulated with CB4II peptide over time.

(00012) FIG. 4 is a diagram showing positive feedback signalling of collagen fragments.

(00013) FIG. 5 shows gelatin zymography of conditioned media from CB4II stimulated MDA-MB-231 cells.

(00014) FIG. 6 shows pictures of comparative osteoclast formation by conditioned media from CB4II stimulated MDA-MB-231 cells versus control media as measured by TRAP staining for large multi-nucleated cells.

(00015) FIG. 7 shows results of a Western Blot demonstrating MMP-1 stimulation in porcine skin fibroblasts by the addition of the CB4II peptide, with a dose-response relationship noted.

(00016) FIG. 8 shows results of a PCR analysis on the suppression of CTGF mRNA in both human and porcine skin fibroblast by the addition of CB4II peptide.

(00017) FIGS. 9A-C show invasion of squamous cell carcinoma in the keratinized component of skin associated with the presence of CB4II as shown by immunostaining with anti-CB4II antibody.

(00018) FIG. 10 shows the results of Western Blot of human MDA-MB-231 breast tumor cell mediated osteolysis and generation of CB4II collagen matrikine fragments in mice.

(00019) FIG. 11 shows the results of Western Blot of human PC3 prostate tumor cell mediated osteolysis and generation of CB4II collagen matrikine fragments in mice.

DETAILED DESCRIPTION

(00020) Without limiting the invention to only those embodiments disclosed herein, and without disclaiming any embodiments, we have discovered and isolated a 24 amino acid peptide derived from Type 1 collagen which is a potent activator of ECM synthesis and remodeling. We also discovered that type I collagen fragments containing the 24 amino acid peptide epitope are produced by tumor cells. Specifically, the peptide is produced in tissues of interest, as shown visually by application of antibodies against the peptide and visualization of the binding areas. Thus, some embodiments comprise, without limitation, the peptide itself as well as active subfragments of same, vectors that contain the peptide and/or its active subfragments, and vectors that may provide for the expression of the peptide and/or its active subfragments, either *in vitro* or *in vivo*. In addition, some embodiments comprise methods of detecting or assessing the presence of tumor cells, or other cells of interest, by detecting or assessing the presence of the peptide and/or its subfragments, as one example only, by addition and detection of labeled antibodies directed against the peptide or its subfragments. Some embodiments also comprise methods for reducing the biological activity of the peptide or its subfragments, as some examples only, by targeting the molecules themselves, by reduction of the expression of the molecules *in vivo*, or by competitive inhibition of the molecules. Conversely, some embodiments comprise methods to increase the biological activity of the molecule, as some examples only, by inducing increased

expression of the peptide or its active subfragments, or by addition and induction of genes coding for the peptide.

(00021) In our work, we evaluated the generation of novel cryptic collagen fragments from the unwinding of helical collagen from proteolysis, resulting in a feedback signal to the cell that initiate cellular changes. In our work in determining what proteases are involved in the collagen turnover and how they are regulated, we focused on the result of their action, the breakdown fragments. In doing so, we discovered, isolated, and mapped a cryptic 24 amino acid sequence (GARGL-HYP-GTAGL-HYP-GMKGHRGFSGLD)(SEQ ID NO. 1)(where HYP is hydroxyproline) that results from the breakdown of the collagen triple helix and that can elicit many cellular responses that promote collagen remodeling. (In some embodiments, without limitation, proline may be substituted selectively for hydroxyproline – see SEQ ID NOS. 2 through 4 herein). We also generated peptide antibodies to this epitope and showed increased detection of this collagen degradation fragment in mouse prostate bone tumors, human skin tumors, synovial fluid and fibroblast cultures. Our discovery shows that the peptide can be generated and is useful as a biomarker (as some examples only, and without limitation, in arthritis, wound healing, development, tumor invasion), and also as a stimulator of proteases to break down matrix (as one example only, fibrotic diseases). As some examples only and without limitation, some embodiments comprise the selective detection of the peptide(s) or subfragments thereof in tissue samples by techniques known to skilled artisans, as one example only, by use of diagnostic antibodies directed to the peptide(s) or any subfragment thereof; the stimulation of protease production in a mammal by administration of a therapeutically effective amount of the peptide or any subfragment thereof; and the detection of the peptide(s) or any subfragment thereof in a sample from the mammal, as some examples only, a urine or blood sample, as an indicator of the disease or injury condition of the mammal.

(00022) In our work, we subjected type 1 collagen to enzymatic cleavage. The resulting fragments were separated and isolated by size, and the resulting fragment populations were tested separately to assess their ability to induce proteases in culture.

(00023) We discovered that a 24 amino acid sequence comprising SEQ ID No. 1 increased the production of MMP1 and MMP3 relative to controls, when cultured in skin fibroblasts. We also tested the effects of the peptide on the expression of connective tissue growth factor (“CTGF”) in skin fibroblasts and human synovial fibroblasts, finding that it suppressed the expression of CTGF.

(00024) We also tested the effect of the peptide on MMP production in breast tumor cultures. We found that breast tumor cells stimulated with the peptide showed increased MMP1, 2, 9, 13, and 14 production, as well as increased expression of parathyroid hormone related peptide ("PTHrP") and osteonectin.

(00025) We also tested the effects of conditioned media from tumors stimulated with the peptide on osteoclast formation and found that the media increased osteoclast production.

(00026) Thus, some embodiments of the invention comprise, without limitation, a peptide comprising SEQ ID NO. 1, 2, 3, or 4 or any of its active subfragments; methods comprising use of the peptide(s) or any of its subfragments as a biomarker for tissues or disease states of interest, including without limitation, metastatic tumors; and methods, compositions, or systems wherein the peptide(s) or its subfragments may be a therapeutic target due to its increased elevation in disease states, either by antibody, drug treatment, competitive inhibition, or other processes.

(00027) **EXAMPLES**

(00028) The following examples of some embodiments of the invention are provided without limiting the invention to only those embodiments described herein and without disclaiming any embodiments.

(00029) **Example 1:**

(00030) The precise mechanisms of osteolysis and targeting of bone by the breast cancer cells are still unknown. The osteolysis that occurs in patients is mediated by factors that come from the tumor cells. The tumor cells provide a microenvironment to the osteoblasts and osteoclasts. Bone remodeling requires the degradation and turnover of type I collagen. Type I collagen is a triple helical fibrillar collagen that can only be cleaved by collagenases of the MMP family or by cathepsin K found only in osteoclasts. The extracellular matrix plays an important role in tissue homeostasis. It regulates cellular differentiation, migration, and proliferation. Recently it has been shown that cleavage products of ECM proteins may have novel biological properties and have been termed matrikines.

(00031) We have discovered a type I collagen degradation fragment (SEQ ID No. 1) in the helical domain that can be generated by either MMPs or cathepsin K cleavage of type I collagen. (Figs. 1(A)-(B)). Through our work, we deduced from cleavage sites of cathepsin K on type I collagen that it produces a peptide fragment containing the entire CB4 of 42 amino acids. We constructed two overlapping peptides of 24 amino acids, CB4I and CB4II, and discovered that CB4II (SEQ ID No. 1) was positive for inducing MMP-1 production.

Among other evaluations, we tested the ability of this peptide fragment to act as a matrikine and stimulate human metastatic breast cancer cells, MDA-MB-231, to stimulate inflammatory cytokines, MMPs and to promote osteoclastogenesis.

(00032) Materials and Methods:

(00033) Peptide synthesis: A 24 amino acid synthetic peptide (GARGL-HYP-GTAGL-HYP-GMKGHRGFSGLD)(SEQ ID No. 1) lacking RGD sequence (CB4II) derived from the helical region of type I collagen was synthesized by Fmoc chemistry.

(00034) Cell culture: Human breast cancer cells - Human breast cancer cells, MDA-MB-231, were cultured in the presence of DMEM + 10% FBS for 2 days. The cells were serum starved for 24h and then stimulated with 0.25, 2.5 or 25 μ g/ml CB4II for 48h in serum-free media. 1 ng/ml rhIL-1 α or 1 ng/ml rhTNF- α was used as positive control for up-regulation of MMPs. Rat monocyte/ macrophages were flushed from the bone marrow of tibias and femurs. Osteoclast formation was visualized by TRAP staining.

(00035) Cell Culture: Human and porcine skin fibroblasts - Human and porcine skin fibroblasts were grown to confluency and cultured in the presence of DMEM plus 10% FBS for 48h. The fibroblasts were then serum starved for 24h followed by stimulation with 0.2, 2.0 or 20 μ g/ml CB4II for 48h in serum-free media. 5 ng/ml and 10 ng/ml human recombinant IL-1 α and TNF- α were used as positive control for upregulation of MMP-1. CB4II, IL-1 α , or TNF- α was not added to negative controls (Ctrl).

(00036) RNA isolation: Total RNA was isolated with Trizol reagent (Invitrogen, CA). The RNA was quantified by UV spectrophotometry and checked on 1.2% agarose gels to evaluate RNA integrity.

(00037) Real time PCR: Total RNA was isolated from cells, converted to cDNA, and subjected to quantitative relative real-time PCR using the ABI PRISM 7700 sequence detection system. Data was computed using the ddCt method.

(00038) In some of our tests, semi-quantitative relative gene expression for CTGF and GAPDH was determined using total RNA extracted from the fibroblasts. 1 μ g of total was reverse transcribed and 10 ng of cDNA was amplified for 40 cycles on an Eppendorf gene thermocycler. PCR products were visualized by ethidium bromide staining and separated on a 1% agarose gel and photographed.

(00039) Protein Quantitation: Amount of protein was quantitated with BCA assay (Pierce, IL).

(00040) Western blot & Gelatin zymography: MMP-1 and MMP-13 proteins from conditioned media were measured by Western blotting. Gelatinase activity was determined using gelatin zymograms.

(00041) For certain Western blots, equal amounts of protein from conditioned media as determined by BCA was separated by SDS-PAGE gels and then transferred to PDF membranes for Western blotting. Blots were incubated with polyclonal rabbit-peptide antibodies against MMP-1(1:2000) overnight and then visualized by chemiluminescence.

(00042) For certain zymography, gelatinase activity was determined using gelatin zymograms. Briefly, conditioned media from MDA-MB-231 cells were separated by SDS-PAGE gelatin zymograms. To determine gelatin activity, gels were stained with Coomassie blue after incubation in substrate buffer and photographed.

(00043) Histology: Paraffin sections of human SSC tumors were incubated with anti-CB4II antibody. Tissue sections are pretreated with a Proteinase K (0.1mg/ml) (1:100) in PBS 1 h at room temperature, then incubated in Citrate buffer (0.1M sodium citrate, 0.1M citric acid) for 2 hours, and then with TBST for 5 min. 4 times at room temperature. Positive staining is visualized with addition of DAB substrate.

(00044) Statistical analysis: Relative gene expression data was analyzed using the non-parametric approach of Friedman using SAS. Differences were declared statistically significant when $P < 0.05$, unless otherwise noted.

(00045) Results:

(00046) Our real time PCR analysis of MDA-MB-231 cells stimulated with 25 $\mu\text{g/ml}$ CB4II, showed an upregulation of MMP-1, 2, 9, 13, and 14, which were all upregulated significantly ($p < 0.05$) (Fig. 2). PTHrP and SPARC, shown to be associated with metastasis, proteolysis and present in bone and breast tumors, were also upregulated significantly ($p < 0.05$).

(00047) MDA-MB-231 cells were stimulated with 24 and 48 hrs with CB4II peptide, IL-1 β or TNF- α . MMP-1 and MMP-13 production was assessed by Western blotting. Both MMP-1 and MMP-13 protein were produced and secreted into the conditioned media. Western Blot of MMP-1 and MMP-13 production from MDA-MB-231 conditioned media showed a dose response stimulation of both MMP-1 and MMP-13 with CB4II. 25 $\mu\text{g/ml}$ CB4II showed similar stimulation levels as 1 ng/ml rhIL-1 β or 1 ng/ml rhTNF- α . (Fig. 3). CB4II stimulated a dose response increase in MMP-1 and MMP-13 in MDA-MB-231 cells. CB4II peptide could stimulate MMP-1 and MMP-13 in a dose-dependant manner and 25 $\mu\text{g/ml}$ of CB4II

could stimulate both to the levels of pro-inflammatory cytokines IL-1 β and TNF- α . This would allow these cells to detach and migrate to bone.

(00048) Zymography of MMP-2 and MMP-9 of MDA-MB-231 conditioned media was used to assay for gelatinase activity. Increasing CB4II stimulated both MMP-2 and MMP-9 activity in MDA-MB-231 human breast cancer tumor cells. (Fig. 5). We also saw an increase in active MMP-2 with increasing peptide concentration.

(00049) To test the ability of CB4II to stimulate MDA-MB-213 cells to secrete factors required to recruit and generate osteoblasts, conditioned media from cells was added to monocyte-macrophage precursors, using purified rat monocyte/macrophages. Adherent cells were plated and cultured in the presence of MDA-MB-231 conditioned media which were grown in the presence or absence of 25 μ g/ml CB4II. Monocyte/macrophage cultures formed large multi-nucleated TRAP positive osteoclasts only in CB4II stimulated conditioned media, suggesting CB4II stimulated osteoclast forming cytokines in MDA-MB-231 cells. (Fig. 6). Western blot of conditioned media from stimulated porcine skin fibroblasts showed MMP-1 stimulation and production by the addition of the peptide, with a dose-response relationship. (Fig. 7).

(00050) Example 2:

(00051) The major site of metastasis of breast cancer cells is bone. Bone metastases occur in 80% of patients with advanced disease and causes significant morbidity. This relationship was first described by Paget in 1889 and the term “seed and soil” was coined to explain this preferential metastasis of breast tumors to bone. The osteolysis that occurs in patients is mediated by factors that come from the tumor cells. The tumor cells thrive and expand in the bone microenvironment and provide the osteoblasts and osteoclasts with factors that promote osteolysis. The precise mechanisms of osteolysis and targeting of bone by the breast cancer cells are still unknown. Bone remodeling requires the degradation and turnover of type I collagen. Type I collagen is a triple helical fibrillar collagen that can only be cleaved by collagenases of the MMP family or by cathepsin K found only in osteoclasts. Recently, studies of breakdown fragments of ECM proteins have shown novel biological activity of the fragments.

(00052) We have identified a type I collagen degradation fragment that can be generated either MMPs or Cathepsin K cleavage of type I collagen. Based on our work, we postulated that a type I collagen matrikine fragment, CB4II, may have several functions in breast tumor metastasis to bone and osteolysis. Bone is an active remodeling tissue and accounts for the

major of type I collagen turnover in the body. We discovered that these fragments may be chemotactic to breast tumor cells. Once the breast tumor cells metastasize to the bone, the tumor cells secrete inflammatory chemokines and cytokines such as PTHrP, receptor activator of nuclear factor kappa B (RANK) and Runx2 that stimulate osteoblasts and osteoclasts formation. These events result in increased osteolysis due to increased protease production and osteoclasts formation. The increased osteolysis of bone leads to increased of type I collagen fragments that signal back to the tumor cells, osteoblasts and osteoclasts perpetuating this cascade. Thus, as part of our discovery, we investigated the production of this matrikine fragment by cathepsin K in an *in vivo* mouse model of breast tumor metastasis.

(00053) Materials and Methods:

(00054) Mouse metastasis model: Human fetal tibiae fragments were implanted subcutaneously into either control or tumor-bearing immunodeficient Rag-1 ^{-/-} or Rag-1 ^{-/-}, CatK ^{-/-} mice (N=3 each). Four weeks later, 0.5x10⁶ MDA-MB-231 breast tumor or PC3 prostate tumor cells were injected through the mouse skin directly into the marrow of previously implanted bone, as described. Four weeks following injection of tumor cells, the mice were sacrificed and the bone implants harvested and extracted for Western blot and immunohistochemical analysis.

(00055) Western Blot analysis: Human tibiae fragments were homogenized in RIPA extraction buffer. Protein concentration was determined by BCA. Equal amounts of protein was loaded and separated by SDS-PAGE. Nylon membranes were probed with anti-CB4II rabbit antibody.

(00056) Immunohistochemistry: Mice tibiae fragments were fixed in formalin and decalcified for immunohistochemistry. Serial sections were stained with anti-CB4II rabbit antibody and staining was visualized by fluorescence microscopy.

(00057) Results:

(00058) Western blotting of control human tibiae fragments (3 different mice) implanted in Rag-1 ^{-/-} mice revealed the presence of CB4II containing type I collagen matrikine fragments from normal bone turnover while MDA-MB-231 tumor-bearing tibiae show increased amounts of CB4II containing type I collagen fragments (Fig. 10A) whereas in both control and MDA-MB-231 tumor-bearing tibiae implanted in Rag-1 ^{-/-}/CatK ^{-/-} mice had a marked decrease in CB4II containing type I collagen matrikine fragments as compared to Rag1 ^{-/-} mice (Fig. 10B). These results indicate that cathepsin K plays an important role in generating CB4II type I collagen fragments and CatK ^{-/-} mice have decreased tumor-

mediated osteolysis due to decrease in generation of CB4II matrikine. The generation of this matrikine was also seen at osteolytic sites by immunohistochemical analysis of the human tibiae injected with MDA-MB-231 cells, and its generation was reduced in Cathepsin K null mice.

(00059) Similarly, Western Blot of Rag-1 ^{-/-} Cat K ^{-/-} mice tibiae extracts with and without PC3 human prostate cancer tumor cells were conducted. Human tibiae fragments were implanted in (3 different mice) in Rag-1 ^{-/-} mice with and without PC3 tumor cells injected. Control mice showed the presence of CB4II containing type I collagen fragments from normal bone turnover while tumor-bearing tibiae showed a large increase in amounts of CB4II containing type I collagen fragments due to tumor mediated osteolysis of the tibiae fragments. (Fig. 11(A)). Human tibiae fragments in Rag-1 ^{-/-}/CatK ^{-/-} mice had large amounts of CB4II containing type I collagen fragments as in control CK null tibiae with or without tumors. (Fig. 11(B)). These results indicate that cathepsin K is not mediating bone resorption in PC3 mediated osteolysis but rather MMPs such as the collagenases from osteoblasts and tumor cells are generating CB4II type I collagen fragments.

(00060) Our discoveries showed unexpectedly that an isolated peptide sequence in the CB4 region of type I collagen can stimulate MMP production in MDA-MB-231 cells. We further evaluated whether this collagen fragment, CB4II, may function in breast tumor metastasis to bone and osteolysis. In our work, we investigated the role of Cathepsin K in the generation of type I collagen matrikine CB4II. We discovered for the first time that Cathepsin K contributes to the generation of the CB4II matrikine during breast tumor mediated osteolysis. In addition, the generation of this matrikine is significantly reduced in Cathepsin K null mice.

(00061) Bone is an active remodeling tissue and accounts for the majority of type I collagen turnover in the body. We evaluated whether the fragment may be chemotactic to breast tumor cells. Once the breast tumor cells metastasize to the bone, the tumor cells secrete inflammatory chemokines and cytokines such as PTHrP, receptor activator of nuclear factor kappa B (RANK) and Runx2 that stimulate osteoblasts and osteoclasts formation. CB4II can induce transcription factors such as NF- κ B in breast tumor cells which in turn stimulate osteoclasts formation. These events result in increased osteolysis due to increased protease production and osteoclasts formation. The increased osteolysis of bone leads to increase of type I collagen fragments that signal back to the tumor cells, osteoblasts and osteoclasts perpetuating this cascade. These observations indicate that the type I collagen fragment, CB4II, may have several functions in breast tumor metastasis to bone and

osteolysis. Recently NF- κ b has been shown to induce GM-CSF and promote osteolytic bone metastasis and CB4II may play a role in this process.

(00062) A schematic of the mechanism of CB4II positive feedback pathway on tumor mediated bone metastasis and osteolysis is shown in Fig. 4, and *in vivo* generation of this matrikine in breast tumor patients may demonstrate this novel pathway in tumor mediated bone osteolysis. CB4II peptide fragments are generated in bone turnover. If a patient develops metastatic prostate or breast tumor, circulating tumor cells travel to bone via marrow and come in contact with CB4II fragments. This in turn stimulates the tumor cells to produce MMPs along with bone resorption signaling molecules to recruit osteoclasts that destroy bone and collagen matrix. This resorption creates more CB4II collagen peptide fragments that can bind tumor cells, osteoblasts, and osteoclasts to mediate osteolysis and bone destruction. Indeed, recently it has been shown that women on bisphosphonate treatments prior to the development of breast tumors have a low incidence of bone metastasis. This observation could be the result of bisphosphonate inhibition of bone remodeling and therefore inhibit the production of matrikine CB4II in bone. Thus, the CB4II matrikine in human breast tumor patients with bisphosphonate treatment may be a target for breast tumor mediated bone metastasis.

(00063) Through our work, we saw generation and increase of collagen fragments with our sequence when we used antibodies in mice bones that have been injected with breast MDA-231 tumor cells or PC3 prostate cells. The generation of fragments decreased in Cathepsin K knockout mice with breast tumor cells due to loss of one of the major enzymes in bone that can create and liberate CB4II.

(00064) Breast cancer is the most common cancer affecting women in the United States and other western countries. The major site of metastasis of breast cancer cells is bone. Bone metastases occur in 80% of patients with advanced disease and causes significant morbidity. In contrast to prostate cancer, which forms osteoblastic lesions, skeletal metastasis of breast cancer typically leads to osteolysis, which is often accompanied by severe pain, pathological fracture and hypercalcemia.

(00065) The precise mechanisms of osteolysis and targeting of bone by the breast cancer cells are still unknown. Nonetheless, the osteolysis that occurs in patients is mediated by factors that come from the tumor cells. Although the molecular mechanism underlying the preferential metastasis of breast cancer to bone is yet to be elucidated fully, it is believed that

osteoclasts activated by breast cancer cells mediate osteolysis. The tumor cells provide a microenvironment to the osteoblasts and osteoclasts.

(00066) Bone remodeling requires the degradation and turnover of type I collagen. Type I collagen is a triple helical fibrillar collagen that can only be cleaved by collagenases of the MMP family or by cathepsin K found in osteoclasts.

(00067) The ECM plays an important role in tissue homeostasis. It regulates cellular differentiation, migration, and proliferation. It has been shown that cleavage products of ECM proteins may have novel biological properties and have been termed matrikines.

(00068) We performed gelatin zymography after MDA-MB-231 were stimulated for 24 hr in serum free DMEM with increasing CB4II peptide or 10 ng/ml IL-1 β or 10 ng/ml TNF- α . The CB4II peptide stimulated both MMP-2 and MMP-9 activity. Also active MMP-2 was stimulated by CB4II peptide. (Fig. 5).

(00069) We also showed that conditioned media from the cell stimulated with CB4II cold stimulation osteoclast formation *in vitro*. Rat monocytes/macrophages were purified from flushing tibias and femurs and plated in monolayer cultures. Adherent monocyte/macrophages were grown in the presence of conditioned medium collected from MDA-MB-231 cells which were grown in the absence or presence of 25 μ g/ml of CB4II for 48 h. Osteoclast formation was measured by TRAP staining for large multi-nucleated cells. As shown in Fig. 6, the conditioned media resulted in osteoclast formation compared to controls.

(00070) Collagen turnover must be carefully regulated, in fibrotic diseases, there is an absence of normal ECM turnover and an accumulation of the ECM. MMP-1 (Collagenase-1) is required for the turnover for type I collagen matrix. Since our peptide is a result of type I collagen turnover and acts on cells to induce turnover, we tested its ability to stimulate MMP-1 (Collagenase-1) in porcine skin fibroblasts. MMP-1 protein was stimulated by addition of the peptide, with a dose-response relationship noted. (Fig. 7).

(00071) In addition, connective tissue growth factor ("CTGF") is a mitogenic protein, a downstream target of TGF-beta recruited during wound healing and known to mediate collagen deposition at the site of repair. TGF-beta and CTGF have been implicated in the pathogenesis of the fibrotic disease Scleroderma or systemic sclerosis ("SSc"), a multisystem disorder of connective tissue. The addition of 25 μ g/ml of CB4II peptide resulted in the suppression of CTGF mRNA in both human and porcine skin fibroblast. (Fig. 8). As CTGF has been implicated to be involved in fibrosis, suppression of its production would be useful

in stopping ECM accumulation. CB4II not only can stimulate collagenase-1 production and downregulate CTGF expression, both pathways needed to reverse fibrotic events.

(00072) We also discovered that CB4II may be a marker for invading tumor cells. Tumors must move to metastasize, they do this by invading into normal tissue and this is mediated by proteases and turnover of collagen matrix. In the skin, we have used our anti-CB4II antibody to show this invasion by squamous cell carcinoma ("SCC") in the keratinized component may be associated with the presence of CB4II. (Fig. 9 A-C).

(00073) Finally, we measured the *in vivo* increase in production of the type I collagen matrikine fragment CB4II in tibiae injected with MDA-MB-231 from Rag -/- as compared to Rag -/-/CatK -/- by Western Blotting. (Figs. 10-11). Our discovery and work supports a new mechanism of breast tumor formation and metastases to bone. We have identified a novel proteolytic cleavage product of type I collagen, CB4II, that can act as a matrikine on breast tumor and bone cells, a mechanism of breast tumor formation and metastases to bone not shown previously.

(00074) Embodiments of the invention may expand the therapeutic window for treatment of injury and diseases involving collagen remodeling or turnover and could be applied to a large patient population who suffer such injury and diseases each year in the United States.

(00075) Thus, in some embodiments, the invention comprises novel methods to prevent, control, or alleviate mammalian injury and disease, including without limitation, human cancer or metastasis, fibrotic conditions or diseases, and conditions where collagen remodeling or turnover are implicated, through the selective application of peptide(s) comprising embodiments of the invention. In accordance with some embodiments, without limitation, one may affect such therapeutic intervention through the use and/or administration of one or more such peptide(s) for a finite interval of time, thereby limiting the effects of such injury or disease.

(00076) In accordance with some embodiments, there is a high likelihood that the duration of drug therapy would be relatively brief and with a high probability of success. Prophylactic administration of efficacious amounts of peptide(s) of some embodiments may greatly reduce the incidence of damage associated with many forms of trauma.

(00077) In accordance with some embodiments, a preferred route of administration in humans is by oral administration. However, any appropriate routes of administering such peptide(s) known to those of ordinary skill in the art also comprise embodiments of the

invention. Since the use of such peptide(s) in accordance with some embodiments specifically targets the evolution and expression of associated pathologies, it is expected that the timing and duration of treatment in humans will approximate those established for animal models. Similarly, the doses established for achieving desired effects using such compounds in animal models, or for other clinical applications, would be expected to be applicable in this context as well.

(00078) The peptide(s) of some embodiments would be administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The "therapeutically effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement, including but not limited to, decreased damage or injury, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art.

(00079) In accordance with some embodiments, such peptide(s) can be used or administered in various ways. It can be administered alone or as an active ingredient in combination with pharmaceutically acceptable carriers, diluents, adjuvants and vehicles. The peptide(s) can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneal, and intranasal administration as well as intrathecal and infusion techniques, or by local administration or direct inoculation to the site of disease or pathological condition. Implants of the compounds are also useful. The patient being treated is a warm-blooded animal and, in particular, mammals including humans. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

(00080) It is noted that humans are treated generally longer than the experimental animals exemplified herein which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses may be single doses or multiple doses over periods of time. The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the patient species being treated.

(00081) When administering the peptide(s) of some embodiments parenterally, it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or

dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

(00082) When necessary, proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such as cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, may also be used as solvent systems for such peptide(s) compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the peptide(s).

(00083) Sterile injectable solutions can be prepared by incorporating the peptide(s) utilized in practicing some embodiments of the present invention in the required amount of the appropriate solvent with various of the other ingredients, as desired. A pharmacological formulation of some embodiments may be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the peptide(s) utilized in some embodiments may be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

(00084) In some embodiments, without limitation, the peptide(s) of some embodiments may be administered initially by intravenous injection to bring blood levels to a suitable level. The patient's levels are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition and as indicated above, can be used.

The quantity to be administered and timing of administration may vary for the patient being treated.

(00085) Any and all references are incorporated in full by reference as though fully set forth herein.

(00086) While the present invention has been particularly shown and described with reference to the foregoing preferred and alternative embodiments, it should be understood by those skilled in the art that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention without departing from the spirit and scope of the invention. A person of ordinary skill in the art would realize that certain modifications would come within the teachings of this invention, and the following claims should be studied to determine the true scope and content of the invention. In addition, the methods and compositions of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are described herein. The described embodiments are illustrative only and do not limit the invention to only those expressly described and do not constitute a disclaimer of other embodiments. Where the claims recite “a” or “a first” element of the equivalent thereof, such claims should be understood to include incorporation of one or more such elements, neither requiring nor excluding two or more such elements.

SEQ ID NO: LISTING

SEQ ID NO: 1:

GARGL-HYP-GTAGL-HYP-GMKGHRGFSGLD

SEQ ID NO: 2:

GARGL-HYP-GTAGLPGMKGHRGFSGLD

SEQ ID NO: 3:

GARGLPGTAGL-HYP-GMKGHRGFSGLD

SEQ ID NO: 4:

GARGLPGTAGLPGMKGHRGFSGLD

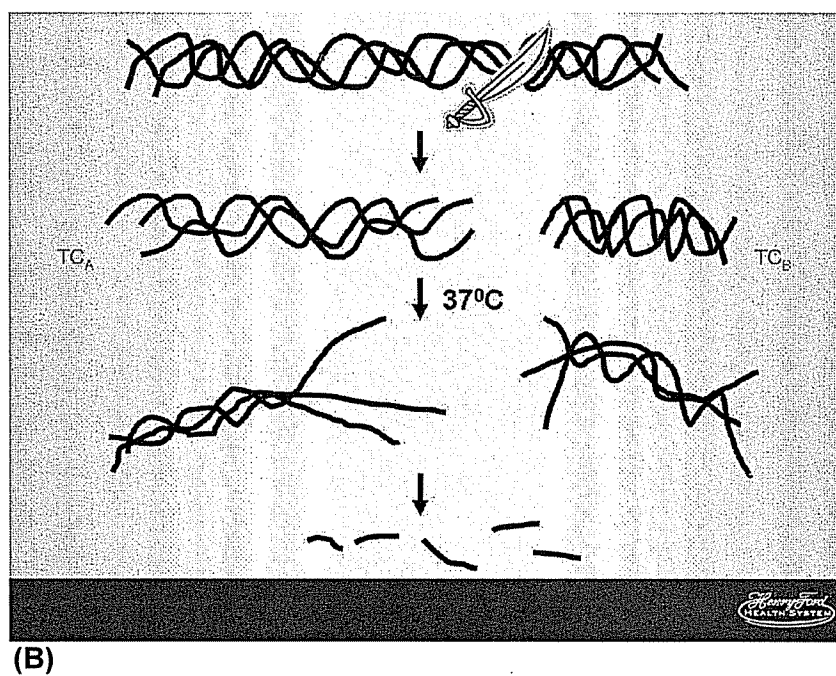
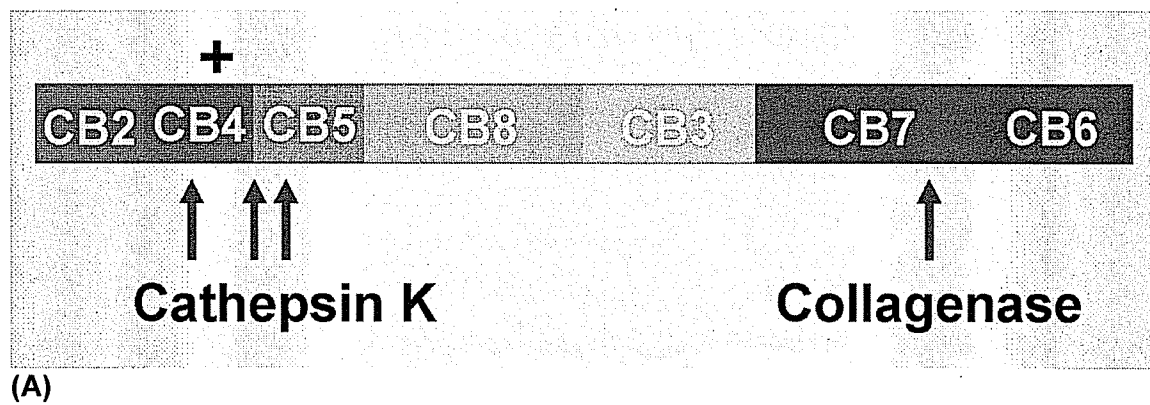
CLAIMS

What is claimed is:

1. An isolated peptide of SEQ ID NO: 1, or any subfragment thereof.
2. A method for detecting the presence of cancer tumor cells in a sample, comprising:
determining the level of a peptide comprising SEQ ID NO: 1, or any subfragment thereof, in the sample, wherein the level of SEQ ID NO: 1 or such subfragment in the sample is indicative of the presence of cancer tumor cells in the sample.
3. The method of Claim 2, wherein the sample is a human sample.
4. The method of Claim 2, wherein the cancer is breast or prostate cancer.
5. A method of stimulating the production of proteases in a mammal, comprising:
administering to the mammal a therapeutically effective amount of a peptide of SEQ ID NO: 1 or any subfragment thereof that stimulates the production of at least one protease in the mammal.
6. The method of Claim 5, wherein the subject is a human.
7. An isolated peptide of SEQ ID NO: 2, or any subfragment thereof.
8. A method for detecting the presence of cancer tumor cells in a sample, comprising:
determining the level of a peptide comprising SEQ ID NO: 2, or any subfragment thereof, in the sample, wherein the level of SEQ ID NO: 2 or such subfragment in the sample is indicative of the presence of cancer tumor cells in the sample.

9. The method of Claim 8, wherein the sample is a human sample.
10. The method of Claim 8, wherein the cancer is breast or prostate cancer.
11. A method of stimulating the production of proteases in a mammal,
comprising:
administering to the mammal a therapeutically effective amount of a peptide
of SEQ ID NO: 2 or any subfragment thereof that stimulates the production of
at least one protease in the mammal.
12. The method of Claim 11, wherein the subject is a human.
13. An isolated peptide of SEQ ID NO: 3, or any subfragment thereof.
14. A method for detecting the presence of cancer tumor cells in a sample,
comprising:
determining the level of a peptide comprising SEQ ID NO: 3, or any
subfragment thereof, in the sample, wherein the level of SEQ ID NO: 3 or
such subfragment in the sample is indicative of the presence of cancer tumor
cells in the sample.
15. The method of Claim 14, wherein the sample is a human sample.
16. The method of Claim 14, wherein the cancer is breast or prostate cancer.
17. A method of stimulating the production of proteases in a mammal,
comprising:
administering to the mammal a therapeutically effective amount of a peptide
of SEQ ID NO: 3 or any subfragment thereof that stimulates the production of
at least one protease in the mammal.
18. The method of Claim 17, wherein the subject is a human.

19. An isolated peptide of SEQ ID NO: 4, or any subfragment thereof.
20. A method for detecting the presence of cancer tumor cells in a sample, comprising:
determining the level of a peptide comprising SEQ ID NO: 4, or any subfragment thereof, in the sample, wherein the level of SEQ ID NO: 4 or such subfragment in the sample is indicative of the presence of cancer tumor cells in the sample.
21. The method of Claim 20, wherein the sample is a human sample.
22. The method of Claim 20, wherein the cancer is breast or prostate cancer.
23. A method of stimulating the production of proteases in a mammal, comprising:
administering to the mammal a therapeutically effective amount of a peptide of SEQ ID NO: 4 or any subfragment thereof that stimulates the production of at least one protease in the mammal.
24. The method of Claim 23, wherein the subject is a human.



FIGURES 1(A)-(B)

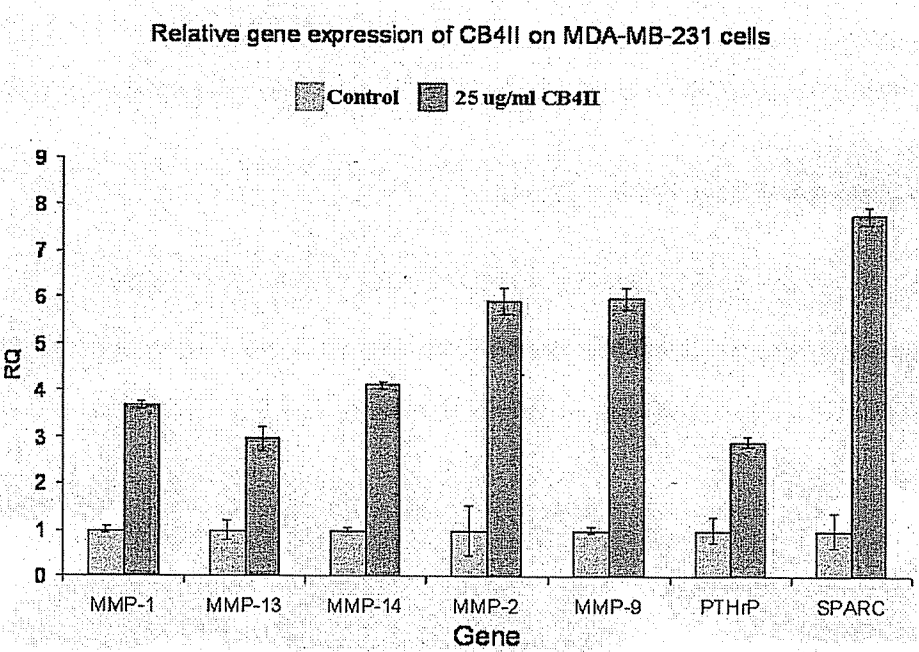


FIGURE 2

MDA-MB-231

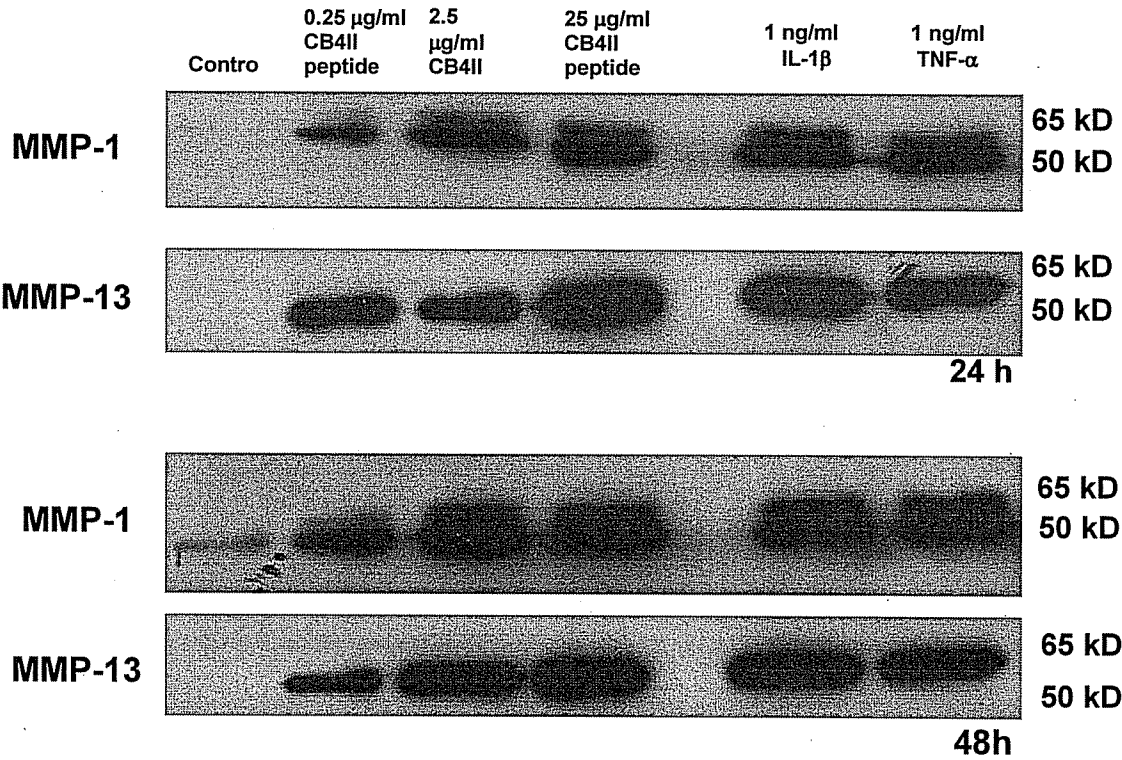
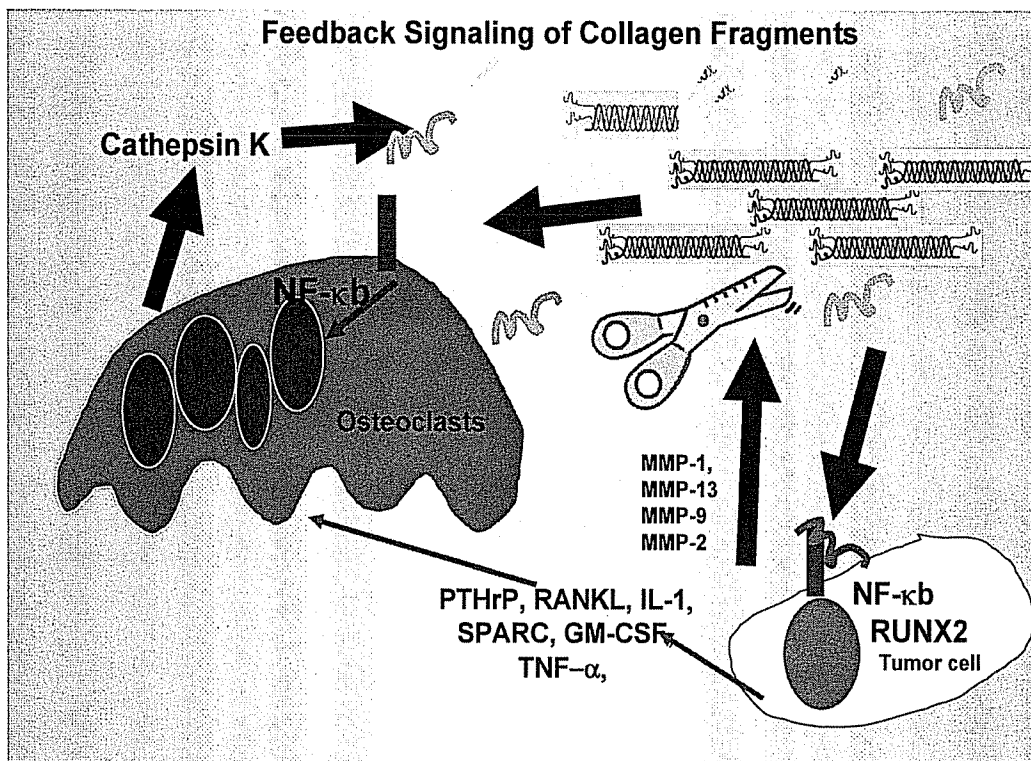


FIGURE 3

**FIGURE 4**

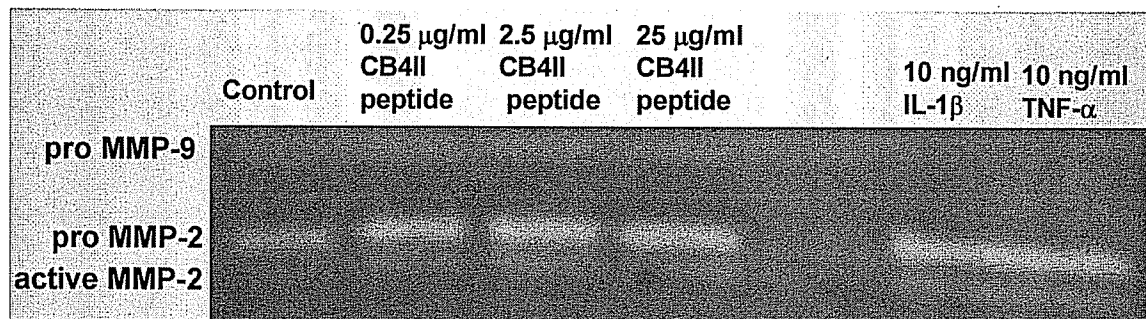


FIGURE 5

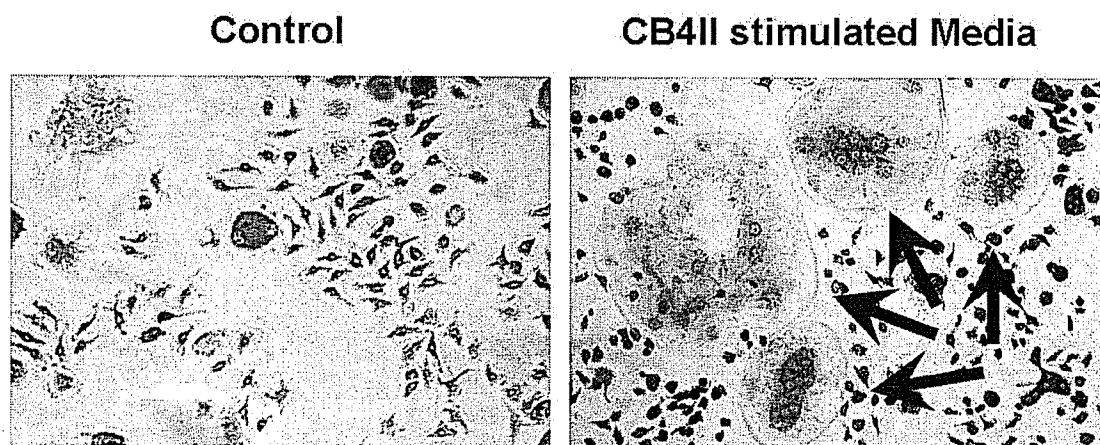


FIGURE 6

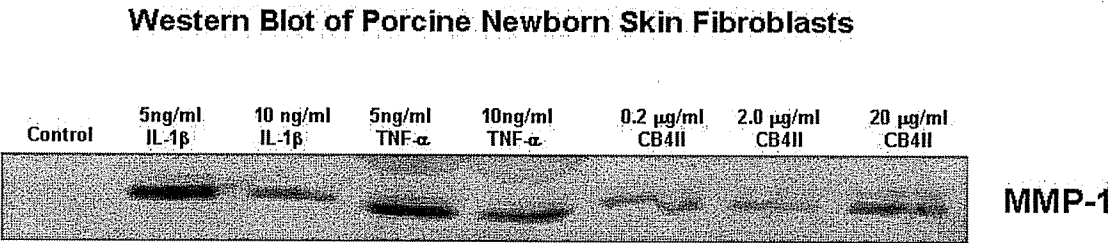


FIGURE 7

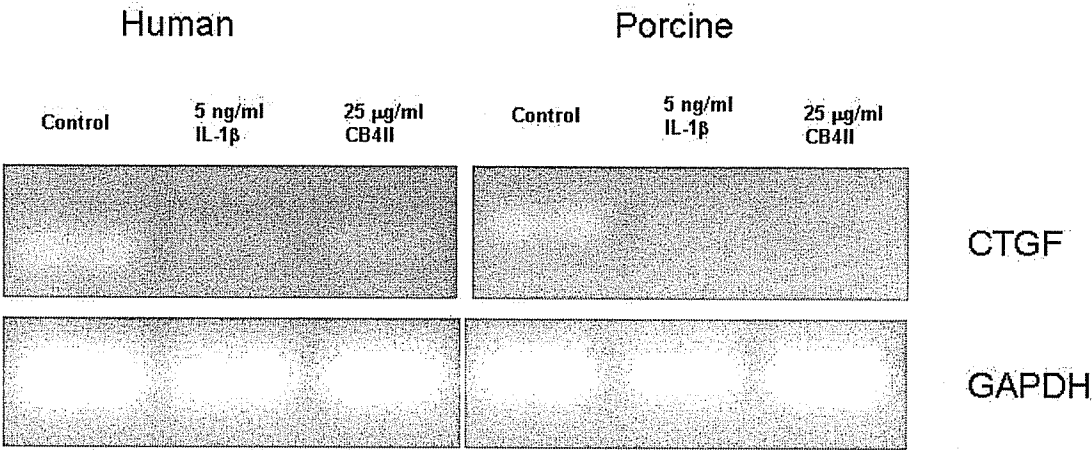
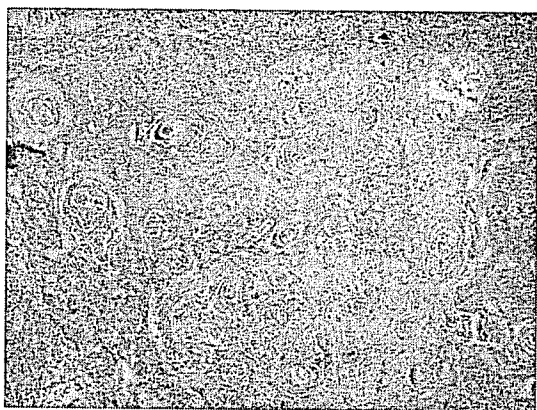
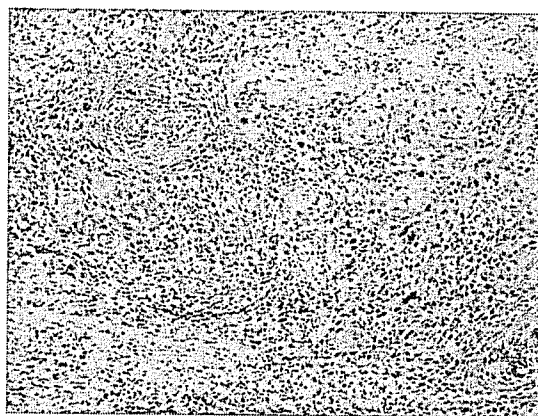


FIGURE 8



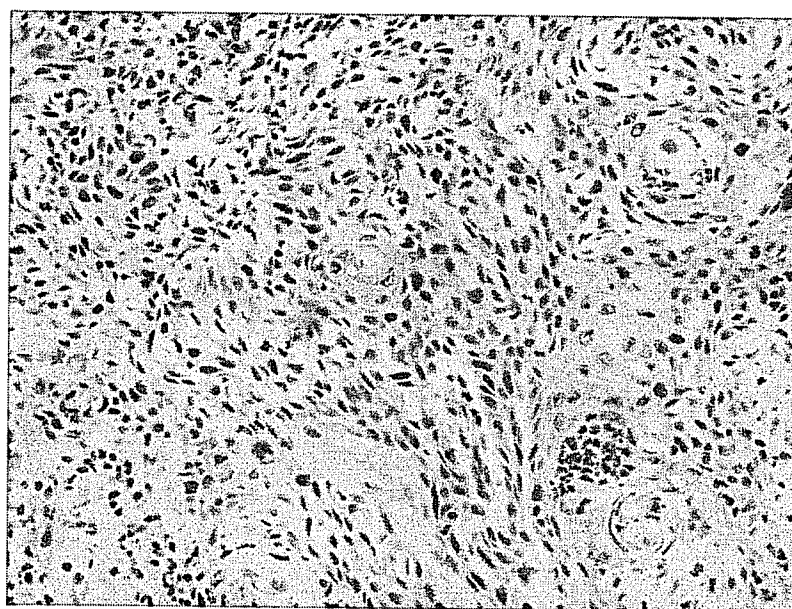
H&E

(9A)



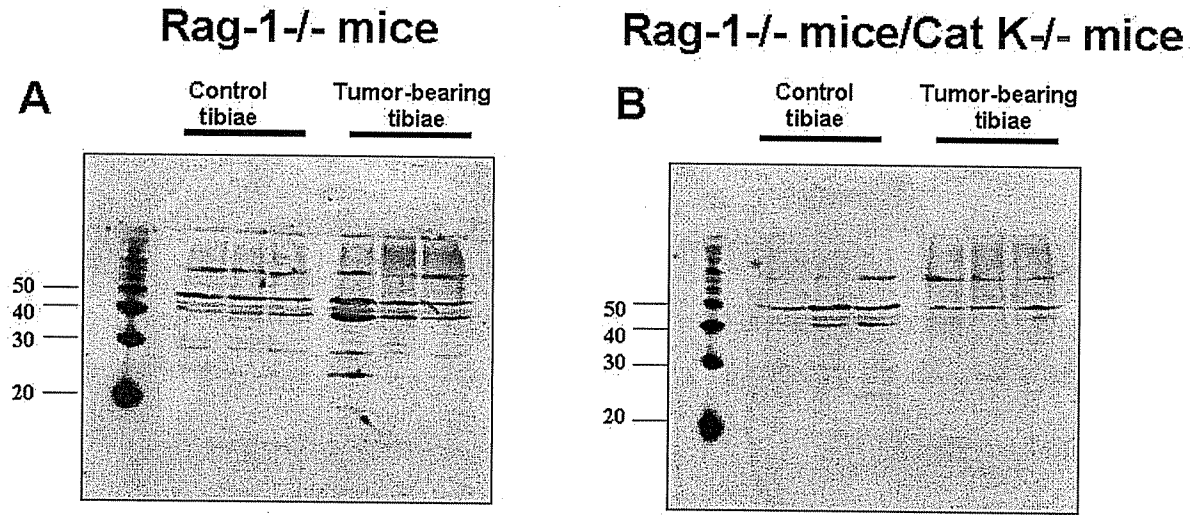
Anti-CB4II

(9B)



(9C)

FIGURES 9(A)-(C)



FIGURES 10(A)-(B)

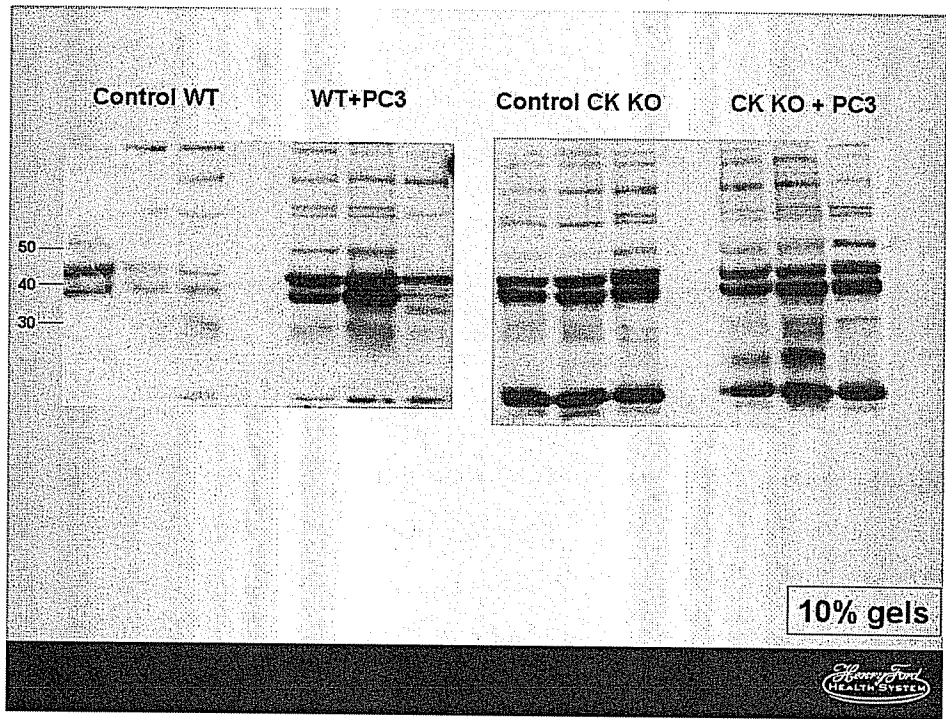


FIGURE 11