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Lefty peptides, derived by MMP2 cleavage, act as a new class of gelatinase A inhibitor

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1. ABSTRACT

MMPs are zinc-dependent endopeptidases that are involved in proteolysis of extracellular matrix in both physiological and pathological processes including cancer. MMPs are involved at all stages of tumor progression, including tumor growth, angiogenesis, and metastasis. We recently showed that overexpression of Lefty in cancer cells restrains tumor growth. Here, we show that small forms of Lefty are generated by MMP2 (gelatinase A) mediated cleavage. In turn, these forms of Lefty strongly inhibit the autocatalytic, gelatinolytic and caseinolytic activities of MMP2 *in vitro*. We show that a short synthesized form of Lefty peptide (CASDGALVP) inhibits gelatinolytic and caseinolytic activities of MMP2 *in vitro* and inhibits tumor growth *in vivo*. Together, these findings show that lefty peptides are a new class of gelatinase A inhibitors that restrain tumor growth.

2. INTRODUCTION

Proteolysis of extracellular matrix (ECM) is essential both to physiological and pathological processes. One of the primary enzyme families involved in breakdown of ECM is matrix metalloproteinase's (MMPs) (1). Members of this family are zinc-dependent endopeptidases and play key roles in embryonic development, neurological processes, wound healing, angiogenesis, arthritis, cardiovascular diseases, and cancer. Since MMPs have been implicated at all stages of tumor progression, including tumor growth, angiogenesis, and metastasis (2), many anti-cancer strategies have been tailored at targeting the activity of this enzyme family. The results of many clinical trials, however, have been less than favorable due to the lack of an objective clinical response, inhibitor selectivity and undesirable side effects (1,3). Despite these early failures, targeting specific

gelatinases remains a desirable approach in cancer therapy and several new inhibitors have shown to be effective in inhibition of tumor invasion and metastasis (4-5). Among MMPs, gelatinase A (MMP-2), is most favored since it is highly expressed in human cancers, and is thought to be a key molecule involved in cancer progression (2,6).

Lefty is a member of TGF-beta family and is one of the four highly enriched genes in embryonic stem cells (7). We recently showed that Ebaf or Lefty B (referred to as Lefty hereafter) overexpression restrains tumor growth in a fibrosarcoma model (8). Here, we show that Lefty is a target for proteolytic cleavage by MMP2. In turn, these proteolytic fragments of Lefty act as strong inhibitors of MMP2. Moreover, a short synthesized peptide of Lefty retains this activity *in vitro* and exhibits anti-cancer effect *in vivo*. Together, these findings show that Lefty peptides might be useful as an inhibitor of gelatinase A.

3. MATERIALS AND METHODS

3.1. Materials, cells, tissues and viruses

Chemicals were from Sigma-Aldrich Company (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). GM6001 was obtained from Chemicon International (Temecula, CA) and MMP2 from EMD Bioscience (La Jolla, CA). Recombinant Human Lefty-A was obtained from R & D Systems, Inc (Minneapolis, MN). The goat polyclonal antibody to the carboxy terminus of mouse Lefty (M-20), and HRP-conjugated donkey anti-goat IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bio-Rad Protein Assay kit was from Bio-Rad Laboratories (Hercules, CA). EnzChek Gelatinase Assay Kit was from Molecular Probes Inc (OR).

GPE+86, 293 and BeWo cells were obtained from ATCC (Manassas, VA). Placenta and colon tissues were obtained under a protocol approved by the institutional Committee on the Use of Human Subjects in Medical Research. Primary cultures of decidual cells were prepared and used after two passages as previously described (9). Ad-Lefty and Ad-CMV (control) adenoviral particles were purified by two successive rounds of cesium chloride equilibrium centrifugation and quantified by Absorbance at 260 nm where 1 O.D. = 1 x 10^{12} virus particles/ml. Cells were transduced or transfected as previously described (10).

3.2. Zymography

Zymographies were carried out as described (8,11). Proteins extracted from cells were separated by electrophoresis in presence of 0.1% sodium dodecylsulfate through 8% polyacrylamide gels, and were co-polymerized with gelatin (1 mg/ml). After 1.5 h of electrophoretic migration at 25 mA, sodium dodecylsulfate was removed by rinsing the gels with 2.5% Triton X-100. The gels were incubated overnight at 37°C in 50 mmol/l Tris-HCl buffer, pH 7.5, containing 200 mmol/l NaCl, 5 mmol/l $CaCl_2$, 0.2 mmol/l sodium azide and stained with 0.25% (wt/vol) Coomassie Brilliant Blue R (Sigma, St. Louis, MO), and destained with 40% ethanol/10% acetic acid (vol/vol). Proteinase activity was evident as cleared (unstained) bands.

3.3. SDS-polyacrylamide gel electrophoresis and Western blotting

The culture media were centrifuged at 7,000 xg, 4°C, for 3 hrs and concentrated by about 20 folds using Centricon 10 (protein molecular size cut-off: 10 kDa, Millipore Corporation, Bedford, MA). Proteins from tissues and cells were prepared by direct lysis in lysis buffer (50 mM Tris.HCl, pH7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail). The concentration of proteins in concentrated media and tissue lysates was determined by Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Protein samples were fractionated in a 12% denaturing gel together with pre-stained protein ladder (Life Technologies, Inc., Rockville, MD) and were subsequently blotted onto nitrocellulose membrane in a Mini-Trans-Blot apparatus (Bio-Rad Laboratories, Hercules, CA). Blots were stained with antibodies to Lefty. The secondary antibody used was donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, CA). Bands were detected by chemiluminescence as described by the manufacturer.

3.4. Quantitative gelatinase assay

MMP activity was measured by EnzChek Gelatinase Assay Kit (Molecular Probes Inc., OR), as per manufacturer's protocol. The assay buffer consists of 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$ 0.2 mM sodium azide, pH 7.6.Assay (200 µl) was performed in a 96 Well Falcon plate using the fluorescent substrate DQ-gelatin (50 µg/ml) at room temperature. MMP2 activity was determined from the fluorescence released from DQ-gelatin digestion. Fluorescence was measured at emission at 530 nm, and excitation at 485 nm, every 15 min for 2 hr period in Cytofluor II (PerSeptive Biosystems, Framingham, MA).

4. RESULTS

We recently showed that overexpression of Lefty in cancer cells restrains tumor growth *in vivo* (8). By site-directed point mutation, we previously identified two bona fide convertase cleavage sites; RGKR (aa^{74-77}) and RHGR ($aa^{132-135}$) in Lefty (12). Cleavage of Lefty precursor (P: 44 kDa) at these sites produces two proteins of 34 (L: Long form) and 28 (S: Short form) kDa. To gain more insight on forms of Lefty released from cells, we assessed Lefty peptides released by 293 cells that were transfected with Lefty A and B. In addition, to the well characterized protein bands of Lefty, other polypeptides, smaller than 28 kDa, were also observed (Figure 1A). To validate these findings by a different approach in a different cell type, primary cultures of a decidual fibroblasts were transduced with adenoviral vectors transducing Lefty (Lefty B). Western blot analysis of the culture supernatants, showed that, in addition to the P, L and S forms of Lefty, smaller polypeptide bands were present in culture media of Lefty transduced cells, in a viral particle number dependent manner (Figure 1B). To determine whether similar products can be found *in vivo*, proteins extracted from colon, known

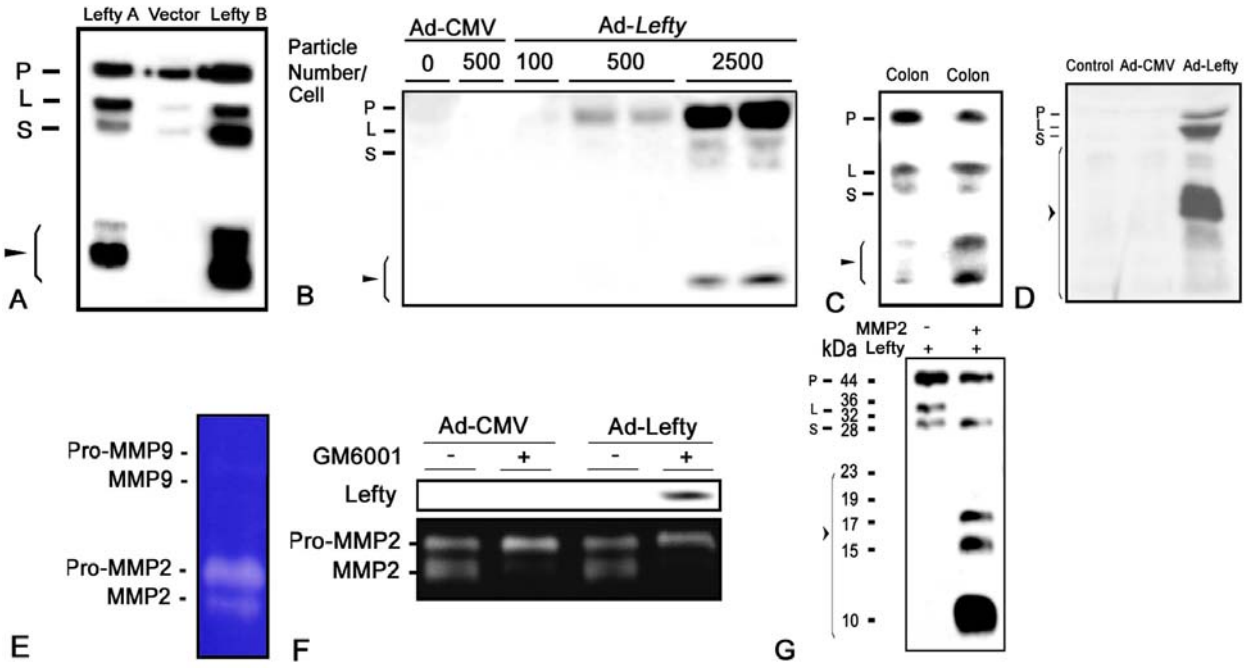

Figure 1. A. Short forms of cleaved Lefty A and B are produced in transfected cells. 293 cells were transfected with Lefty A, empty vector (Vector) and Lefty B and cultured for 48 hr in serum free medium. Conditioned media were subjected, after concentration, to Western blot analysis for Lefty. B. Short forms of cleaved Lefty are produced in Lefty transduced cells. Human decidual fibroblasts were transduced with Ad-CMV (control) and Ad-Lefty adenovirus particles and cultured for 48 hr in serum free medium. Conditioned media were subjected, after concentration, to Western blot analysis for Lefty. C. Short cleaved forms of Lefty are present in tissues. Tissue lyastes of two human colons were subjected to Western blot analysis for Lefty. D. Short forms of cleaved Lefty are produced in Lefty transduced BeWo cells. BeWo cells were transduced without (Control) and with Ad-CMV (Control) and Ad-Lefty (5000 particle/cell) for 48 hr and then culture media were subjected to Western blot analysis. E. BeWo cells release MMP2 into culture medium. Culture medium of BeWo cells were subjected to gelatin zymography. F. Inhibition of MMP2 prevents Lefty cleavage. BeWo cells were transduced with Ad-CMV (Control) and Ad-Lefty (5000 particle/cell) for 48 hr in the absence (-) and presence (+) of 5 μM inhibitor of MMPs, GM6001. The culture media were then subjected to Western blot analysis for Lefty (upper panel) and gelatin zymography (lower panel). G. Recombinant MMP2 leads to Lefty cleavage. 400 ng of recombinant Lefty was incubated without (lane 1) and with (lanes 2) 400 ng recombinant MMP2 for 48 hr. An aliquot of the mixture was subjected to Western blot analysis for Lefty. P: Precursor (44 kDa), L: Long form (34 kDa), S: Short form (28 kDa), Arrowhead points to short cleaved forms of lefty (<17 kDa).

to express Lefty, were subjected to Western blot analysis. Similar small forms of Lefty polypeptides were found in these protein extracts (Figure 1C). These findings show that these short polypeptides are bona fide products of Lefty, independent of cell type both *in vitro* and *in vivo*.

Since Lefty has only two known convertase cleavage sites, we hypothesized that the smaller protein bands of Lefty might result from a convertase independent cleavage. Since Lefty is known to induce MMPs, we considered that Lefty might be a substrate for MMP cleavage (12). Analysis of amino acid sequence of Lefty protein according to MMP cleavage site motifs showed several potential sites for cleavage by MMP2 (13). To examine the potential of MMP2 in cleaving Lefty, BeWo cells which are known to produce significant quantities of MMP2 were transduced to express Lefty. Other than the expected P, L and S forms of Lefty, there were several smaller bands in the culture supernatant of BeWo cells transduced to express Lefty (Figure 1D). Gelatin zymogram of BeWo cells showed abundant MMP2 and not MMP9 (Figure 1E). This finding suggested that Lefty is subject to cleavage by MMP2 and not MMP9. To determine whether MMP2 released from BeWo cells leads to Lefty cleavage, cells were transduced in presence of the hydroxamic acids, GM6001 (3-(N-hydroxycarbamoyl)-(2R)-isobutylpropionyl-L-tryptophan methylamide). GM6001 is a general inhibitor of all MMPs with K_i values of less than 100 nM (14-15). In the absence of GM6001, pro-MMP2 from BeWo cells was converted to MMP2, and Lefty precursor was completely cleaved while in presence of GM6001, Lefty cleavage was inhibited (Figure 1F). To directly demonstrate cleavage of Lefty by MMP2, recombinant Lefty proteins were incubated with recombinant MMP2 and then the mixture was subjected to Western blot analysis for Lefty (Figure 1G). Incubation of Lefty with MMP2 for a period shorter than 48 hr showed transitional bands > 17 kDa (not shown). With additional incubation, several distinct protein bands, smaller than 17 kDa were detected by an antibody to a Lefty C-terminal

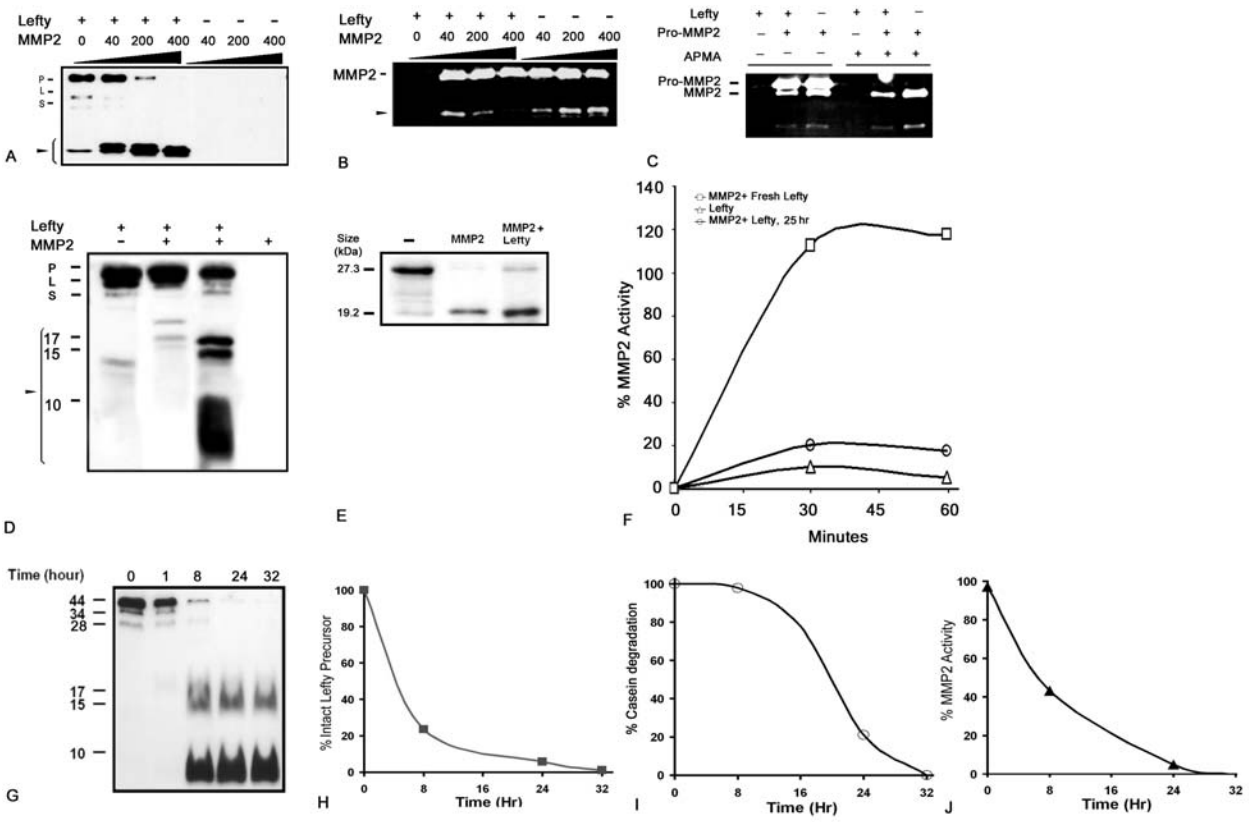

Figure 2. A. Dose dependent cleavage of Lefty by MMP2. MMP2 (0, 40, 200 and 400 ng) was incubated with recombinant Lefty A (400 ng) (+Lefty) and without Lefty (-Lefty) for 17 hr at 37 ^{o}C and then the mixture was subjected to Western blot analysis for Lefty. Arrowhead points to short cleaved forms of Lefty. B: Autocatalysis of MMP2 is inhibited with the formation of MMP2 cleaved Lefty peptides. A sample of the mixture shown in A was subjected to gelatin zymography. Arrowhead points to the 42.5 kDa autocleaved form of MMP2. C. Lefty inhibits gelatinolysis and autocatalytic production of 42.5 kDa MMP2. Recombinant pro-MMP2 (400 ng) was incubated in presence (+) and absence (-) of Lefty A (400 ng) and without (-) and with (+) APMA (1 mM) for 17 hr at 37^{o}C. Samples were then subjected to gelatin zymography. D. Cleaved forms of Lefty inhibit MMP2 activity. Lefty and MMP2 were incubated alone and in equimolar concentration. An aliquot of the MMP2 incubated without and with Lefty was subjected to Western blot analysis for Lefty after 8 (lane 2) and 25 (lanes 1, 3, 4) hr. E. After 25 hr, an aliquot of the mixture shown in D was incubated for two hours with 2 mg of casein at 37^{o}C and then the mixture was subjected to SDS-PAGE followed by staining on Coommassie blue staining. F: Gelatinolytic activity of MMP2 was measured on an aliquot of sample shown in D after 25 hr of incubation. Each Reading was performed at 15, 30, 45 and 60 minutes. Reading from Lefty alone was used as background. G. Cleaved forms of Lefty inhibit MMP2 activity. Recombinant Lefty A (100 ng) and MMP2 (100 ng) were incubated for 0, 1, 8, 24 and 32 hr at 37^{o}C and then the mixture was subjected to Western blot analysis for Lefty (Top, left panel). H. Densitometric analysis of the lefty precursor band shown in I. Percentage of casein degradation from sample in G was determined by carrying out caseionlytic assay of the mixture. J: EnzChek Gelatinase Assay was carried out on sample of the mixture shown in G for determination of MMP2 activity. P: Precursor, L: Long form, S: Short form

peptide (Figure 1G). The size of the bands matched those predicted from MMP2 cleavage motif analysis. Together, these findings show that Lefty is subject to cleavage by MMP2.

To gain an insight on the biologic activity of these small Lefty products, Lefty was incubated with MMP2 and then, the cleavage of Lefty (Figure 2A) and autocatalytic activity of MMP2 (Figure 2B) was assessed by gelatin zymography. The autocatalytic cleavage of the NH2-terminal, 80 amino acid fragment of MMP2 releases a 42.5 kDa truncated enzyme that retains its gelatinolytic activity (16-18). Appearance of the small cleaved Lefty products, led to inhibition of autocatalytic activity of MMP2 in a dose dependent manner (Figure 2B). To validate these findings, Lefty was incubated with pro-MMP2 in presence and absence of APMA (p-amino-phenol-mercuric acetate) which converts the pro form of MMP2 to active MMP2. Complete conversion of pro-MMP2 to MMP2 yielded more autocatalytic, 42.5 kDa MMP2 in the absence rather than presence of Lefty (Figure 2C).

To quantitate the level of inhibition of MMP2 upon production of cleaved Lefty products, Lefty was incubated with MMP2 and then the mixture was subjected

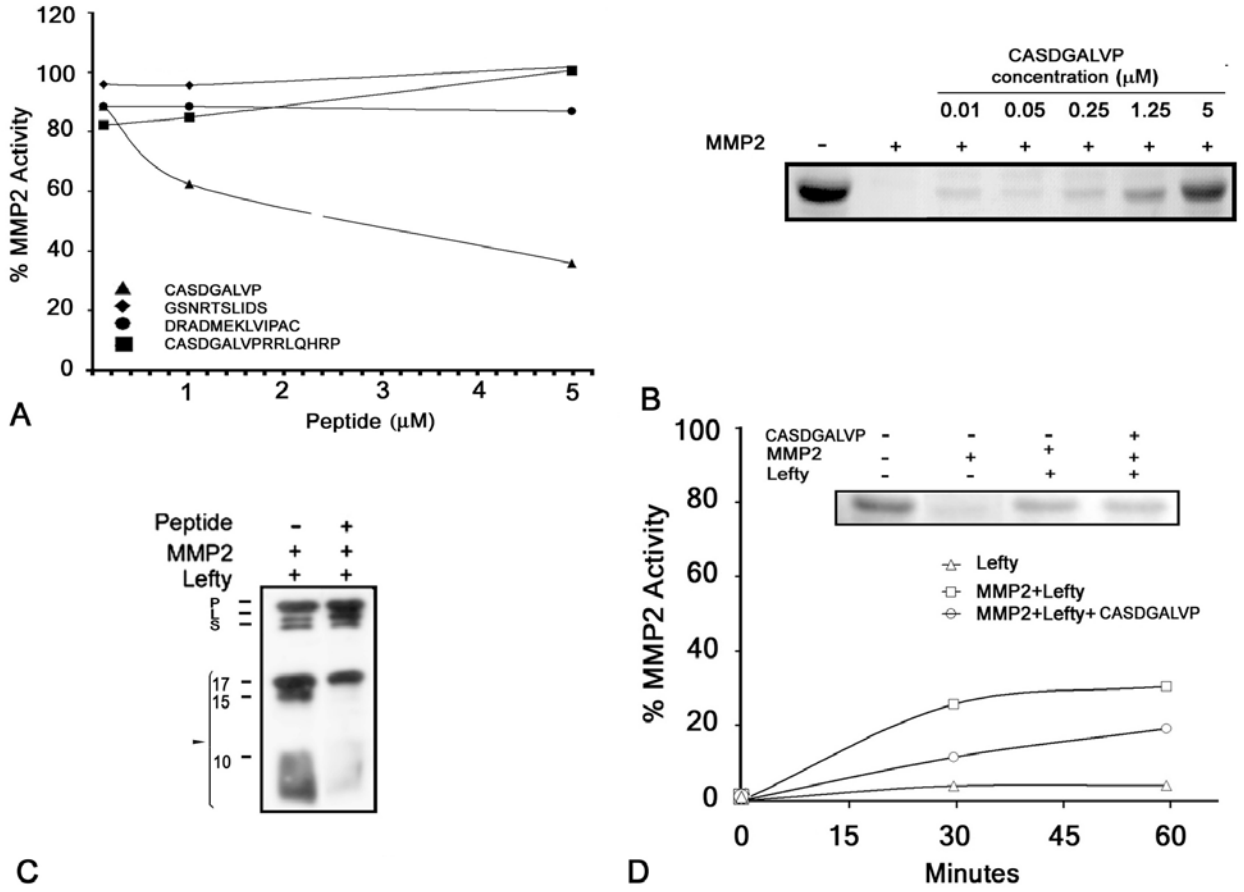

Figure 3. Inhibition of the MMP2 activity by Lefty peptides *in vitro*. A: 100 ng of MMP2 was incubated with various lefty peptides for 24 hr and then the mixture was subjected to EnzChek Gelatinase Assay. MMP2 incubated without peptide was used as positive control. Percent of total MMP2 activity is shown. B: CASDGALVP peptide inhibits the MMP2 mediated caseinolysis in a dose dependent manner. 70 ng of MMP2 was incubated with various concentrations of CASDGALVP peptide for 24 hours and then the mixture was incubated with casein to determine its ability to degrade casein. Casein (2 μg) incubated without MMP2 served as positive control. C. CASDGALVP peptide inhibits MMP2 mediated Lefty cleavage. Lefty (100 ng) was incubated with MMP2 (200 ng) in a total volume of 20 μl without and with CASDGALVP (5 μM) for 20 hr at 37^{o}C. Following incubation, a sample was subjected to Western blot analysis of Lefty. Arrowhead points to short cleaved forms of Lefty. D. CASDGALVP peptide inhibits the MMP2 mediated caseinolysis in a time dependent manner. 70 ng of MMP2 was incubated alone, with lefty (100 ng) and with CASDGALVP peptide (0.25 mM) for two hours. An aliquot of mixture was subjected to EnzChek Gelatinase Assay. Another sample of the mixture was subjected to caseinolysis (Inset). Casein (2 μg) incubated without MMP2 served as positive control.

Table 1. List of peptides

Name	Size (kDa)	Sequence
C-Peptide	0.832	CASDGALVP
C-Peptide, modified [1]	0.873	CASDGALVP
C-Peptide, Extended	1.816	CASDGALVPRRLQHRP
Irrelevant -Peptide	0.962	GSNRTSLIDS
N-Peptide	1.502	DRADMEKLVIP

[1] N-terminal Acetylated and C-Terminal Amidated

to Western blot analysis for Lefty to confirm Lefty cleavage (Figure 2D). The caseinolytic activity of the mixture was also assessed (Figure 2E). An aliquot of the same mixture was subjected to a quantitative gelatinolytic assay (Figure 2F). The findings showed that accumulation of cleaved Lefty products led to a significant inhibition of caseinolytic and gelatinolytic activities of MMP2. Since the MMP2 cleaved Lefty products accumulate over time, the same analyses were carried out over an extended time period (Figure 2G-J). The findings showed that cleavage of Lefty by MMP2 was time dependent and that the accumulation of these cleaved products, led to a time dependent decrease in both caseinolytic and gelatinolytic activities of MMP2.

To further provide validation for the bioactivity of MMP2 cleaved Lefty fragments, we then, synthesized several Lefty polypeptides (Table 1). One C-peptide (C terminus peptide: CASDGALVP) inhibited the gelatinolytic (Figure 3A) and caseinolytic (Figure 3B) activities of MMP2, in a dose dependent manner. On the other hand, other Lefty peptides (GSNRTSLIDS, DRADMEKLVIP) did not exhibit any biological activity. Moreover, when the C peptide was modified by acetylation

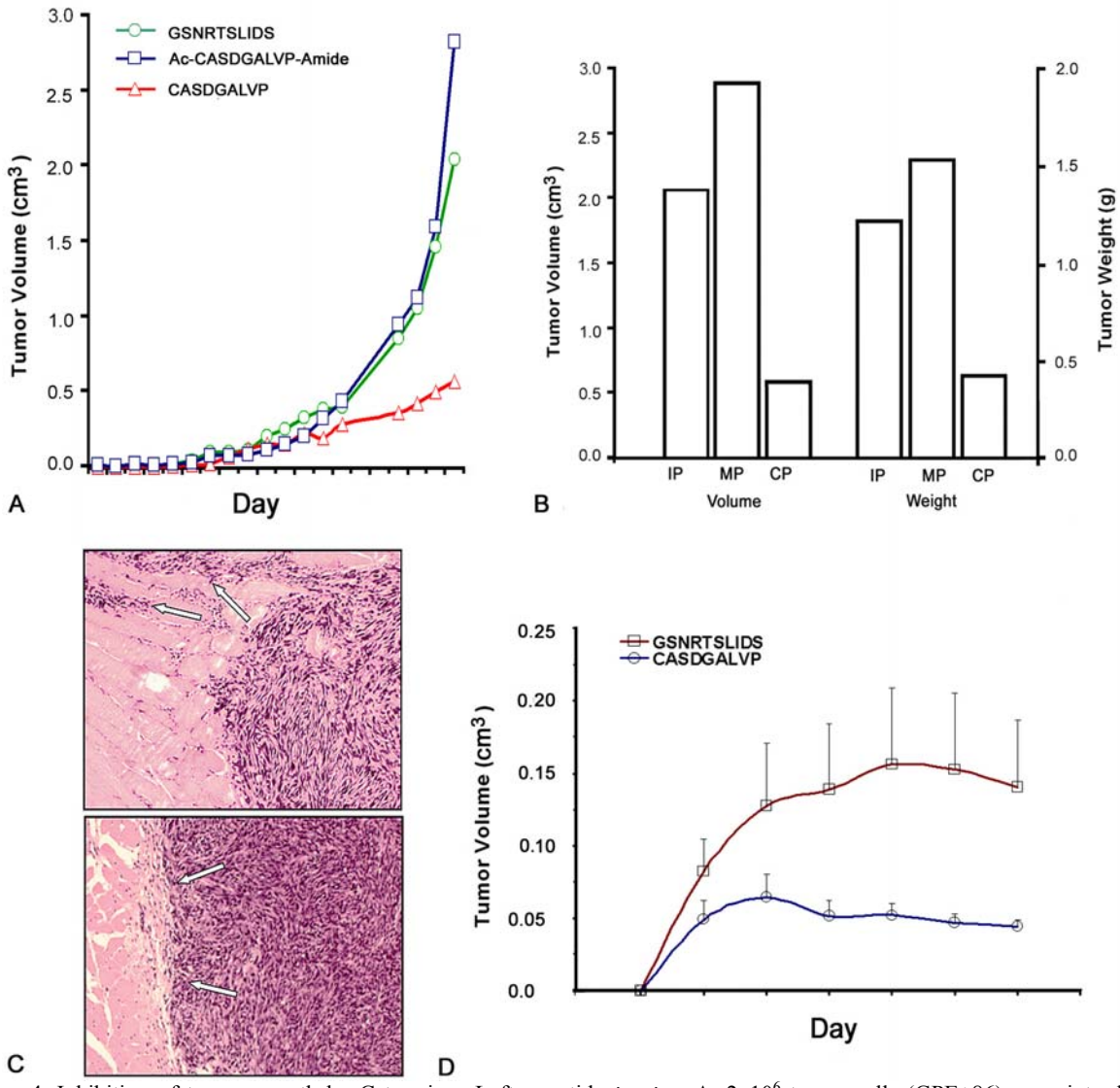

Figure 4. Inhibition of tumor growth by C terminus Lefty peptide *in vivo*. A. $2x10^6$ tumor cells (GPE+86) were introduced subcutaneously into athymic nude mice (n=6 per group). Mice received an intra-peritoneal injection of 200 µg of irrelevant (IP: GSNRTSLID), Modified C peptide (MP: Ac-CASDGALVP-Amide) and C peptide (CP: CASDGALVP) daily. Presented data are the tumor total volumes of six tumors from one of three experiments that showed similar results. B. The total weight and volumes of tumors were measured after removal of the tumors at the end of the study. C. Histologic sections of GPE+86 tumors treated without (upper panel) and with C terminus peptide (lower panel). Arrows in the upper panel point to the invasive borders of tumors showing tumor cells invading adjacent myofibers. Arrows in the lower panel show the smooth border of tumors with minimal invasion of adjacent muscle fibers. D: $2x10^6$ MDA-MB 435 cells were introduced subcutaneously into athymic nude mice (n=6). Mice received daily (100 µg) intraperitoneal injection of irrelevant (GSNRTSLID), or C peptide (CASDGALVP). Presented data are the mean of tumor volumes of six tumors from one of two experiments that showed similar results. Error bars are shown. *p* values are as follows: Day 1: 0, Day 2: 0.145986, Day 3: 0.101864, Day 4: 0.051352, Day 5: 0.049033, Day 6: 0.044811, Day 7: 0.040015.

and amidation at N-terminal and C-terminal respectively, or its length was increased (CASDGALVPRRLQHRP), the inhibitory biological activities were lost (Figure 3A). These findings show that the Lefty C peptide exerts specific inhibitory activity on bioactivity of MMP2. The CASDGALVP peptide also inhibited the caseinolytic activity of MMP2, cleavage of lefty by MMP2 and gelatinolytic activity of MMP2 (Figure 3C-D). These findings show that the Lefty C peptide exerts specific inhibitory activity on bioactivities of MMP2.

From MMPs, the activity of MMP2 and MMP9 has been closely correlated with local growth and metastatic potential. To determine that the Lefty peptides can prevent tumor growth and or invasion, fibrosarcoma cells (GP+E86) were introduced subcutaneously into athymic nude mice and the tumor growth was assessed over a period of two weeks without and with administration of peptides. Administration of C peptide (CASDGALVP) significantly inhibited tumor growth while the irrelevant peptide (GSNRTSLIDS) had no effect (Figure 4A-B).

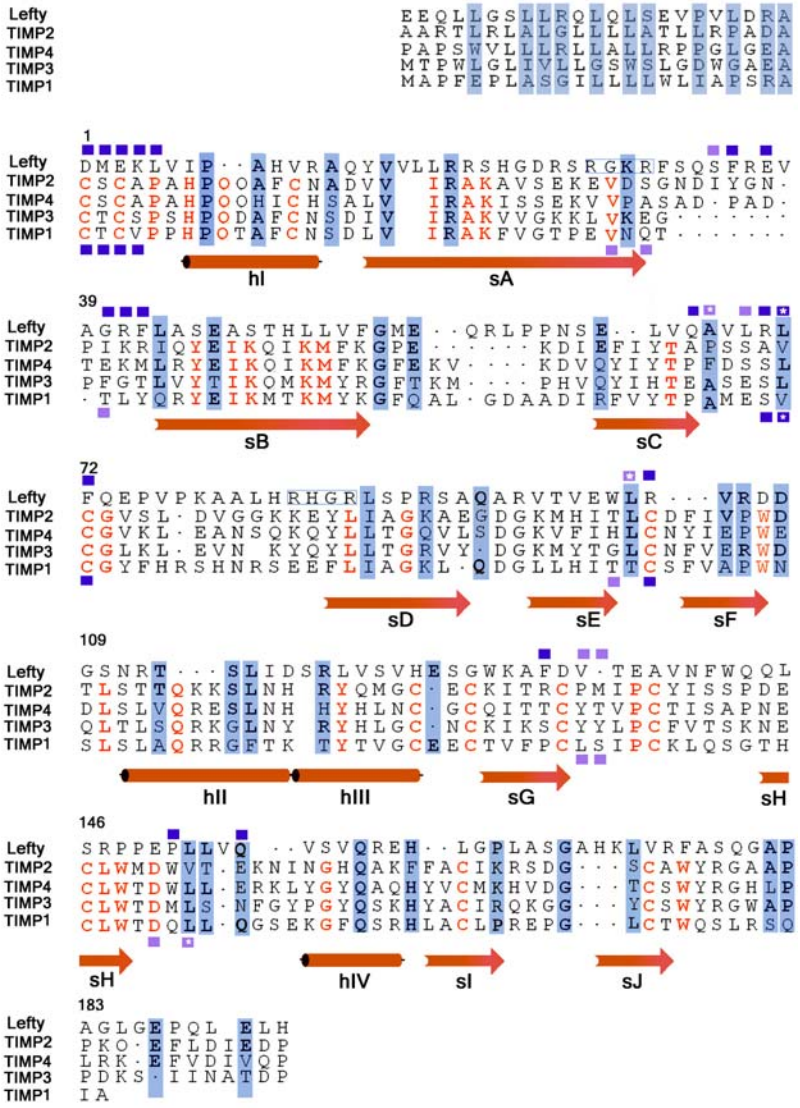

Figure 5. Alignment of N terminus of human Lefty A (EBAF/Lefty 2/Lefty B), TIMP2, TIMP4, TIMP3 and TIMP1. Strictly Conserved residues in TIMPs are shown in orange and strictly conserved residues shared between Lefty and at least one member of the TIMP family is boxed in blue. Residues strongly and weakly involved in sub 4.0 Å intermolecular atom-atom contacts of TIMP1-MMP3 (19) and TIMP2-MT1-MMP (20) are marked respectively by dark and light purple boxes and those shared with Lefty are marked by asterisk. The numbering is that of mature TIMP2. Strands of the TIMP 1 are shown by arrows and helices by cylinders. The cleavage sites in Lefty are boxed. Lefty shares 43 strictly conserved residues with TIMPs which all reside in the N and not in C terminus of Lefty. Ten of these homologous residues reside in beta strands and eight in alpha helices (cylinders). Lefty shares only two residues which are involved in sub 4.0 Å intermolecular atom-atom contacts in TIMP2-MT1-MMP. Lefty also shares only 2 of 18 residues involved in sub 4.0 Å intermolecular atom-atom contacts in TIMP1-MMP3.

Histological examination of tumor tissues showed an invasive border in wild type tumors (Figure 4C, upper panel). On the other hand, the tumors in the C-peptide treated tumors had a smooth border showing a limited invasive property (Figure 4C, lower panel). Then, the experiments were repeated using the MDA-MB 435. These cells were introduced subcutaneously into athymic nude mice and the growth of the tumors was assessed during a 7 day period in animals receiving daily intraperitoneal injection of 100 µg of either the C-peptide (CASDGALVP) or irrelevant peptide (GSNRTSLIDS). The C-peptide inhibited tumor growth of MDA-MB 435 cells as compared to that obtained by the irrelevant peptide (Figure 4D).

Since the proteolytic activity of matrix metalloproteinases is held in check by tissue inhibitors of metalloproteinase's (TMP), we compared the amino acid sequence of Lefty with the known TIMPs 1-4 (Figure 5). This analysis shows that Lefty bears a restricted homology to TIMPs. The limited homology with known members of

TIMPs, including sharing of residues that participate in TIMP-MMP2 binding, places Lefty peptides in a new class of tissue inhibitors of MMP.

5. DISCUSSION

The overexpression of MMP2 has been correlated with an invasive phenotype and as compared to their non-metastatic counterparts, metastatic tumor cell lines express higher levels of MMP2 (21). In addition, MMP2 is secreted by the host cells including, the mesenchymal cells, endothelial cells and tumor infiltrating macrophages (22-25). For this reason, MMP2 is useful in tumor targeting since the enzyme overexpressed in the tumor tissue is accessible in the tumor stroma. MMP2 inhibitors have been shown to prevent tumor growth, invasion, metastasis and angiogenesis (5, 26). The findings presented here show that Lefty is a substrate for MMP2 dependent cleavage. In turn, cleaved Lefty products inhibit the enzymatic activity of MMP2. The finding from this study is significant because they provide a broader understanding as how peptides produced through enzymatic action of MMP2 on Lefty regulate the bioactivity of MMP2 and are likely candidates for homeostasis of the extracellular matrix. Furthermore, the synthetic Lefty peptide which acts to inhibit MMP2, might be clinically useful in cancer treatment as MMP2 inhibitor.

The mechanistic view that is provided here, is likely only a glimpse of actions that are exerted by Lefty on tumor cells or their stroma. Microarray analysis has shown that Lefty, is the most abundant inhibitor in embryonic stem cells (7). Lefty regulates expression of TGF-beta family proteins such as Nodal and provides a blanket inhibition of the activity of members of this family that require EGF-CFC as a co-receptor (7). Consistent with such actions, a recent study shows that exposure of the tumor cells to a stem cell microenvironment containing Lefty leads to a dramatic down-regulation in Nodal expression, reduction in clonogenicity and tumorigenesis and an increase in apoptosis. Furthermore, this ability to suppress the tumorigenic phenotype is directly associated with secretion of Lefty (27). Interestingly, such effects are observed in both breast carcinomas and melanomas (27). The study also reveals that over-expression of Lefty limits tumorigenic potential of breast carcinoma cells. These findings are consistent with our results in Lefty inhibition of tumor growth both in fibrosarcoma and melanomas since although, MD-MB-435 was originally defined as a breast carcinoma, based on more recent gene expression profile, these cells have been re-classified as melanoma (28). Together, these results suggest that Lefty suppresses the tumorigenic phenotype, reduces invasive property and diminishes tumor growth of a variety of tumors and may offer new therapeutic strategies in treatment of tumors.

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