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Advances in Brief

Inhibition of Tumor Cell Invasion by a Highly Conserved Peptide Sequence from the Matrix Metalloproteinase Enzyme Prosegment[1]

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Abstract

The metastasis associated 72-kDa type IV collagenase is secreted as a latent proenzyme which is converted to an active 62-kDa form by autoproteolytic removal of an amino terminal profragment. The region immediately upstream from the cleavage site contains a highly conserved peptide sequence, MRKPRCGNPDV, which is present in all known members of the matrix metalloproteinase family. Evidence implicates the cysteine residue of this sequence as critical for maintenance of the latent form through coordination with the catalytic zinc atom of the active site. A synthetic peptide, TMRKPRCGNPDVAN (peptide 74), encompassing this conserved sequence, has been shown to inhibit the activated form of the 72-kDa type IV collagenase *in vitro*. In the present study we examine the ability of this peptide inhibitor to modulate tumor cell invasiveness. Peptide 74 and the control peptide 78, which contains a single substitution of serine for the "critical" cysteine residue, were added at 30 μM concentrations to the upper compartment of the Boyden chamber in the chemoinvasion assay using HT1080 and A2058 human tumor cells. In this assay a layer of reconstituted basement membrane, Matrigel, is coated onto chemotaxis filters and acts as a barrier to the migration of cells in the Boyden chambers. Only cells with invasive capacity can cross the Matrigel barrier. Peptide 74 containing the cysteine residue inhibited the invasion of both the HT1080 and A2058 cells through the Matrigel barrier; control peptide 78 was not inhibitory. Both peptides were shown to be without cytotoxic action and did not inhibit chemotaxis or affect cell number. This study demonstrates that addition of an excess peptide containing the matrix metalloproteinase prosegment inhibitory sequence can inhibit invasive activity at the cellular level and suggests that this may be a useful strategy to modulate tumor cell invasiveness *in vivo*.

Introduction

Metalloproteinases, as well as proteases from all other enzyme classes (serine, cysteine, and aspartyl), have been implicated in tumor cell invasion of extracellular matrix compartments (1–4). However, much of the work on the role of these various enzymes in tumor invasion has been correlative without direct demonstration of their involvement. Recently, we and others have used specific inhibitors of the matrix metalloproteinases, tissue inhibitors of metalloproteinases, to directly block tumor cell invasion *in vitro* (5–8). These studies suggest that metalloprotease activity in general, and specifically the 72-kDa type IV collagenase, is required for tumor cell invasion. However, it is possible that the tissue inhibitors of metalloproteinases could also act directly on the tumor cells to modify their invasive behavior.

The matrix metalloproteinase (MMP) enzymes are secreted as latent zymogens that must be activated prior to attaining proteolytic activity (9). Studies have shown that activation can be initiated *in vitro* by a variety of agents including organomercurial compounds (9, 10). These studies have also shown that the stability of the latent form of the proenzymes is probably due to the specific interaction of a highly conserved cysteinyl residue with the zinc metal atom of the active site (10–12). This hypothesis has been alternately referred to as the cysteine switch hypothesis (10) or the Velcro mechanism (11). The putative cysteine residue involved in this interaction is contained within a highly conserved region of the enzyme profragment. Recently, we have shown that a synthetic peptide encompassing this region can inhibit the activated form of the 72-kDa type IV collagenase (12). Substitution of the cysteinyl residues with a seryl residue abrogates this inhibitory activity. These profragment peptides provide specific inhibitors of the metalloproteinases and allow direct testing of the role of these enzymes in tumor cell invasion. In the present study we have examined the effect of the conserved profragment peptide sequences on tumor cell invasion *in vitro*. Our results demonstrate that these profragment peptides, which inhibit metalloproteinase activity, also block tumor cell invasion *in vitro*.

Materials and Methods

Cells. Human A2058 melanoma and HT1080 fibrosarcoma cells were cultured in DMEM[3] supplemented with 10% fetal calf serum, glutamine, and antibiotics. Cells were passaged by trypsinization and replating in supplemented DMEM. Balbc/3T3 fibroblasts were grown under identical culture conditions for the production of conditioned media used as a chemoattractant in the chemoinvasion assay (see below).

72 kDa Type IV Collagenase/Gelatinase Production as Detected by Zymography. Subconfluent cultures (80% confluent) of A2058 and HT1080 cells were rinsed 3 times with Dulbecco's phosphate buffered saline and cultured with serum free DMEM for 1 h. The medium was then changed and the cells were cultured for 24 h. The conditioned media from these cultures were collected and passed through a 0.2-Mm filter and were then stored at −20°C. Identical amounts of protein were loaded onto gelatin-containing acrylamide gels and zymography was performed as described (1). The zymograms were incubated at 37°C for 16 h in 50 mM Tris-HCl-200 mM NaCl-5 mM $CaCl_2$-0.02% Brij 35, pH 7.5, containing either peptide 74 or peptide 78 were indicated.

Synthesis and Purification of Peptides. Peptide 74, sequence TMRKPRCGNPDVAN, and peptide 78, sequence TMRKPRSGNPDVAN, were synthesized on a Biosearch 9600 solid phase peptide synthesizer using *t*-butoxycarbonyl methodology. Peptides were deblocked in HF in the presence of *p*-thiocreosol to protect sulfhydryl groups. Following ether extraction, peptides were further purified by reverse phase high performance liquid chromatography. Composition and concentration were determined by amino acid composition analysis.

Chemoinvasion Assay and Chemotaxis. Basement membrane matrigel was extracted from the Engelbreth-Holm-Swarm tumor as described

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[3] The abbreviation used is: DMEM, Dulbecco's modified medium.

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previously (13), and protein concentration was determined using standard methods. Chemoinvasion assays were performed as reported previously (14). Polycarbonate filters (8 μm pore diameter; Nucleopore) were first coated with 5 μg/filter of type IV collagen (Collaborative Research) and air-dried. Twenty-five μg of Matrigel from a 500 μg/ml dilution of Matrigel in distilled cold water were then layered onto each filter. The coating was dried at room temperature and reconstituted with DMEM. This procedure produces a thin, continuous, even coating of basement membrane material on the filters (14).

Serum free 3T3 conditioned medium, obtained by incubation of the cells for 24 h, was used as a source of chemoattractants. In certain experiments peptides 74 or 78 were added directly to the chemoattractant which was placed in the lower compartment of the Boyden chambers (see below).

A2058 or HT1080 cells were harvested by trypsinization and suspended in supplemented DMEM containing 0.1% (w/v) bovine serum albumin. Cells (8×10^4) were suspended in a final volume of 400 μl of supplemented DMEM with 0.1% bovine serum albumin. Boyden chambers were assembled using freshly prepared Matrigel coated polycarbonate filters and 3T3 fibroblast conditioned media in the lower chamber. A 400-μl sample of the human tumor cell suspension was then placed in the upper compartment of the Boyden chambers with or without peptide 74 or peptide 78 at the indicated concentrations.

The assembled chambers were incubated for 6 h at 37°C in a 10% CO_2 atmosphere. At the end of the incubation period the cells on the upper surface of the filters were mechanically removed, and the filters were fixed, stained with toluidine blue, and counted under a microscope. Five fields were counted per filter, and experiments were run in triplicate. Each triplicate assay was repeated at least twice on separate occasions.

Chemotaxis in response to 3T3 conditioned medium was assayed in the Boyden chambers using type IV collagen coated filters and omitting the Matrigel. This coating is permissive for cell attachment and migration. The effects of peptides 74 and 78 on cell migration were assessed by addition of these peptides to the upper compartment of the Boyden chamber.

Results

Production of Type IV Collagenases/Gelatinases by Tumor Cells. Analysis of A2058 melanoma and HT1080 fibrosarcoma conditioned media by gelatin zymography reveals that both cell lines secrete type IV collagenases (Fig. 1). HT1080 cells produce both the 72- and 92-kDa type IV collagenases in latent form, while A2058 cells secrete only the latent 72-kDa form. This is consistent with reports observing the production of either one or both collagenase IV forms by invasive tumor cells.

Fig. 1 also shows that the addition of peptide 74 (cysteine containing) at a final concentration of 1 mg/ml to the incubation buffer during gelatin zymography inhibits all gelatinolytic activity ascribed to the 72-kDa and 92-kDa enzymes. Addition of peptide 78 (serine containing) at identical concentrations causes no loss in gelatinolytic activity when compared to the control.

Inhibition of Tumor Cell Invasion by a Peptide Sequence from the Matrix Metalloproteinase Prosegment. A2058 and HT1080 cells were stimulated to migrate and invade through a reconstituted basement membrane in the chemoinvasion assay in response to Balbc/3T3 conditioned medium, a potent chemoattractant. Chemotaxis assays in which the Boyden chambers are assembled with filters lacking the matrigel barrier were also performed. These experiments control for agents which may disrupt tumor cell invasion by interfering with tumor cell attachment and migration.

The peptide TMRKPRCGNPDVAN, derived from a highly conserved cysteine containing sequence from the prosegment of the 72-kDa type IV collagenase (Peptide 74) was added along with the human tumor cells in the upper compartment of Boyden chambers to evaluate the effect of these reagents on tumor cell invasion. In the absence of added peptide both cell lines were highly invasive even when low numbers of tumor cells were placed in the upper compartment. Addition of peptide 74 at a concentration of 30 μM reduced both A2058 and HT 1080 tumor cell invasion by 60–80% (Figs. 2 and 3). The maximal reduction was achieved by these concentrations inasmuch as increasing the concentration of peptide 74 to 100 μM did not potentiate inhibition above the 80% seen at 30 μM (Fig. 3). Peptide 74 was not effective in blocking tumor cell invasion if added to the lower compartment of the Boyden chamber. Peptide 78, with the sequence TMRKPRSGNPDVAN in which the critical cysteine is replaced with a serine residue, did not inhibit A2058 or HT1080 tumor cell invasion when added to either the upper (Fig. 2) or lower compartment of the Boyden chamber. Furthermore, neither peptide altered the chemotactic response of either A2058 or HT1080 cells to the 3T3 cell conditioned medium (Table 1; data shown only for A2058 cells).

Peptide 74 inhibited HT1080 cell invasion of the Matrigel barrier in a dose dependent fashion (Fig. 3). HT1080 cells were

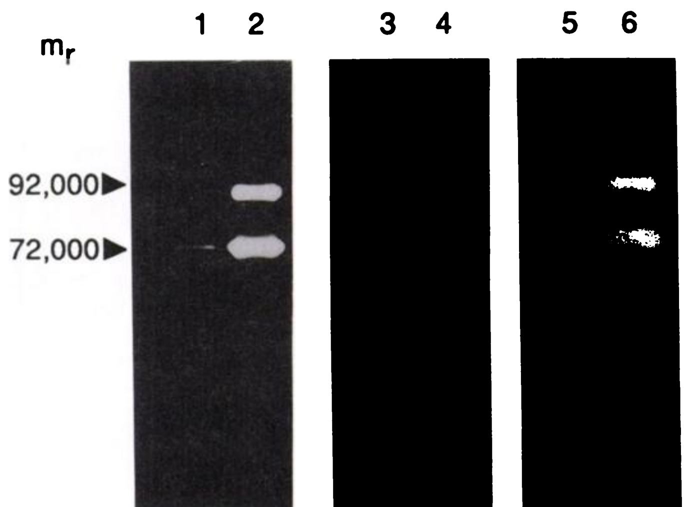

Fig. 1. Zymogram analysis of media conditioned with A2058 and HT1080 cells and inhibition of gelatinase activity by peptide 74. Conditioned media from A2058 melanomas (*lanes 1, 3,* and *5*) and HT1080 fibrosarcoma cells (*lanes 2, 4,* and *6*) were subjected to zymography as described in "Materials and Methods." The gel was incubated at 37°C for 16 h without peptide (*lanes 1* and *2*) or with 1 mg/ml peptide 74 (*lanes 3* and *4*) or 78 (*lanes 5* and *6*). *Left ordinate,* migration of molecular weight markers.

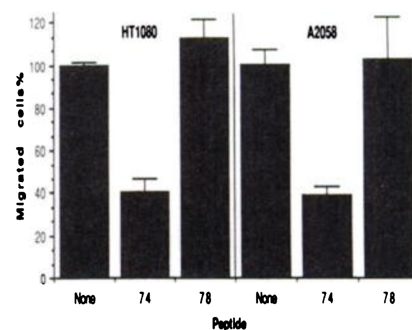

Fig. 2. Inhibition of HT1080 and A2058 cell chemoinvasion by peptides (*pep*) from the conserved sequence of the matrix metalloproteinase enzyme prosegment. Peptide 74 or control peptide 78 were added to the upper compartment of Boyden chambers at 30 μM. Experiments were run in triplicate and repeated three times. Average values ± SD of a typical experiment are shown. The results demonstrate that peptide 74 specifically inhibits chemoinvasive activity of both HT1080 and A2058 cell lines.

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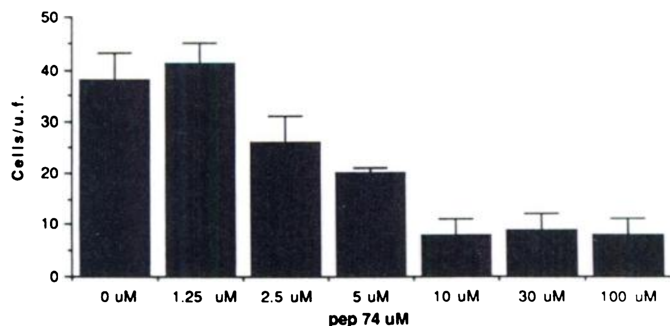

Fig. 3. Dose dependent response for peptide 74 inhibition of chemoinvasion by HT1080 cells. Experiments were performed in triplicate and repeated twice. Average values ± SD of a single experiment are shown. The lowest dose of peptide 74 which showed inhibitory activity was 2.5 μM.

Table 1 *Chemotaxis of A2058 cells in the presence of peptides 74 and 78*

Peptide	Chemotaxis to conditioned medium (migrated cells/field)
No added peptide (control)	439 ± 18[a]
30 μM peptide 74	445 ± 6
100 μM peptide 74	456 ± 15
30 μM peptide 78	452 ± 10
100 μM peptide 78	440 ± 15

[a] Mean ± SD.

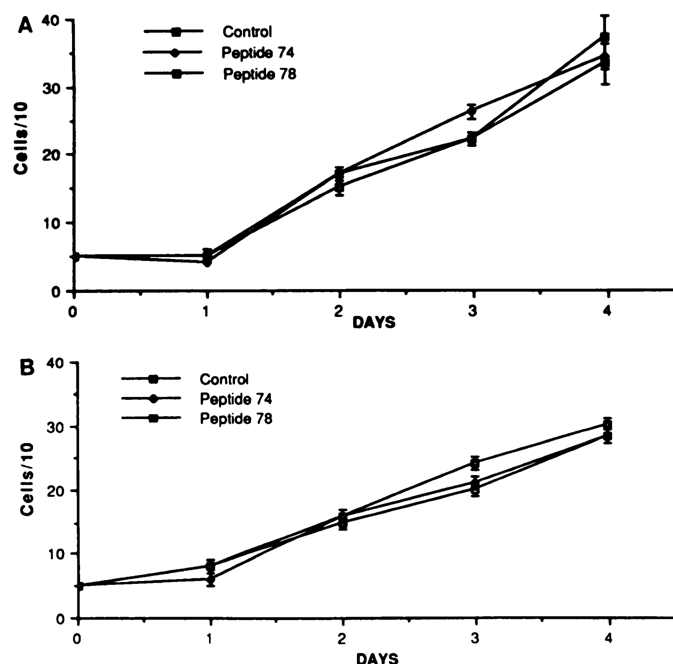

Fig. 4. Growth curves for HT1080 (*A*) and A2058 (*B*) cells in the absence of added peptide, 30 μM peptide 74, or 30 μM peptide 78. Each experiment was run in triplicate and repeated twice. The results show that growth is not affected by the presence of the added peptides.

inhibited by concentrations as low as 2.5–5 μM peptide 74, whereas with A2058 cells the inhibitory effect was lost below 10 μM (data not shown). The nature of the differential sensitivity to peptide 74 inhibition of Matrigel invasion by these cells lines is not known. However, this effect may be due to either the levels of enzymes produced by these cells or the redox environment of these cells in culture. Excessive enzyme production would rapidly overwhelm a given level of added inhibitor. Alternatively, an excessively oxidative environment could lead to the effective inactivation of the free sulfhydryl of the inhibitor peptide sequence.

Effects of Inhibitor Peptides on Cell Growth. The absence of any effects of peptide 74 or 78 on tumor cell chemotaxis (Table 1) suggested that the effects of tumor cell invasion were probably not the result of a cytotoxic action. However, this possibility was examined directly by following the growth of both A2058 and HT1080 cells in the presence of a 30 μM concentration of peptides. Cell counts were performed daily on both HT1080 (Fig. 4*A*) and A2058 (Fig. 4*B*) cells grown in the continuous presence of 30 μM concentrations of peptide 74 or 78. As shown in Fig. 4 these added peptides showed no demonstrable cytotoxic effect during 96 h in cell culture.

Discussion

Previous studies have shown that the conserved peptide sequence MRKPRCG(V)PD present in the profragment of all matrix metalloproteinase enzymes is responsible for the maintenance of the latency of the proenzyme forms (10–12). The importance of this profragment sequence has also been demonstrated by site directed mutagenesis studies which have shown that alteration of this sequence results in enhanced autoactivation of members of this enzyme family (15).

We have recently shown that addition of a peptide containing the sequence TMRKPRCGNPDVAN (peptide 74) to solutions of the activated form of the 72-kDa type IV collagenase results in inhibition of the *in vitro* gelatinolytic activity of this enzyme (12). An identical peptide in which the single cysteine residue was substituted with a serine residue (peptide 78) was also tested and showed no inhibitory activity. This suggested that enzyme inhibition was dependent on the presence of an unpaired cysteine residue. In the present study we demonstrate that this same highly conserved peptide sequence TMRKPRCGNPDVAN, when added to human cancer cells *in vitro* in the form of peptide 74, blocks tumor cell invasion. A concentration of 30 μM peptide 74 was sufficient to inhibit up to 80% of invasion by both human tumor cell lines tested. A further increase in peptide 74 concentrations failed to enhance this inhibitory effect beyond the 80% seen at 30 μM. Furthermore, the A2058 cell line was refractory to peptide 74 inhibition of Matrigel invasion at concentrations of 10 μM or below. These effects are attributed to either the excessive overproduction of enzyme by A2058 cells or sensitivity of peptide 74 to rapid oxidation and loss of inhibitory activity.

Addition of an excess of propeptide to solutions of activated 72 kDa type IV collagenase is thought to mimic restoration of the proenzyme form through a conformational change that results in enzyme inactivation (12). The addition of peptide 74 to human tumor cells in culture results in a down-modulation of the degradative activities necessary for these cells to hydrolyze basement membrane collagen and invade the Matrigel structures. The observation that peptide 74 does not inhibit migration in the absence of reconstituted basement membrane favors a specific role of metalloproteinases in the degradation and subsequent invasion of the Matrigel matrix. These observations suggest that peptides corresponding to the conserved sequence in the prosegment of the matrix metalloproteinase family could be used as inhibitors of tumor cell invasion *in vivo.* The lack of cytotoxic effects of these peptide inhibitors has led us to initiate these *in vivo* experiments to test the efficacy of peptide 74 and related peptides as potential antimetastatic agents.

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