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Monoclonal antibodies to type IV collagenase recognize a protein with limited sequence homology to interstitial collagenase and stromelysin

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Type IV collagenase is a metalloproteinase associated with metastatic tumor cells. It specifically cleaves the triple helical basement membrane (type IV) collagen molecule at a single site. Monoclonal antibodies which block the activity of the human type IV collagenase were developed and used to purify this antigen. The purified type IV collagenase was partially sequenced following cyanogen bromide and trypsin cleavage. The amino acid sequence of the human type IV collagenase fragments revealed a region homologous to the human interstitial collagenase and stromelysin. However, several sequences in type IV collagenase were identified which are distinct from the latter. Polyclonal antibodies were raised against a synthetic peptide derived from such a sequence. Following affinity purification, the antibodies recognized the denatured human type IV collagenase in Western immunoblotting. These data indicate that type IV collagenase is a distinct member of a general family of metalloproteinases.

Type IV collagenase; Metalloproteinase; Basement membrane; Metastasis

1. INTRODUCTION

Type IV collagen, a major structural component of the basement membrane, is cleaved by a 70 kDa type IV collagenase, a metalloproteinase produced by invasive tumor cells [1–4]. Type IV collagen is resistant to cleavage by interstitial collagenase, which degrades collagens I, II, III and X. In contrast, type IV collagenase produces a specific cleavage in type IV collagen but does not degrade other collagen types or laminin, a major glycoprotein of basement membranes [3,4]. Type IV collagen monomers are about 350 nm long triple helical molecules that contain a noncollagenous globular knob at the carboxyl-terminal end. The molecules are thought to form a network structure with two individual molecules joined at the carboxyl-terminus and four molecules joined at the amino-terminus [6]. Type IV collagenase cleaves the triple helical molecule approx. 25% of the distance from the amino-terminus in a pepsin resistant domain [4]. Type IV collagenase has a potential, therefore, to disrupt the type IV collagen network in the basement membrane that is a prerequisite for cancer invasion and metastasis [2].

A previously observed [2,5] biochemical linkage of the metastatic phenotype to the secretion of type IV collagenase has been supported by studies employing rat embryo fibroblasts [7] and NIH 3T3 cells [8] transfected by the *ras* oncogene. Transfection of c-Ha-*ras* into the cells produced fully metastatic tumor cell clones. In contrast, cotransfection of c-Ha-*ras* with adenovirus 2E1a oncogene resulted in fully tumorigenic cells which

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were not metastatic [9]. All nine metastatic tumor clones in this study produced high levels of type IV collagenase. However, all five nonmetastatic clones produced very low or undetectable levels of this proteinase.

In order to study the molecular properties of type IV collagenase and its relationship to other known metalloproteinases, it is necessary to have specific antibodies which recognize the enzyme, alter its activity, and distinguish this proteinase from other known metalloproteinases such as interstitial collagenase and stromelysin [10–12]. We have developed monoclonal antibodies to the enzyme and immunopurified and partially sequenced the antigen. The results show that type IV collagenase contains sequences related to stromelysin and interstitial collagenase suggesting that these three enzymes belong to the same family of metalloproteinases.

2. MATERIALS AND METHODS

2.1. *Purification of antigen and production of monoclonal antibodies*

For the preparation of monoclonal antibodies type IV collagenase was purified from culture media of human melanoma cells (A2058, Meloy Laboratories, Springfield, VA) using $(NH_4)_2SO_4$ precipitation, molecular sieve (BioGel A1.5m) and affinity chromatography on type IV collagen-Sepharose as described elsewhere [3]. Additionally, a DEAE ion-exchange chromatography step was used prior to the molecular sieve instead of concanavalin A-Sepharose. A 6-week-old female BALB/c mouse was immunized with the purified antigen (40 μg s.c. + 20 μg i.p. + 20 μg i.v.) and the spleen cells were fused with mouse myeloma cells NS-1 [13] using the standard protocols [14] and plated on 96-well microtiter plates (Flow).

ELISA and immunoblot assays were performed with the same procedure using either Tris- or phosphate-buffered saline. The nonspecific binding sites were blocked with 1% bovine serum albumin, immune antibodies were incubated for 2 h and finally horseradish peroxidase conjugated antimouse IgG antibodies for 1 h at room temperature. The peroxidase reaction was visualized using 4-chloro-1-naphthol and H_2O_2 as substrate.

2.2. *Enzyme purification with a monoclonal antibody immunoaffinity column*

Ascites from mice were dialyzed in TBS, pH 8.5 and chromatographed on a protein A-Sepharose column (Pharmacia) and the bound IgG was eluted with 0.1 M glycine-HCl, pH 3.0, dialyzed against 0.5 M NaCl, 0.1 M $NaHCO_3$ and coupled to cyanogen bromide-activated Sepharose (Pharmacia). The conjugated matrix was washed with 0.05 M Tris-HCl, pH 7.4, containing 3.5 M NaSCN to remove nonspecific bound proteins. The enzyme was precipitated with 60% saturated ammonium sulphate, dialyzed, applied to the column and washed with 0.05 M Tris-HCl, 1.0 M NaCl, 0.01 M $CaCl_2$, pH 7.4. The bound enzyme was eluted with 0.05 M Tris-HCl, 3.5 M NaSCN, pH 7.4.

2.3. *Preparation of antibodies to synthetic peptides*

The synthetic peptides used in the immunization procedures were made on a Biosearch 9600 peptide synthesizer. The peptides were conjugated to bovine serum albumin using glutaraldehyde. Two rabbits for each antigen were immunized i.d. using complete Freund adjuvant in all immunizations. Serum from the fourth bleed onwards was used to prepare IgG. This step was carried out by heating the serum first to 56°C for 0.5 h, and absorbing the pertinent antibody then on the appropriate peptide-AffiGel 10 resin at 4°C for 18 h with gentle agitation. After thorough washing, the absorbed antibody was eluted from the resin with 0.1 M acetic acid. The eluate was neutralized to pH 7.0 with NaOH, and the resulting solution was concentrated and exchanged to PBS (Amicon YM-10 diaflo).

2.4. *Cell cultures and biosynthetic radiolabeling of tumor cell proteins*

Human melanoma (A2058) cells were cultured to confluency after which the culture flasks were washed twice with PBS. The cells were then grown in serum-free DMEM supplemented with 250 μCi of [^{35}S]methionine per flask for two days. The medium was collected and the proteins were precipitated with ammonium sulphate at 60% saturation. The precipitate was centrifuged, the pellet dissolved and dialyzed in the same buffer.

2.5. *Amino acid sequencing*

Trypsin digestion was performed at an enzyme to substrate ratio of 1:100, 37°C, pH 8.2 for 6 h. Alternatively, the purified type IV collagenase protein was digested with cyanogen bromide [15], dissolved into 400 μl of 0.1% trifluoroacetic acid/acetonitrile and chromatographed as two 200 μl injections on a reverse-phase Hamilton PRP-1 column (25 cm × 4.1 mm) with a flow rate of 2 ml/min. Amino acid sequencing of the purified fragments was performed on a model 470A gas-phase sequencer (Applied Biosystems Inc.) with an attached model 120A-PTH analyzer using the manufacturer's program 03RPTH. The sensitivity was 20 pM and the repetitive yield was 95%.

2.6. *Other procedures*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 9% separating gels and 4% stacking gels [16] or 10–15% gradient separating gels on a Pharmacia PhastSystem. Gelatin zymogen gels described previously [17]. Type IV collagenase activity was measured using [^{3}H]proline-labeled type IV collagen as substrate [2,3]. Inhibition of enzymic activity was determined using purified IgG, which was added to the reaction mixture before the substrate.

3. RESULTS

3.1. *Characterization of monoclonal antibodies to human type IV collagenase*

The purified antigen from the melanoma cell

line A2058 contained the 68 kDa type IV collagenase polypeptide described previously [3] and additionally another protein of about 100 kDa (not shown). Following the immunization procedure, the hybrid cells were plated into 360 microtiter plate wells and tested for production of antibodies in an ELISA assay using the same antigen as for immunization. A total of 17 positive cloned hybrids were derived from the primary cultures, and the IgG produced by these clones was screened for inhibition of type IV collagenase activity (Höyhtyä et al., in preparation). One antibody (6B2-A) inhibited the enzyme activity in a dose-dependent manner up to 60% at 10 μg/ml (not shown) whereas control IgG had no effect. This antibody did not stain the human enzyme protein in Western blots of the SDS-denatured protein indicating that it only recognizes the native form of the enzyme. This was verified using a dot blot assay with native and SDS-treated enzyme where only the native protein stained positively (fig.1). In this assay, the antibody detected type IV collagenase from human melanoma (A2058) and human breast carcinoma (MCF-7) cells (fig.2).

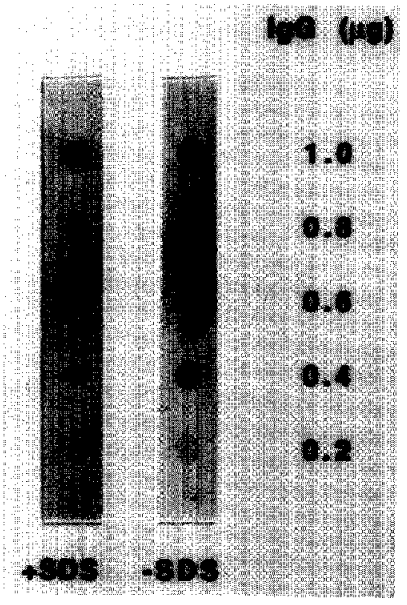

Fig.1. Dot blot analysis showing the reactivity of monoclonal anti-type IV collagenase IgG (6B2-A) with the native (– SDS) and denatured (+ SDS) enzyme derived from human breast carcinoma.

3.2. *Immunoaffinity purification of type IV collagenase*

The purified 6B2-A IgG was immobilized on activated Sepharose and used for affinity purification of the type IV collagenase. Cell culture media containing the enzyme, secreted by the human melanoma A2058 cell line, were applied to the column in collagenase buffer and the bound antigen eluted with 0.05 M Tris-HCl, 3.5 mM NaSCN, pH 7.4. As shown in fig.3A, the protein migrated as a single 68 kDa band in SDS-PAGE. In vivo labeled proteins secreted by A2058 were applied to the column and the bound material was eluted. This procedure also resulted in a single-step purification of the antigen with an identical migration pattern in SDS-PAGE after reduction (fig.3B).

3.3. *Sequence analysis of human type IV collagenase*

Purified preparations of type IV collagenase from the human melanoma A2058 and breast carcinoma (MCF-7) cells that were recognized by the specific monoclonal antibody were subjected either to cyanogen bromide or trypsin cleavage. The resulting peptides were separated by HPLC, and five were partially sequenced (fig.4A). As seen in fig.4B, one of the sequences (TP1) exhibits clear homology to human stromelysin and human in-

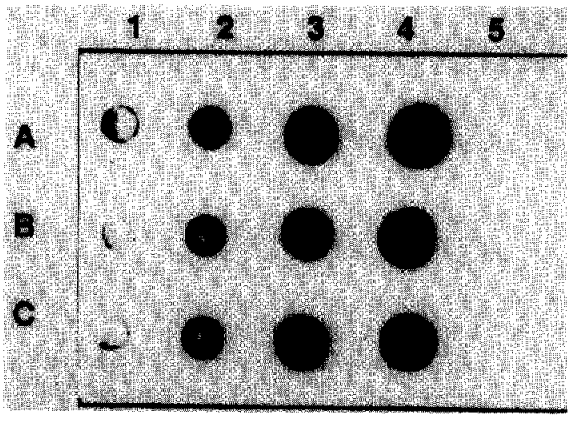

Fig.2. Dot blot of human type IV collagenase using a monoclonal antibody (dilution 100 μg/ml). Rows: A, purified human A2058 melanoma cell type IV collagenase (fig.5, lane B); B, purified human *ras*-transfected MCF-7 type IV collagenase; C, crude A2058 metalloproteinase (fig.5, lane A). Concentrations: A1, B1, 10 ng; A2, B2, 50 ng; A3, B3, 100 ng; A4, B4, 250 ng; column 5, control BSA 250 ng; C1, 100 ng; C2, 500 ng; C3, 1 μg; C4, 2 μg.

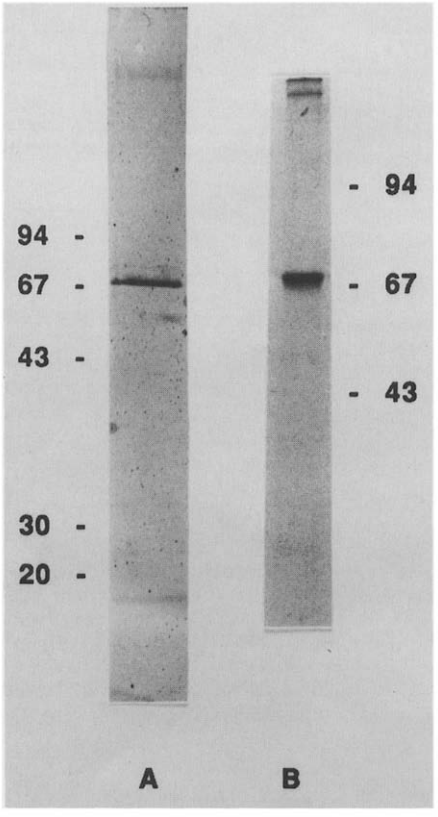

Fig.3. Immunoaffinity purification of human melanoma cell (A2058) type IV collagenase. Serum-free cell culture media were applied to an IgG-Sepharose column and eluted with 3.5 M NaSCN, 0.5 M Tris-HCl, pH 7.4. The eluate was analyzed by SDS-PAGE. (A) Unlabeled medium protein. PhastSystem gradient gel 10–15%. (B) Analysis of the protein metabolically labeled with [^{35}S]methionine using a 9% separating gel.

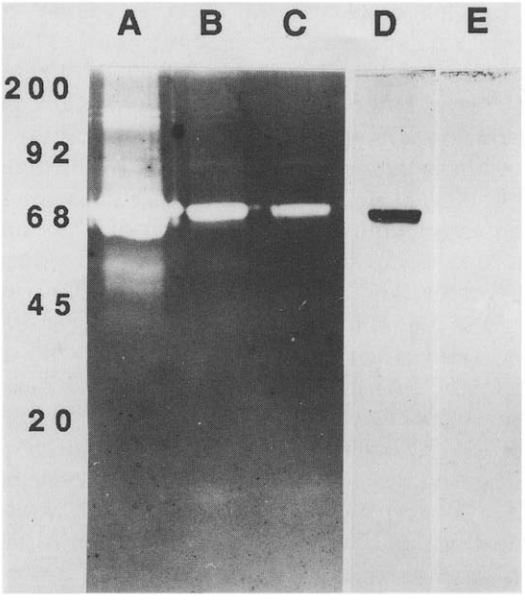

Fig.5. Immunoprecipitation and immunoblotting of human type IV collagenase using affinity purified rabbit anti-synthetic peptide (CB 4) IgG antibodies. Lanes: A–C, gelatin substrate gel; D–E, Western immunoblotting; polyacrylamide gel electrophoresis without reduction. Molecular sizes in kDa are shown on the left. (A) Crude preparation from conditioned media of human melanoma A2058 metalloproteinase activity; (B) purified type IV collagenase (100 ng) from A2058; (C) specific immunoprecipitation of type IV collagenase using antisynthetic peptide antibodies. Source material is shown in lane A. (D) Immunoblotting of purified A2058 type IV collagenase (100 ng antigen), using 100 μg/ml of affinity purified anti-peptide antibodies; (E) immunoblot control using 500 μg/ml of pre-immune IgG.

A

CB1	M K Y G F C P E T A M
CB2	M S T V G G N S E G A
CB3	M I H F G K M
CB4	M G P L L V A T F W P E L P E K
TP1	I I G Y T P D L D P E I V D D A F A R

B

Interstitial collagenase:	I E N Y T P D L P R A D V D H A I E K
Type IV collagenase:	I I G Y T P D L D P E I V D D A F A R
Stromelysin:	I V N Y T P D L P K D A V D S A V E K

Fig.4. (A) Amino acid sequences of cyanogen bromide (CB)- or trypsin (TP)-cleaved peptides of human type IV collagenase. (B) Sequence homology of the TP1 peptide with regions of human interstitial collagenase (residues 118–136, [11,12]) and human stromelysin (residues 118–136 [11]). Identical amino acids are shown by boxes and conserved substitutions between type IV collagenase and the other enzymes are shown by dots.

terstitial collagenase [11]. The other sequences were unique. Synthetic peptides corresponding to sequence CB4 were coupled to albumin and antibodies raised in rabbits. The antibodies were affinity-purified on columns containing the immobilized synthetic peptide. They recognized the denatured antigen following SDS-PAGE and immunoblotting (fig.5). This was the same antigen which was recognized in its native state by 6B2-A. The antipeptide antibodies are able to recognize it as well since they specifically immunoprecipitated the type IV collagenase antigen from a heterogeneous mixture of metalloproteinases secreted by human melanoma cells. The presence of gelatin degrading activity of type IV collagenase was shown in zymogen gels (fig.5).

4. DISCUSSION

The monoclonal antibody (6B2-A) to human melanoma-derived type IV collagenases that are characterized in the present study, exhibit inhibitory effect on the activity of the enzyme. However, the maximum inhibition was only 60% of the original activity which indicates that the antibody does not bind directly to the active site of the enzyme. The antibody presumably recognizes a conformational epitope because it does not react with the SDS-treated enzyme. Conformation specificity as opposed to amino acid sequence specificity is a common finding for monoclonal antibodies. The antibody detects type IV collagenase from several human tumors.

The reactivity of the monoclonal antibody with the native enzyme could be utilized for a single step purification procedure based on IgG affinity chromatography. Both unlabeled and biosynthetically labeled proteins from culture media of human melanoma cells that bound to the anti-type IV collagenase IgG column, migrated as a single 68 kDa band in SDS-PAGE after reduction. This demonstrated high binding specificity which enabled quantitative purification of the antigen.

Previous reports have shown that interstitial collagenase [12] and stromelysin (transin-1 [10,11,18]) as well as a still unidentified related metalloproteinase, protein transin-2 [19] are homologous. The highest similarity is present in a region that has been identified as the zinc-binding site in the enzyme [11]. This is in keeping with the fact that all of these metalloproteinases have EDTA-inhibited gelatinase activity. Substrate specificity may be related to regions in the metalloproteinases separate from the zinc-binding domain. Amino acid sequence analysis of peptides from the type IV collagenase from A2058 melanoma and *ras*-transfected MCF-7 breast carcinoma cells revealed a 19 residue sequence that has homology to both human stromelysin and interstitial collagenase. In this sequence, there are 9 identical residues both in human stromelysin and interstitial collagenase and 5 conservative substitutions. This suggests that type IV collagenase belongs to the same family of metalloproteinases.

Polyclonal antibodies made against the CB4 peptide (fig.4) that does not share homologous sequence with either stromelysin or interstitial collagenase only recognize the type IV collagenase protein. This further confirms that the sequenced peptides are derived from the true type IV collagenase. These unique sequences may play a role in the substrate specificity of type IV collagenase.

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