Cloning and Characterization of Human Tumor Cell Interstitial Collagenase

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ABSTRACT

A full-length complementary DNA (cDNA) for interstitial collagenase was isolated from an A2058 melanoma cDNA library using the pCD-X Okayama-Berg vector. The tumor interstitial collagenase cDNA was sequenced and compared to the published sequences for human fibroblast collagenase. The sequence for the tumor collagenase has two DNA base pairs which differ from the sequence of normal fibroblast collagenase. Restriction enzyme digestion of a specific DNA fragment produced by polymerase chain reaction amplification of genomic DNA from human placenta resolves a discrepancy in the previously reported DNA and amino acid sequences for the fibroblast collagenase. A high level of expression of interstitial collagenase message was found in human A2058 melanoma cells by Northern blot analysis, and this level was slightly increased by phorbol ester (phorbol myristate acetate) stimulation. Intersitial collagenase mRNA expression was significantly decreased by treatment with either transforming growth factor-β1 or retinoic acid in A2058 melanoma cells. A high level of the collagenase protein secreted into conditioned media was identified by Western blotting. As shown by gelatin zymogram analysis interstitial collagenase was one of at least two metalloproteinases secreted by this same cell line. Thus, human melanoma cells can directly produce interstitial collagenase without a requirement for host cell interaction.

INTRODUCTION

The collagenase gene family consists of a number of matrix-degrading metalloproteinases. Each member of the family has a different substrate specificity. Type IV collagenase, one member of this family, is thought to play a role in basement membrane degradation and has been closely linked to the metastatic phenotype (1). However, basement membranes are only one barrier which must be crossed by invading tumor cells. These cells must also traverse interstitial stroma composed of types I and III collagen (2-10). Furthermore, tumor cells may also invade bone and cartilage which are composed principally of types I and II collagen.

Collagens pose a significant mechanical barrier to migrating cells (11). Several investigators have proposed that interstitial collagenase is active in migrating cells (12, 13) and is induced in human tumors. Because tumors are heterogeneous with respect to cellular composition, the cell type responsible for the origin of type I collagenase in tumors has not been definitively established for all tumor types. Several immunohistochemical studies suggest that tumor cells do not produce interstitial collagenase and that, instead, it is produced by the surrounding fibroblast cells possibly in response to tumor cell factors (12, 14, 15). Alternatively, other immunohistochemical data propose that some tumor cells, including melanoma, could produce interstitial collagenase (16).

Interstitial collagenolytic activity has been identified in some tumor cell preparations (11, 17). However, the identity of the type I collagen-degrading enzyme associated with tumor cells and its possible relationship to interstitial fibroblast collagenase has not been established. The purpose of the present study was to identify and characterize tumor cell-associated type I collagenase activity and compare this enzyme with the well-characterized fibroblast interstitial collagenase.

MATERIALS AND METHODS

mRNA Preparation and cDNA Library Construction. Total cellular RNA was prepared from A2058 cells by the guanidine isothiocyanate method (18). The A2058 melanoma cell line was derived from a brain metastasis in a 43-year-old man (19). A2058 RNA was used to prepare a cDNA library constructed in the pCD-X Okayama-Berg vector as previously described (20).

Screening and Analysis of cDNA Clones. Oligonucleotide probes were synthesized which have homology to the complementary strand of the metal-binding domain of human fibroblast collagenase. These probes include: WSS-15, GAGTCCAAGAGAATGGCCGATTCATAGCCG; WSS-16, GAGTCCAAGAGAATGGCCGATTCCACTAGCCG; and WSS-20, GAGTCCAAGAGAATGGCCGATTCCACTAGCCG. The probes were 5'-end-labeled (specific activity, 2.5 x 10⁶ cpm/ml) with T4 polynucleotide kinase (18) and [γ-32P]ATP (6000 Ci/mm; New England Nuclear) and were used to screen 2.5 x 10⁹ colonies. Hybridization and wash conditions were as described previously (18), with the temperature of the stringent 6× SSC/0.2% SDS wash set at 58°C. Positive clones were selected, purified, and rescreened.

DNA was prepared from selected clones according to the standard protocol (18). The DNAs were digested with Xhol to determine the size of the inserts.

Northern Blot Analysis. Total cytoplasmic RNA was prepared according to the guanidine isothiocyanate method (18) from A2058 melanoma and the following human breast carcinoma cell lines: MDA 231, MDA 435, MCF 7, ras-transformed MCF7, and T47D. All cell lines were obtained from the American Type Culture Collection with the exception of the A2058 melanoma cell line which was developed at the NIH (19) and the ras-transformed MCF-7 cell line which was a gift from Dr. E. Gelman, Lombardi Cancer Center, Georgetown University, Washington, DC. The cell lines were selected to represent a wide spectrum of invasive potential (both estrogen dependent and estrogen independent).

Total cytoplasmic RNA (5.0 μg/lane) was electrophoresed on an agarose gel containing 2 M formaldehyde. The RNA was transferred to a GeneScreenPlus membrane (DuPont) overnight using 10× SSC. The RNA blot was washed in 2× SSC and UV cross-linked in a UV Stratalinker 2400 (Stratagene). Prewashing was at 42°C in 1 M NaCl, 10 mM Tris, 1 mM EDTA, and 0.1% SDS for 2 h. Prehybridization was at 42°C in 10% dextran sulfate, 4× SSC/40% formamide, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA. The p35–1 insert was labeled with [α-32P]CTP (3000 Ci/mm; New England Nuclear) by the random primer method, and the blot was hybridized overnight at 42°C with 1 x 10⁶ cpm/ml of the labeled probe. A stringent wash was done for 30 min at 65°C in 0.2× SSC/1% SDS.

Treatment of A2058 Cells. Human melanoma A2058 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 0.5% (v/v) ITS* (Collaborative Research, Inc.), for 24 h prior to treatment. Fresh medium containing either 16 nm PMA (Sigma Chemical Co.), 200 pm TGF-β1 (R&D Systems Inc.), or 1 μM all-trans-RA.
was performed.

Restriction Map of p35-1. A restriction map of clone p35-1 was generated by digestion with the following enzymes: BamHI, CiaI, Hind III, Sal I, XbaI, and XhoI. Many restriction digests were done using one of these restriction enzymes per digest or using two and three different enzymes simultaneously in a digest (data not shown). There are no restriction sites present within the insert for Hind III, Sal I, and XhoI.

DNA Sequencing. The sequence analysis was performed on the Genesis 2000 automated sequencer (DuPont) using the fluorescent chain-terminating deoxyribonucleotide method (21, 22). The double-stranded DNA dye was followed. Twenty-two oligonucleotide primers (18mers; NT-1–NT-22) were synthesized which are homologous to various regions found on both strands of the human fibroblast collagenase sequence (4, 23). These oligonucleotides were used as primers for the sequencing reactions.

Polymerase Chain Reactions and Primers. The polymerase chain reaction (24, 25) was carried out in a total volume of 100 μl with 2 μg genomic DNA, 10 μl of each oligonucleotide primer in 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM MgCl2, 0.01% gelatin, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, and 2.5 units of Thermus aquaticus polymerase (Perkin-Elmer/Cetus) for 62 cycles in an automated thermal cycler (Perkin-Elmer/Cetus). The first cycle was 94°C denaturation (30 s), 55°C annealing (15 s), and 72°C extension (1 min). Cycles 2–61 were 94°C denaturation (15 s), 55°C annealing (15 s), and 72°C extension (1 min). The last cycle was 72°C extension (10 min). The primers used were: NT-4, GGTGATGA-ACGACGCCCAG; and NT-15, GTCCACATCTGCCTTGG. The 5' end of NT-4 begins at base pair 323 of the collagenase cDNA, and the 5' end of NT-15 is found on the complementary strand beginning at base pair 461. On the interstitial collagenase genomic DNA, intron 2, which consists of 90 base pairs, is present after base pair 418 of the fibroblast collagenase cDNA. Therefore, it was predicted that upon amplification of genomic DNA with primers NT-4 and NT-15 a DNA fragment of approximately 229 base pairs would be generated.

After amplification, the polymerase chain reaction mixtures were extracted with phenol/chloroform, precipitated with ethanol, washed with 70% ethanol, and resuspended in TE, pH 7.5, as described previously (18). Each amplified DNA fragment was digested with Rsal and electrophoresed on a 3% NuSieve/1% agarose gel. Amplified DNA fragments which were not digested with restriction enzyme and HaelIII-digested φX174RF DNA marker were electrophoresed on the same gel with the Rsal-digested DNA fragments. The gel was stained with ethidium bromide and visualized with UV.

Western Blot Analysis. A2058 melanoma cell-conditioned media (20 μl/lane) was electrophoresed on a 10–20% SDS-polyacrylamide gradient gel. The samples were electroblotted onto Immobilon P membranes (Millipore) at 30 V constant voltage overnight at 4°C using a methanol/Tris-glycine transfer buffer (26). The membrane was incubated with polyclonal anti-human type I collagenase antibody. The antibody to collagenase antibody was a gift from Dr. H. Briedel-Hansen, University of Alabama at Birmingham, Birmingham, AL. Rabbit anti-sheep horseradish peroxidase conjugate (BioRad) was used as the secondary antibody.

Gelatin Zymogram. Samples of A2058 melanoma cell-conditioned media (20 μl/lane) were analyzed directly by SDS-polyacrylamide gel electrophoresis on 9% acrylamide gels containing 0.1% gelatin as described previously (27).

RESULTS

Cloning of Human Melanoma Interstitial Collagenase. An A2058 melanoma cDNA library was constructed in the Okayama-Berg vector, pCD-X. The metal-binding domain of the collagenase enzyme family is highly conserved among all members of this family. To identify any possible collagenases secreted by the human melanoma cell line A2058, we screened a cDNA library with three oligonucleotide probes which have homology to the metal-binding domain of the human fibroblast collagenase. Thirty-seven positive clones were selected after the initial screening. These clones were rescreened several times, and 13 positive clones were selected after the final screen. DNA was prepared from each of the 13 clones and was digested with XhoI to yield the intact insert. The DNA inserts also include 50 base pairs near the AT tail region and 175 base pairs near the GC stretch of the pCD-X vector because of the location of XhoI sites within this vector. The length of the human fibroblast collagenase cDNA is 1.97 kilobases. Clone p35-1 yielded the largest insert with a size of 2.2 kilobases upon digestion with XhoI. This clone was selected for further analysis, including DNA sequencing.

Expression of Type I Interstitial Collagenase in Tumor Cell Lines. Total cytoplasmic RNA from a variety of tumor cell lines was hybridized to the p35-1 probe by Northern blot analysis (Fig. 1A). The results show that the p35-1 message is expressed at a high level in A2058 melanoma cells (Lane 2) and at a lower level in MDA 231 cells, a non-estrogen-dependent breast carcinoma cell line (Lane 6). However, other human breast carcinoma cell lines, both non-estrogen dependent (MDA 435) and estrogen dependent (T47D, MCF-7, and ras-transformed MCF-7) showed no detectable levels of p35-1 transcripts.

The regulation of interstitial collagenase expression in the A2058 melanoma tumor cell line was studied by treatment of these cells for 24 h with PMA, TGF-β1, and retinoic acid. These agents have been shown to modulate the mRNA levels of interstitial collagenase in fibroblast cell lines (28, 29), and we used these same agents in order to determine whether tumor cell interstitial collagenase responded in a fashion similar to that reported for the fibroblast message. Total cytoplasmic RNA was isolated and an autoradiograph of the Northern blot analysis is shown in Fig. 1B. Untreated tumor cells show a high steady state level of interstitial collagenase mRNA, in contrast to the extremely low levels observed in human fibroblast cell lines (28). This level is increased slightly by PMA treatment.
and is markedly decreased by treatment with either TGF-β1 or retinoic acid. In these respects, the regulation of interstitial collagenase mRNA expression in human tumor cells appears to be similar to that previously described in human fibroblasts (28–30).

Restriction Map of p35-1. The restriction map of p35-1 was compared to a restriction map of the human fibroblast collagenase cDNA (Fig. 2B). The restriction map of the fibroblast collagenase cDNA was produced by mapping the restriction sites predicted by the published sequence. The comparison of the two restriction maps demonstrated that they were identical.

Characterization of p35-1 by DNA Sequence Analysis. Since the restriction maps of p35-1 and human fibroblast collagenase cDNA proved to be identical, we decided to facilitate the sequencing of p35-1 by synthesizing 22 oligonucleotide primers (NT-1–NT-22) which are homologous to regions of the human fibroblast collagenase found on both strands. These primers are spaced at approximately 200-base pair intervals along the fibroblast collagenase cDNA. The DNA sequencing was performed on the automated Genesis 2000 DNA sequencer (DuPont). The p35-1 DNA sequence and the predicted amino acid sequence are displayed in Fig. 2. This cDNA sequence is identical to that reported for interstitial fibroblast collagenase (4, 23) with the exception of the changes noted in Table 1.

The differences at base pairs 383 and 899 do not change the encoded amino acids, base pair 10 is found in the 5′-untranslated region, and base pair 1891 is found in the 3′-untranslated region. The differences at base pairs 412, 413, and 1296 do not make changes in the predicted amino acids encoded. The p35-1 sequence and the sequence published by Whitham et al. (23) predict a Thr115 and a Ser410. The sequence published by Goldberg et al. (4) predicts an Arg115 and an Arg410. The differences at amino acid 115, Thr versus Arg, is a nonconservative substitutio...
collagenase is directly produced by the highly metastatic human melanoma cell line, A2058. This is demonstrated by (a) the presence of a cDNA for the interstitial collagenase in an A2058 cDNA library, (b) verification of the identity of the cDNA as the interstitial collagenase cDNA by sequence analysis (Fig. 2),

**DISCUSSION**

These results provide evidence that the type I interstitial collagenase is directly produced by the highly metastatic human fibroblast collagenase.

**Fig. 2.** A, nucleotide sequence of the full-length cDNA for the type I interstitial collagenase isolated from an A2058 Okayama-Berg library. This sequence was determined by automated DNA sequencing with fluorescent chain-terminating deoxyribonucleotides (21, 22) performed on the DuPont Genesis 2000 sequencer. The double-stranded DNA dye-exchange protocol was utilized. The deduced amino acid sequence for the tumor type I collagenase is shown below the DNA sequence. B, comparison of restriction maps for the human fibroblast collagenase cDNA and the p35-1 cDNA. bp, base pair.

**Human fibroblast collagenase**

<table>
<thead>
<tr>
<th>XbaI</th>
<th>SpcI</th>
<th>BamHI</th>
</tr>
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<tbody>
<tr>
<td>150 bp</td>
<td>200 bp</td>
<td>200 bp</td>
</tr>
</tbody>
</table>

**TUMOR INTERSTITIAL COLLAGENASE**

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The amino acid sequences predicted by our p35-1 sequence data are indicated at the point of each arrow.

Fig. 3. Rsal restriction enzyme digests of genomic DNA amplified by the polymerase chain reaction (24, 25). Specific primers were used (see "Materials and Methods" for details) so that 229-base pair DNA fragments were produced. The samples were electrophoresed on a 20% polyacrylamide gel and stained with ethidium bromide. Lane 1, human placental DNA fragment; Lane 2, A2058 DNA fragment; Lane 3, human placental DNA fragment digested with Rsal; Lane 4, A2058 DNA fragment digested with Rsal; Lane 5, HaelIII-digested y,174RF DNA marker. Lanes 1–4 contain 25% of the total DNA fragment produced by 100 μl of each polymerase chain reaction. Ordinate, base pairs.

Table 1 Summary of base changes for published sequences of interstitial collagenase cDNA clones

<table>
<thead>
<tr>
<th>Base pair position</th>
<th>p35-1 [Templeton et al. (present study)]</th>
<th>Fibroblast [Goldberg et al. (4)]</th>
<th>Fibroblast [Whitham et al. (23)]</th>
<th>Amino acid position and difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>A</td>
<td></td>
<td>5'-untranslated region</td>
</tr>
<tr>
<td>2</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>5'-untranslated region</td>
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<tr>
<td>10</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>105, no change</td>
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<tr>
<td>383</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>412</td>
<td>G</td>
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<td>413</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>115 Arg-&gt;Thr*</td>
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<tr>
<td>666</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>200 His-&gt;Asp</td>
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<tr>
<td>691</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>208 Thr-&gt;Arg</td>
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<tr>
<td>899</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>277, no change</td>
</tr>
<tr>
<td>1018</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>317 Thr-&gt;Ile</td>
</tr>
<tr>
<td>1029</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>410 Ser-&gt;Gly</td>
</tr>
<tr>
<td>1891</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td>3'-untranslated region</td>
</tr>
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</table>

* The amino acid sequences predicted by our p35-1 sequence data are indicated at the point of each arrow.

Fig. 4. A, gelatin zymogram of A2058 melanoma cell-conditioned media (20 μl). The activities of type I interstitial collagenase and type IV collagenase are shown at M, 53,000 and 72,000, respectively. B, Western blot of type I interstitial collagenase immunoblotted with anti-type I interstitial collagenase antibody (20 μl of A2058 melanoma cell-conditioned media).

(e) proof that the message for this cDNA is expressed in A2058 melanoma cells (Fig. 1A), (d) Western blot data which show the presence of the type I collagenase in A2058 cell-conditioned media (Fig. 4B), and (e) zymogram data which demonstrate gelatinase activity of the type I interstitial collagenase in A2058 cell-conditioned media (Fig. 4A). Furthermore, the Northern blot analysis of various tumor cell lines shows that interstitial collagenase message is produced by the breast carcinoma cell line, MDA 231, which is also a highly metastatic human tumor cell line (34). This suggests that certain aggressive tumor cells, such as A2058 and MDA 231, may directly possess the enzymatic mechanisms to disrupt the collagen barriers which block invasion. In addition, since interstitial collagenase can be produced by both mesoderm-derived cells (A2058) and epithelial cells (MDA 231), this enzyme is not restricted to one type of cellular origin.

However, not all tumor cell lines tested showed detectable levels of interstitial collagenase mRNA transcripts (Fig. 1A) or protein detectable by Western blotting (data not shown). The effects of the presence or absence of interstitial collagenase production by tumor cells on their invasive phenotype is not known. The invasive properties of the estrogen-independent cell lines (MDA231 and MDA 435) are very similar in in vitro invasion assays yet show very different interstitial collagenase transcript levels. However, these assays measure invasiveness on or over type IV collagen-containing matrices which are not susceptible to cleavage by interstitial collagenase. It would be of interest to observe the relative invasiveness of such cell lines on type I collagen matrices, which may better reflect the composition of the interstitial compartment, as well as the effects of type I collagen on the levels of interstitial collagenase expression in various tumor cell lines.

Previous studies have suggested that invading tumor cells induce surrounding stromal fibroblasts to secrete collagenase (14, 15), thereby facilitating tumor cell invasion. It was unclear whether tumor cells were capable of producing interstitial collagenase themselves or whether tumor cell-associated collagenase was actually produced by tumor cells or stromal fibroblasts. The present study clearly demonstrates that tumor cells of both epithelial and mesodermal origin are capable of synthesizing interstitial collagenase mRNA. This suggests that tumor cells have at least two possible mechanisms for dissolution of the type I collagenous stroma: (a) direct production of interstitial collagenase enzyme or (b) stimulation of tumor-associated or stromal fibroblasts to produce this enzyme.

Our sequence data of human melanoma interstitial collagenase show that this sequence is identical to that of the human fibroblast interstitial collagenase, with the exception of two base pair differences. This suggests that there are no functional differences found in interstitial collagenases of other cellular origins, and therefore this enzyme most likely functions in a similar manner in various cells. Studies suggest that other

* R. Thompson, personal communication.
metalloproteinases are identical in both the normal and transformed phenotypes (35, 36). Results of the Northern blot analysis show an extremely high level of expression of the interstitial collagenase message in A2058 melanoma cells. Therefore, activity of interstitial collagenase present in these human tumor cells is most likely the result of increased production of this enzyme within these cells and not due to an alteration in the collagenase protein structure.

Comparison of the regulation of interstitial collagenase mRNA expression in the human melanoma tumor cell line and in normal human fibroblasts reveals many similarities. The level of interstitial collagenase mRNA in untreated A2058 cells is much higher than in human fibroblasts which exhibit only barely detectable levels of this mRNA (37). However, PMA treatment of both A2058 cells and fibroblasts results in increased interstitial collagenase mRNA expression, although the induction is far more dramatic in the latter (38). TGF-β1 has been shown to decrease interstitial collagenase mRNA in fibroblast growth factor-stimulated human fetal lung fibroblasts (29). These results demonstrate that the interstitial collagenase gene is under similar negative transcriptional controls in both the human A2058 melanoma tumor cell line and normal human fibroblasts but that the tumor cell line must have some constitutive positive element resulting in enhanced transcription of interstitial collagenase mRNA.

In summary we have shown that interstitial collagenase is constitutively expressed in two highly metastatic human tumor cell lines, A2058 melanoma and MDA 231 breast carcinoma. The cDNA for this enzyme was isolated, sequenced, and shown to be identical to the human fibroblast collagenase sequence. Several discrepancies were noted at the cDNA level which were further investigated using the polymerase chain reaction technique. These differences were ascribed to either sequencing errors or polymorphic divergence. Studies of the regulation of human tumor interstitial collagenase demonstrated that this transcript was negatively regulated by TGF-β1 and retinoic acid in a fashion that is identical to that reported for the fibroblast collagenase cDNA. The constitutively high levels of expression in the metastatic cell lines suggest that this enzyme may contribute to the highly invasive phenotype which these cells demonstrate.

ACKNOWLEDGMENTS

We thank T. Zarucki for preparation of the A2058 Okayama-Berg library, Dr. Q-X. Sang for performing the Western blot analysis, Dr. M. Sobel for the pAPC-2 clone, Dr. C. Brinckerhoff for the I cell pupK9 clone, Dr. H. Birkedahl-Hansen for the anti-interstitial collagenase antibody, E. Unsworth for synthesis of the oligonucleotide primers, and B. Berghoffer for performing the cell culture. We also thank Drs. S. Berger and R. Manrow for helpful discussions and for their polymerase chain reaction protocol.

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