[CANCER RESEARCH 54, 2091–2094, April 15, 1994]

Advances in Brief

Structure and Expression in Breast Tumors of Human TIMP-3, a New Member of the Metalloproteinase Inhibitor Family[1]

José A. Uría, Adolfo A. Ferrando, Gloria Velasco, José M. P. Freije, and Carlos López-Otín[2]

Departamento de Biología Funcional, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain

Abstract

A new member of the metalloproteinase inhibitor family of proteins has been cloned from a complementary DNA library derived from a human breast tumor. The isolated complementary DNA contains an open reading frame 633 base pairs long, encoding a polypeptide of 211 amino acids, which has been called tissue inhibitor of metalloproteinase 3 (TIMP-3). This protein displays low sequence similarity to the previously known human TIMPs but shows a high degree of similarity with chicken inhibitor of metalloproteinase 3, a recently described metalloproteinase inhibitor stimulated during oncogenic transformation of chicken fibroblasts and with the ability to promote some phenotypic properties of transformed cells. Northern blot analysis of RNA from human tissues revealed that the *TIMP-3* gene is expressed in placenta and uterus but not in liver and ovary. In addition, TIMP-3 transcripts were detected in all breast carcinomas examined. On the basis of these expression data in breast tumors, together with its high degree of structural homology with chicken inhibitor of metalloproteinase 3, a possible role for human TIMP-3 in the regulation of connective tissue turnover and remodeling is proposed.

Introduction

The ability of tumor cells to metastasize from the primary site of growth to distant sites in the body seems to be the result of the concerted action of different gene products (1). Among these proteins with potential involvement in the spread of cancer, MMPs[3] have attracted considerable interest because they are able to degrade the different components of the extracellular matrix, thereby facilitating tumor growth, invasion, and metastasis (2, 3). Consistent with this concept, increased expression of several of these enzymes produced either by the tumor cells or by the surrounding stromal cells has been found in several types of human carcinomas (4, 5). Additional evidence supporting the role of MMPs in cancer comes from the fact that specific TIMPs can block tumor invasion in several model systems. Thus, it has been observed that exogenously added TIMPs can inhibit human amnion invasion and lung colonization by murine melanoma cells (6) as well as degradation of smooth muscle cell matrices by fibrosarcoma or c-Ha-*ras*-I transfected rat embryo cells (7). In addition, DeClerck *et al.* (8) have reported that inducible expression of metalloproteinase inhibitors in these c-Ha-*ras*-I expressing cells markedly reduces tumor growth rate *in vivo* after s.c. injection and completely suppresses their invasive behavior. Furthermore, down-regulation of TIMP levels confers invasive, tumorigenic, and metastatic properties to murine 3T3 cells (9). Based on structural and functional comparisons, these metalloproteinase inhibitors constitute a protein family that in humans is composed at present by two members, TIMP-1 and TIMP-2. TIMP-1 is an ubiquitous M_r 28,000 glycoprotein that binds tightly to the active form of multiple MMPs and is also found associated specifically with the latent form of M_r 92,000 gelatinase (10–12). TIMP-2 is a nonglycosylated protein with a molecular weight of about 20,000 that shares about 40% homology with TIMP-1 at the protein level and displays similar inhibitory activities against active MMPs. In addition, TIMP-2 binds with high affinity to the latent form of M_r 72,000 gelatinase (13, 14). Over the last years, animal homologues of human TIMPs have been isolated from a number of species including bovine, rabbit, and murine TIMP-1 and bovine TIMP-2. More recently, an additional member of the TIMP family, called ChIMP-3, has been isolated from chicken fibroblasts (15). According to its amino acid sequence, its unusual solubility properties, and its exclusive location in the extracellular matrix, it has been proposed that ChIMP-3 is the third member of the TIMP family, distinct from TIMP-1 and -2 (16). The availability of TIMP sequences of different sources has raised the possibility of finding new members of this protein family by using a PCR-based homology cloning strategy with degenerate oligonucleotides derived from conserved sequences. Following this approach and using a breast tumor cDNA library as starting material, we have cloned a cDNA coding for a new member of the metalloproteinase inhibitor family. In this work, we describe the complete nucleotide sequence of this putative metalloproteinase inhibitor, here designated as TIMP-3. We also show that this gene is expressed by placenta, uterus, and all examined breast carcinomas but not by other human tissues like liver and ovary.

Materials and Methods

Materials. Samples of human breast tumors were obtained from women who had undergone surgery for primary breast carcinoma; human placenta was obtained immediately after delivery and the remaining tissue specimens were from autopsies performed within 15 h after death. Tissue samples were frozen in liquid nitrogen and stored at −70°C until used. Restriction endonucleases and other reagents used for molecular cloning were from Boehringer Mannheim (Mannheim, Germany). Oligonucleotides were synthesized by the phosphoramidite method in an Applied Biosystems DNA synthesizer, model 381 A, and used directly after synthesis. The RNA-PCR kit used for the reverse transcription of total RNA and cDNA amplification was purchased from Perkin-Elmer Cetus (Norwalk, CT). The poly(A)$^+$ RNA purification and cDNA synthesis kits were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Double-stranded DNA probes were radiolabeled with [^{32}P]dCTP (3000 Ci/mmol) using a commercial random-priming kit from Pharmacia LKB.

PCR Amplification of Breast Carcinoma RNA. Total RNA from a breast carcinoma was isolated by guanidinium thiocyanate-phenol-chlorophorm extraction. cDNA synthesis was performed with the RNA-PCR Kit from Perkin-Elmer Cetus. The reverse transcription was carried out for 1 h at 42°C with 1 μg of total RNA and random hexamers as primer. The whole mixture was used for PCR with two degenerate oligonucleotides corresponding to conserved regions in TIMP-1, TIMP-2, and ChIMP-3 [5′-AT(ACT)CA(CG)CCNCA-(GA)GA(CT)GC and 5′-TCNGTCCA(GAT)AT(AG)C(TC)TC, respectively]. PCR reaction was carried out in a Techne Termal Cycler PHC-3 for 40 cycles

Received 1/12/94; accepted 3/4/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología (SAL91-0617) and Plan FEDER-European Community. J. A. U and J. P. F are recipients of a fellowship from FICYT-Asturias (Spain). The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with Accession No. X76227.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: MMP, matrix metalloproteinase; cDNA, complementary DNA; PCR, polymerase chain reaction; TIMP, tissue inhibitor of metalloproteinases; ChIMP-3, chicken inhibitor of metalloproteinase 3; poly(A)$^+$ RNA, polyadenylated RNA; SSC, standard saline-citrate (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0); SDS, sodium dodecyl sulfate.

of denaturation (94°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 1 min). The PCR products were phosphorylated with T4 polynucleotide kinase and the DNA band of the expected size (approx 0.4 kilobase) was gel purified and ligated in the *Sma*I site of pEMBL19. DNA from 24 independent clones was isolated and sequenced.

Preparation and Screening of a Breast Carcinoma cDNA Library. Breast carcinoma poly(A)$^+$ RNA was selected by oligodeoxythymidylate cellulose chromatography using a commercial kit from Pharmacia. Double-stranded cDNA was synthesized with the You-Prime cDNA Synthesis Kit (Pharmacia) using oligodeoxythymidylate as primer and ligated into the *Eco*RI site of μgt11. About 3×10^5 plaque forming units of the resulting library were plated using *Escherichia coli* Y1088 as host and screened using the partial TIMP cDNA cloned by RNA-PCR as probe. Hybridization to the radiolabeled probe was carried out at 60°C for 18 h in 6× SSC, 5× Denhardt's (1× is 0.02% bovine serum albumin, 0.02% polyvinylpirrolidone, and 0.02% Ficoll), 0.1% SDS, and denatured herring sperm DNA (100 μg/ml). Subsequently, the filters were washed twice for 1 h at 60°C in 1 × SSC-0.1% SDS and subjected to autoradiography. Following plaque purification, the cloned insert was excised by *Eco*RI digestion and subcloned in pEMBL19.

Nucleotide Sequencing and Computer Sequence Analysis. Appropriate DNA fragments were inserted in the polylinker region of phage vectors M13 mp18 and M13 mp19 and sequenced by the dideoxy terminator method using either M13 universal primer or cDNA specific primers and the Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH). In all cases, both strands were analyzed in order to confirm the sequence results. Sequence ambiguities were solved by substituting dITP for dGTP in the sequencing reactions. Computer analysis of DNA and protein sequences was performed with the software package of the University of Wisconsin Genetics Computer Group.

Northern Blot Analysis. Total RNA (about 30 μg) was separated by electrophoresis in 1.4% agarose-formaldehyde gels. After assessing RNA integrity and equal loading by observing the appearance of the rRNAs, blotting onto Hybond N nylon filters was carried out. Filters were prehybridized at 42°C for 3 h in 50% formamide, 5x saline-sodium phosphate-EDTA (1× is 150 mM NaCl-10 mM NaH_2PO_4-1 mM EDTA, pH 7.4), 2× Denhardt's, 0.1% SDS, and 100 μg/ml of denatured herring sperm DNA and then hybridized for 48 h under the same conditions, using the complete TIMP-3 cDNA. Filters were washed with 0.2× SSC-0.5% SDS for 2 h at 65°C and subjected to autoradiography for 3 h (normal tissues) or 24 h (breast carcinomas). In a parallel experiment, filters were also hybridized with a 23-mer oligonucleotide specific for TIMP-3 (5′-TTACCAGCTTCTTCCCCACCACC), end-labeled with polynucleotide kinase and [γ-^{32}P]ATP. In this case, filters were hybridized at 55°C for 18 h in 6× SSC, 5× Denhardt's, 0.1% SDS, and 100 μg/ml of denatured herring sperm DNA. Finally, filters were washed with 2× SSC-0.1% SDS for 2 h at 55°C and subjected to autoradiography for 48 h.

Results and Discussion

We have recently found that some breast tumors produce a novel human MMP that has been called collagenase 3.[4] Since it has been reported previously that the same cells which produce these proteolytic enzymes are capable of synthesizing and secreting specific tissue inhibitors, we were prompted to examine the possible presence of new members of the TIMP gene family in these mammary carcinomas. To do that, we first synthesized degenerate oligonucleotides the sequence of which was derived from conserved domains among different members of this protein family. After RNA-PCR of total RNA isolated from a breast tumor producing large amounts of collagenase 3, a band of the expected size (about 0.4 kilobase) was obtained. This material was cloned in the plasmid vector pEMBL19 and DNA from 24 independent clones was isolated and sequenced. Nucleotide sequencing analysis of these clones revealed that three of them corresponded to a cDNA similar to, but distinct from, the two human TIMPs previously isolated and characterized. Then, and in order to obtain a full-length cDNA for this putative new metalloproteinase inhibitor, a cDNA library was prepared using as starting material poly(A)$^+$ RNA from the same breast carcinoma used for the original RNA-PCR experiment. Upon screening of approximately 3×10^5 plaque forming units using the PCR generated cDNA as probe, two positive clones were identified. One of them, named T7–1, had an insert of about 1.0 kilobase, which could be large enough to contain the complete coding information for a TIMP. This insert was subcloned in pEMBL19, and its complete nucleotide sequence was determined as described in "Materials and Methods." Computer analysis of the obtained sequence (Fig. 1) revealed an open reading frame 633 base pairs long, starting with an ATG codon at position 71 and ending with a TGA codon at position 704. Assuming that translation starts at this ATG codon, the identified open reading frame codes for a protein of 211 amino acids and a predicted molecular weight of 24,117. In this sequence, the presence of a stretch of hydrophobic residues close to the initial methionine strongly suggests the existence of a secretory signal peptide that must be cleaved off to liberate the mature protein. Cleavage of this signal peptide is likely to occur after the second alanine residue located in the consensus sequence Ala–X–Ala, usually recognized by the eukaryotic cell signal peptidase. This cleavage would result in cysteine as the putative NH_2-terminal residue of the mature protein and allows the best sequence alignment with the remaining TIMPs. If this cleavage occurs, mature TIMP-3 would be composed of 188 residues with a predicted size of 21,665, similar to those corresponding to the previously characterized TIMPs.

A detailed comparison of this amino acid sequence with that corresponding to the other human TIMPs showed that it shares 40.1% identity with TIMP-1 and 44.9% with TIMP-2, including the 12 cysteine residues which are conserved among all members of the family (Fig. 2). However, when the comparison was performed with all TIMPs with complete sequences available, the highest degree of identity (82.4%) was found with ChIMP-3, a tissue metalloproteinase inhibitor produced by transformed fibroblasts from chicken (15, 16). According to these structural comparisons, it seems clear that the putative new metalloproteinase inhibitor identified in this work is the human counterpart of the previously described ChIMP-3; consequently, it has been called TIMP-3 since it represents the third human member of this protein family. It is worthwhile mentioning that TIMP-3 contains one potential *N*-glycosylation motif (Asn–Ala–Thr) in the carboxyl-terminal part of the molecule which is also present in ChIMP-3 at an identical position. In addition, TIMP-3 contains a 9-amino acid sequence at the carboxyl-terminal end, present in TIMP-2 but absent in TIMP-1, which has been proposed to exist in TIMP-2 as an exposed tail responsible for binding to M_r 72,000 gelatinase and for increasing the rate of inhibition of the active form of the enzyme (17). Finally, it is also noteworthy that the TIMP-3 sequence reported herein is very similar to an unpublished partial cDNA sequence from human placenta recently submitted to the GenBank database by Apte and Olsen (Accession No. L15078). When compared with the sequence of the breast carcinoma cDNA described in this work, the partial cDNA sequence from placenta lacks 303 nucleotides at the 3′-flanking region and 109 at the 5′-end, including the coding information for 13 amino acid residues of the putative TIMP-3. In addition, it lacks three nucleotides in a part of the coding region (positions 130, 131, and 136 of the breast carcinoma cDNA sequence) and displays a transition (C to T) at position 319. These changes originate a short alteration in the open reading frame, finally resulting in some variations in the amino acid sequence predicted for the corresponding proteins. The origin of these variations is presently unclear, and further studies will be required to establish whether they are the result of allelic variants, sequencing errors or differences between normal and tumoral metalloproteinase inhibitors.

In this work we have also studied the expression of TIMP-3 gene in

[4] J. P. Freije, I. Diez-Itza, M. Balbín, and C. López-Otín. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas, submitted for publication.

```
CCCGCCGGCGGCGCGCACGGCAACTTTGGAGAGGCGAGCAGCAGCCCCGGCAGCGGCGGCAGCAGCGGCAATGAC   75
                                                                      M  T

CCCTTGGCTCGGGCTCATCGTGCTCCTGGGCAGCTGGAGCCTGGGGGACTGGGGCGCCGAGGCGTGCACATGCTC  150
 P  W  L  G  L  I  V  L  L  G  S  W  S  L  G  D  W  G  A  E  A  C  T  C  S

GCCCAGCCACCCCCAGGACGCCTTCTGCAACTCCGACATCGTGATCCGGGCCAAGGTGGTGGGGAAGAAGCTGGT  225
 P  S  H  P  Q  D  A  F  C  N  S  D  I  V  I  R  A  K  V  V  G  K  K  L  V

AAAGGAGGGGCCCTTCGGCACGCTGGTCTACACCATCAAGCAGATGAAGATGTACCGAGGCTTCACCAAGATGCC  300
 K  E  G  P  F  G  T  L  V  Y  T  I  K  Q  M  K  M  Y  R  G  F  T  K  M  P

CCATGTGCAGTACATCCACACGGAAGCTTCCGAGAGTCTCTGTGGCCTTAAGCTGGAGGTCAACAAGTACCAGTA  375
 H  V  Q  Y  I  H  T  E  A  S  E  S  L  C  G  L  K  L  E  V  N  K  Y  Q  Y

CCTGCTGACAGGTCGCGTCTATGATGGCAAGATGTACACGGGGCTGTGCAACTTCGTGGAGAGGTGGGACCAGCT  450
 L  L  T  G  R  V  Y  D  G  K  M  Y  T  G  L  C  N  F  V  E  R  W  D  Q  L

CACCCTCTCCCAGCGCAAGGGGCTGAACTATCGGTATCACCTGGGTTGTAACTGCAAGATCAAGTCCTGCTACTA  525
 T  L  S  Q  R  K  G  L  N  Y  R  Y  H  L  G  C  N  C  K  I  K  S  C  Y  Y

CCTGCCTTGCTTTGTGACTTCCAAGAACGAGTGTCTCTGGACCGACATGCTCTCCAATTTCGGTTACCCTGGCTA  600
 L  P  C  F  V  T  S  K  N  E  C  L  W  T  D  M  L  S  N  F  G  Y  P  G  Y

CCAGTCCAAACACTACGCCTGCATCCGGCAGAAGGGCGGCTACTGCAGCTGGTACCGAGGATGGGCCCCCCCGGA  675
 Q  S  K  H  Y  A  C  I  R  Q  K  G  G  Y  C  S  W  Y  R  G  W  A  P  P  D

TAAAAGCATCATCAATGCCACAGACCCCTGAGCGCCAGACCCTGCCCCACCTCACTTCCCTCCCTTCCCGCTGAG  750
 K  S  I  I  N  A  T  D  P  *

CTTCCCTTGGACACTAACTCTTCCCAGATGATGACAATGAAATTAGTGCCTGTTTTCTTGCAAATTTAGCACTTG  825

GAACATTTAAAGAAAGGTCTATGCTGTCATATGGGGTTTATTGGGAACTATCCTCCTGGCCCCACCCTGCCCCTT  900

CTTTTTGGTTTTGACATCATTCATTTCCACCTGGGAATTTCTGGTGCCATGCCAGAAAGAATGAGGAACCTGTAT  975

TCCTCTTCTTCGTGATAATATAATCTCTATTTTTTTAGGAAAAAAA
```

Fig. 1. Nucleotide sequence of TIMP-3 cDNA from a breast carcinoma. The deduced amino acid sequence in standard one-letter code is shown below the nucleotide sequence. The complementary sequence of the oligonucleotide used for Northern blot analysis is *underlined*.

```
          1                                                  50
TIMP-1    .....MAPFE PLASGILLLL WLIA.PSRAC TCVPPHPQTA FCNSDLVIRA
TIMP-2    MGAAARTLRL ALGLLLLATL L...RPADAC SCSPVHPQQA FCNADVVIRA
TIMP-3    .....MTPWL GLI.VLLGSW SLGDWGAEAC TCSPSHPQDA FCNSDIVIRA
ChIMP-3   .....MTAWL GFLAVFLCSW SLRDLVAEAC TCVPIHPQDA FCNSDIVIRA

          51                                                 100
TIMP-1    KFVGTPEVNQ TTLY...... .QRYEIKMTK MYKGFQALGD AADIRFVYTP
TIMP-2    KAVSEKEVDS GNDIYGNPIK RIQYEIKQIK MFKGPEK... ..DIEFIYTA
TIMP-3    KVVGKKLVKE G......PFG TLVYTIKQMK MYRGFTKM.. .PHVQYIHTE
ChIMP-3   KVVGKKLMKD G......PFG TMRYTVKQMK MYRGFQIM.. .PHVQYIYTE

          101                                                150
TIMP-1    AMESVCGYFH RSHNRSEEFL IAGKLQDGLL HITTCSFVAP WNSLSLAQRR
TIMP-2    PSSAVCGVSL DVGGKKEYLI AGKAEGDGKM HITLCDFIVP WDTLSTTQKK
TIMP-3    ASESLCGLKL EV.NKYQYLL TGRVY.DGKM YTGLCNFVER WDQLTLSQRK
ChIMP-3   ASESLCGVKL EV.NKYQYLI TGRVY.EGKV YTGLCNWYEK WDRLTLSQRK

          151                                                200
TIMP-1    GFTKTYTVGC EECTVFPCLS IPCKLQSGTH CLWTDQLLQG SEKGFQSRHL
TIMP-2    SLNHRYQMGC .ECKITRCPM IPCYISSPDE CLWMDWVTEK NINGHQAKFF
TIMP-3    GLNYRYHLGC .NCKIKSCYY LPCFVTSKNE CLWTDMLSNF GYPGYQSKHY
ChIMP-3   GLNHRYHLGC .GCKIRPCYY LPCFATSKNE CIWTDMLSNF GHSGHQAKHY

          201                          229
TIMP-1    ACLPREPGLC TWQSLRSQIA .........
TIMP-2    ACIKRSDGSC AWYRGAAPPK QEFLDIEDP
TIMP-3    ACIRQKGGYC SWYRGWAPPD KSIINATDP
ChIMP-3   ACIQRVEGYC SWYRGWAPPD KTIINATDP
```

Fig. 2. Alignment of amino acid sequences for members of the human family of tissue metalloproteinase inhibitors. The amino acid sequences of TIMP-1, TIMP-2, and ChIMP-3 were extracted from the Swiss-Prot database and the multiple alignment was performed with the PILEUP program of the GCG package. Residues which are common to all sequences are in *boldface*.

both normal and pathological human tissues. To do that, samples from several tissues (uterus, placenta, liver, ovary, and breast carcinomas) were collected, and total RNA was isolated from them and analyzed by Northern blot, using the full-length TIMP-3 cDNA as probe. As can be seen in Fig. 3, a strong hybridizing band of about 2.4 kilobases was recognized by the probe in the lane corresponding to RNA from placenta. Additional bands corresponding to mRNA species of about 5, 2.7, 1.6, and 1.1 kilobases were also detected in this tissue. Similar bands although with clear differences in their relative intensity were also obtained in ovary as well as in all examined breast carcinomas. By contrast, none of them was present in other tissue specimens analyzed like liver and ovary (Fig. 3). In order to rule out the possibility of cross-hybridization with either TIMP-1 or TIMP-2, an additional filter was hybridized with a 23-mer oligonucleotide which represents a nucleotide sequence specific for TIMP-3. As shown in Fig. 3, the oligonucleotide probe recognized the same bands in placenta RNA as the complete cDNA probe, thus confirming that the different transcripts correspond to TIMP-3. Several possibilities could contribute to explain the occurrence of multiple TIMP-3 transcripts, including alternative utilization of polyadenylation sites, variable extension of the 5′ flanking region or existence of additional genes with a high degree of sequence similarity with TIMP-3. Interestingly, the presence of two different transcripts in both normal and tumor tissues has been also reported for TIMP-2. The origin of these two transcripts is also unknown, although it has been suggested that they are probably the result of alternate 5′ untranslated regions (18).

Finally, the availability of the cDNA coding for TIMP-3 opens the possibility of studying its potential role in both normal and pathological conditions, with special interest in defining the significance of its expression in breast carcinomas. In this regard, it is remarkable that in addition to their inhibitory activity on metalloproteinases, growth factor properties have also been ascribed to TIMPs (11, 19). It is also noteworthy that the synthesis of ChIMP-3, the putative chicken homologue of the human TIMP-3 here described, is stimulated during the early stages of oncogenic transformation of chicken fibroblasts. Furthermore, purified ChIMP-3 is able to promote some of the phenotypic properties of transformed cells (20). Additional studies with purified native or recombinant protein will be helpful in examining the possibility that TIMP-3 may play similar functions in human

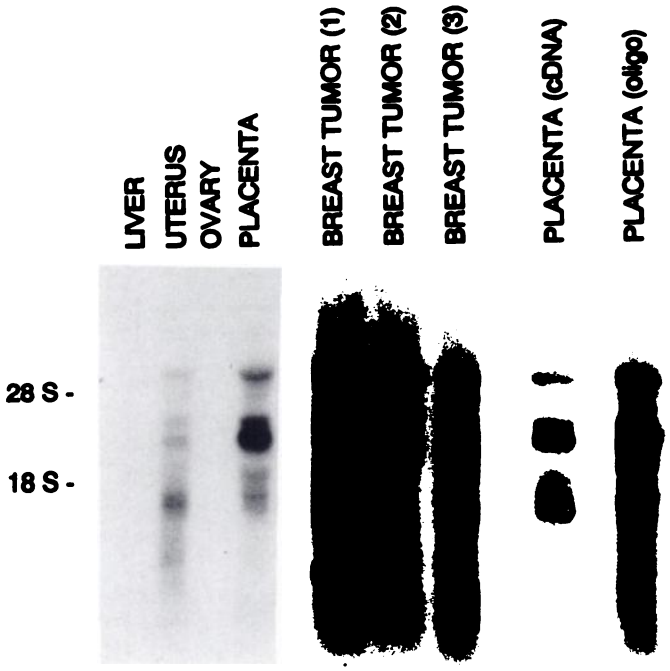

Fig. 3. Northern blot analysis of TIMP-3 mRNA in normal and pathological human tissues. About 30 μg of total RNA were loaded in each lane and analyzed by hybridization with the complete TIMP-3 cDNA probe or with a 23-mer oligonucleotide specific for TIMP-3. The positions of 28 and 18S rRNA are shown. Results are representative of 20 different breast carcinomas examined.

tissues and will contribute to the definition of its precise role in the maintenance of connective tissue homeostasis.

Acknowledgments

We thank Dr. S. Gascón for support, Drs. F. Vizoso and I. Díez-Itza for supplying tissue specimens, Drs. Y. S. López-Boado and M. Balbín for RNA samples, and Dr. S. G. Granda (Scientific Computer Center, Universidad de Oviedo) for computer facilities.

References

1. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, *64:* 327–336, 1991.
2. Matrisian, L. M. The matrix-degrading metalloproteinases. BioEssays, *14:* 455–463, 1992.
3. Woessner, J. F. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J., *5:* 2145–2154, 1991.
4. Stetler-Stevenson, W. G. Type IV collagenases in tumor invasion and metastasis. Cancer Metastasis Rev., *9:* 289–303, 1990.
5. Basset, P., Bellocq, J.-P., Wolf, C., Stoll, I., Hutin, P., Limacher, J. M., Podhajcer, O. L., Chenard, M. P., Rio, M. C., and Chambon, P. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature (Lond.), *348:* 699–704, 1990.
6. Schultz, R. M., Silberman, S., Persky, B., Bajkowski, A. S., and Carmichael, D. F. Inhibition by human recombinant tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells. Cancer Res., *48:* 5539–5545, 1988.
7. DeClerck, Y. A., Yean, T. D., Chan, D., Shimada, H., and Langley, K. E. Inhibition of tumor invasion of smooth muscle cell layers by recombinant human metalloproteinase inhibitor. Cancer Res., *51:* 2151–2157, 1991.
8. DeClerck, Y. A., Pérez, N., Shimada, H., Boone, T. C., Langley, K. E., and Taylor, S. M. Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer Res., *52:* 701–708, 1992.
9. Khokha, R., Waterhouse, P., Yagel, S., Lala, P. K., Overall, C. M., Norton, G., and Denhardt, D. T. Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science (Washington DC), *243:* 947–950, 1989.
10. Stricklin, G. P., and Welgus, H. G. Human skin fibroblast collagenase inhibitor. Purification and biochemical characterization. J. Biol. Chem., *258:* 12252–12258, 1983.
11. Docherty, A. J. P., Lyons, A., Smith, B. J., Wright, E. M., Stephens, P. E., Harris, T. J. R., Murphy, G., and Reynolds, J. J. Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature (Lond.), *318:* 66–69, 1985.
12. Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A., and Goldberg, G. SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. J. Biol. Chem., *264:* 17213–17221, 1989.
13. Stetler-Stevenson, W., Krutzsch, H. C., and Liotta, L. A. Tissue inhibitor of metalloproteinases (TIMP-2): a new member of the metalloproteinase inhibitor family. J. Biol. Chem., *264:* 17374–17378, 1989.
14. Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S., and He, C. Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2. Proc. Natl. Acad. Sci. USA, *86:* 8207–8211, 1989.
15. Staskus, P. W., Masiarz, F. R., Pallanck, L. J., and Hawkes, S. P. The 21-kDa protein is a transformation-sensitive metalloproteinase inhibitor of chicken fibroblasts. J. Biol. Chem., *266:* 449–454, 1991.
16. Pavloff, N., Staskus, P. W., Kishnani, N. S., and Hawkes, S. P. A new inhibitor of metalloproteinases from chicken: ChIMP-3. J. Biol. Chem., *267:* 17321–17326, 1992.
17. Willembrock, F., Crabbe, T., Slocombe, P. M., Sutton, C. W., Docherty, A. J. P., Cockett, M. I., O'Shea, M., Brocklehurst, K., Phillips, I. R., and Murphy, G. The activity of the tissue inhibitors of metalloproteinases is regulated by C-terminal domain interactions: a kinetic analysis of the inhibition of gelatinase A. Biochemistry, *32:* 4330–4337, 1993.
18. Stetler-Stevenson, W. G., Brown, P. D., Onisto, M., Levy, A. T., and Liotta, L. A. Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J. Biol. Chem., *265:* 13933–13938, 1990.
19. Gasson, J. C., Golde, D. W., Kaufman, S. E., Westbrook, C. A., Hewick, R. M., Kaufman, R. J., Wong, G. G., Temple, P. A., Leary, A. C., Brown, E. L., Orr, E. C., and Clark, S. C. Molecular characterization and expression of the gene encoding human erythroid-potentiating activity. Nature (Lond.), *315:* 768–771, 1985.
20. Yang, T. T., and Hawkes, S. P. Role of the 21-kDa protein TIMP-3 in oncogenic transformation of cultured chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA, *89:* 10676–10680, 1992.

Structure and Expression in Breast Tumors of Human TIMP-3, a New Member of the Metalloproteinase Inhibitor Family

José A. Uría, Adolfo A. Ferrando, Gloria Velasco, et al.

Cancer Res 1994;54:2091-2094.