Human tissue inhibitor of metalloproteinase-2 (TIMP-2) was cloned and sequenced from an A2058 human melanoma cell cDNA library. When the sequence was compared with that of human TIMP-1 at both the nucleotide and deduced amino acid levels, the homology appeared lower at the protein level than at the nucleotide level, suggesting that these inhibitors diverged early in the evolution of this gene family. Comparison of the deduced amino acid sequence for TIMP-2 with that of human TIMP-1 shows that there are two regions in which the similarity is below the overall average of 66%. It is postulated that these regions are responsible for the unique ability of TIMP-2 to bind to the latent form of the 72-kDa type IV collagenase. Polyclonal anti-TIMP-2 antisera recognized TIMP-2 but not TIMP-1 on immunoblotting.

Northern blot analysis of RNA from A2058 human melanoma, HT-144 human melanoma, HT-1080 human fibrosarcoma, and WI-38 fetal lung fibroblast cell lines demonstrated two distinct transcripts of 1.0 and 3.5 kilobases (kb) for timp-2 mRNA. Both transcripts are down-regulated in response to transforming growth factor-β but are unchanged in response to phorbol ester treatment. This is in contrast to the up-regulation of timp-1 transcripts by these agents and indicates that timp-2 and timp-1 are independently regulated in cell culture. Northern blot analyses of matched normal and tumor tissue samples from five cases of human colorectal carcinoma were performed. Normal and tumor tissues contain both the 1.0- and 3.5-kb transcripts. However, in the tissue samples the ratio of the 3.5-kb transcript to the 1.0-kb transcript was markedly elevated. No evidence of down-regulation of timp-2 transcript levels was noted in the tumor tissues. This is in contrast to the elevated timp-1 transcript levels seen in these tumor samples. Thus, timp-2 mRNA transcript levels are differentially regulated from timp-1 levels in vivo as well as in cell culture.

The collagenase family enzymes are a group of metalloproteinases which are secreted in the zymogen form and degrade both the collagenous and noncollagenous components of the extracellular matrix. The overproduction and unrestrained activity of these enzymes have been linked to a variety of pathologic conditions such as rheumatoid arthritis and malignant conversion of tumor cells (Okada et al., 1986; Harris et al., 1984; Werb et al., 1977; Liotta et al., 1980; Kalebic et al., 1983). The down-regulation of metalloproteinase collagenolysis and proteolysis may occur through naturally occurring inhibitor proteins, such as the tissue inhibitors of metalloproteinases (TIMPs).1

TIMP-1 is a glycoprotein with an apparent molecular size of 28.5 kDa which forms a complex of 1:1 stoichiometry with activated interstitial collagenase, activated stromelysin, and the 92-kDa type IV collagenase (Welgus and Stricklin, 1983; Welgus et al., 1986a; Wilhelm et al., 1989). The gene coding for TIMP-1 has been cloned, sequenced, and mapped to the X chromosome (Carmichael et al., 1985; Docherty et al., 1985; Mullins et al., 1988; Mahtani and Willard, 1988). The secreted protein has 184 amino acids and six intramolecular disulfide bonds. The same cells which produce interstitial collagenase are capable of synthesizing and secreting TIMP-1 (Welgus et al., 1985b; Herron et al., 1986). Thus, the net collagenolytic activity for these cell types is the result of the balance between activated enzyme levels and TIMP-1 levels. Studies have shown an inverse correlation between TIMP-1 levels and the invasive potential of murine and human tumor cells (Khokha et al., 1989).

Recently we have isolated, purified, and determined the complete primary structure of a second member of the TIMP family, TIMP-2 (Stetler-Stevenson et al., 1989). TIMP-2 is a 21-kDa protein which selectively forms a complex with the latent proenzyme form of the 72-kDa type IV collagenase (Stetler-Stevenson et al., 1989; Goldberg et al., 1989). The secreted protein has 192 amino acid residues and is not glycosylated. TIMP-2 shows an overall 71% similarity to TIMP-1 at the amino acid sequence level. The positions of the 12 cysteine residues are conserved with respect to those present in TIMP-1, as are three of the four tryptophan residues. TIMP-2 inhibits the type IV collagenolytic activity and the gelatinolytic activity associated with the 72-kDa enzyme (Stetler-Stevenson et al., 1989). Inhibition studies demonstrated that complete enzyme inhibition occurred at a 1:1 molar ratio of TIMP-2 to activated 72-kDa type IV collagenase (Stetler-Stevenson et al., 1989). Thus, unlike TIMP-1, TIMP-2 is capable of binding to both the latent and activated forms of type IV collagenase. Cell culture studies using cell lines that produce a variety of collagenase family enzymes, as well as both TIMP-1 and TIMP-2 suggest that TIMP-2 preferentially interacts with the 72 kDa type IV collagenase (Stetler-Stevenson et al., 1989; Goldberg et al., 1989).
Augmented type IV collagenolytic activity has been associated with the metastatic phenotype in a number of experimental systems. This could possibly be due to the increased production and activation of the 72-kDa type IV collagenase enzyme. However, decreased production of TIMP-2 could also result in greater effective enzyme activity. To examine the regulation of TIMP-2 we have isolated and sequenced a cDNA clone for human TIMP-2. Comparison of the cDNA sequence of *timp-2* with that of *timp-1* suggests that these inhibitors diverged early in the evolution of the TIMP family. We have used this probe to measure the levels of *timp-2* mRNA in human tumor cell lines and the effects of phorbol ester and transforming growth factor β (TGF-β1) treatment on *timp-2* mRNA levels. These effects were contrasted with those noted for *timp-1* mRNA levels. Finally, we have examined *timp-2* and *timp-1* mRNA levels in a series of human colon adenocarcinomas and adjacent normal colonic mucosa.

**EXPERIMENTAL PROCEDURES**

**Human Melanoma Cell cDNA Library Preparation, Screening, and DNA Sequencing**—Oligo(dT)-select poly(dA) mRNA was prepared from human A2058 melanoma cells using standard methods. 1 μg of purified mRNA was used to prepare double-stranded cDNA using a commercially available cDNA synthesis kit (Amersham Corp.). This cDNA was methylated using EcoRI methylase (Promega). linked to EcoRI linkers (Promega), restricted with EcoRI, and ligated to EcoRI-digested λ-GEM-4 (Promega). The ligations were packaged (Gigapack Gold, Stratagene) and the optimal reactions were pooled to give 1.5 × 10^10 recombinants. 7.5 × 10^8 recombinants were screened using oligonucleotide 27-40. Oligonucleotide 27-40, a 45-mer, with the sequence, 5'-GAGAAGGAGGTGGACTCTGGCAATGACATCTATGGGACCGCAGGGACTGCCAGGT3', corresponds to the reverse translation of residues 27 through 40 of the previously sequenced TIMP-2 protein.

The predicted amino acid sequence is shown under the DNA sequence. The human TIMP-2 cDNA. Comparison of the cDNA sequence with that of *timp-1* indicates that these inhibitors diverged early in the evolution of the TIMP family. We have used this probe to measure the levels of *timp-2* mRNA in human tumor cell lines and the effects of phorbol ester and transforming growth factor β (TGF-β1) treatment on *timp-2* mRNA levels. These effects were contrasted with those noted for *timp-1* mRNA levels. Finally, we have examined *timp-2* and *timp-1* mRNA levels in a series of human colon adenocarcinomas and adjacent normal colonic mucosa.

**RESULTS**

We have previously determined the primary structure of the TIMP-2 protein by direct amino acid sequencing (Stetler-Stevenson et al., 1988). This information was used to prepare a synthetic oligonucleotide probe, oligo 27-40, which was used to screen a cDNA library constructed from mRNA isolated from human A2058 melanoma cells. Ten clones were isolated, and the nucleotide sequence of the cDNA insert in the longest clone, pT2-MO1, is presented in Fig. 1.

![Fig. 1. Nucleotide sequence of human TIMP-2 cDNA.](image-url)
sequence includes a 26-residue signal peptide sequence and a mature TIMP-2 protein of 194 amino acids. The 130-nucleotide-long 3′-untranslated region contains a polyadenylation signal 30 bases upstream from the 3′ end of the RNA.

Comparison of the amino acid sequence of TIMP-2 deduced from the cDNA clone with that determined by direct amino acid sequencing of overlapping endoproteinase-derived peptide fragments shows excellent agreement. The original sequence contained only 192 amino acids. The previously unidentified residues correspond to the glycyl residue at position 92 and the prolyl residue at the carboxyl terminus. Other changes are noted in Fig. 2. The homology of mature TIMP-2 with TIMP-1 at the predicted protein level is 37.6% identity and 65.6% overall similarity.

Rabbit anti-human TIMP-2 polyclonal antibodies were used in an immunoblot comparison of bovine TIMP-1 and human TIMP-2. As shown in Fig. 3, bovine TIMP-1 migrates at approximately 28 kDa, as previously reported for this glycosylated protein, compared with TIMP-2 which migrates at 21 kDa and is unglycosylated. Rabbit polyclonal anti-TIMP-2 antibodies failed to detect TIMP-1 but showed excellent reactivity with TIMP-2.

Northern blot analysis of oligo(dT)-selected mRNA isolated from the A2058 human melanoma cell line revealed two specific timp-2 mRNA species with approximate sizes of 3.5 and 1.0 kb (Fig. 4). These timp-2 transcripts are clearly distinguished from the 0.9-kb timp-1 transcript detected with the P551 probe. No evidence of cross-hybridization between the temps was observed under the hybridization and stringent wash conditions utilized. Both timp-2 mRNA species were also detected in RNA isolated from human Wi-38 fibroblasts, HT-1080 fibrosarcoma, and HT-144 melanoma cells. The 3.5-kb timp-2 transcript predominated in the fibroblast and fibrosarcoma cell lines with only low levels of the 1.0-kb species detectable (Fig. 5).

Treatment of cells with TPA (10 ng/ml) for 48 h failed to significantly modulate either timp-2 transcript level in the melanoma cell lines tested (Fig. 5). Both timp-2 transcripts showed a 2-fold induction by TPA in the normal fibroblast cell line Wi-38 and a 2-fold reduction in the fibrosarcoma cell line HT-1080. In contrast timp-1 transcript levels were increased 2-fold by TPA treatment in both melanoma cell lines as well as in the HT-1080 fibrosarcoma cells. Phorbol ester treatment of the Wi-38 fibroblast cell line resulted in an 8-fold induction of the timp-1 transcript level. These findings are consistent with the induction of timp-1 by this agent as previously reported (Edwards et al., 1985; Murphy et al., 1985; Welsch et al., 1985).

Treatment of cells with TGF-β1 for 48 h resulted in a clearly detectable decrease in timp-2 mRNA levels in all cell lines tested except the Wi-38 fibroblasts (Fig. 5). The 3.5- and 1.0-kb timp-2 transcripts showed equal TGF-β1-induced decreases in steady-state levels, and there was no indication
of differential repression by TGF-β1. Treatment of A2058 melanoma cells with TGF-β1 reduced steady-state timp-2 transcript levels to 46 and 59% of control values for the 1.0 kb and 3.5-kb transcripts, respectively. Treatment of HT-144 cells with TGF-β1 resulted in reduction of timp-2 transcripts to 42 and 47% of control levels for the 1.0 and 3.5-kb message, respectively. Both timp-2 transcripts showed a more moderate TGF-β1-induced reduction in the HT-1080 fibrosarcoma cell line of 25% from control levels. These effects are in contrast to the results observed for timp-1 message levels. In the tumor cell lines (HT-144, HT-1080, and A2058) TGF-β1 induced an increase in timp-1 steady-state transcript levels to 150% of control values. Treatment of Wi-38 fibroblasts with TGF-β1 resulted in a 6-fold increase of message levels, respectively.

Northern blot analyses of tissue from five primary human colorectal tumors and patient-matched adjacent normal mucosa were performed using both the timp-1 and timp-2 probes (Fig. 6). Ethidium bromide staining of the formaldehyde gels prior to transfer demonstrated equal loading of RNA in all lanes (data not shown). The results demonstrate that both the 1.0- and 3.5-kb timp-2 transcripts are present but that the 1.0-kb message level is markedly reduced compared with that seen in the RNA isolated from cultured human tumor cells. Transcript levels for timp-2 show little correlation with tissue origin (normal versus tumor). In most tumor samples the timp-2 transcript levels show no difference from the adjacent normal tissue, with the exception of tumor T2 in which there is a marked decrease in timp-2 message levels compared with that seen in the adjacent normal colonic mucosa N2. These data suggest that primary colon adenocarcinomas may be heterogeneous with respect to the levels of timp-2. However, timp-1 transcript levels are obviously elevated in all tumor tissue samples, including sample T2, compared with normal adjacent mucosa. Again, these observations suggest that timp-2 and timp-1 are independently regulated.

**DISCUSSION**

Isolation and sequencing of a cDNA clone for human TIMP-2 was performed. Characterization of the TIMP-2 cDNA clone pT2-MO1 confirmed that TIMP-2 is a unique gene product independent of TIMP-1. The protein sequence predicted from the TIMP-2 cDNA is in excellent agreement (95%) with the amino acid sequence obtained for this protein by direct protein sequencing (Stetler-Stevenson et al., 1989). Pustell Matrix analysis of the homology distribution between these two predicted protein sequences using a cut-off value of 66% and an 8-amino acid overlap demonstrates that there are two areas in which the homology falls below this average value (Fig. 7A). TIMP-2 shows a distinct preference for binding to the latent form of the 72-kDa type IV collagenase in the presence of both other latent metalloproteinases and TIMP-1 (Stetler-Stevenson et al., 1989; Goldberg et al., 1989). However, both forms of TIMP will inhibit activated type IV collagenase. Thus regions of amino acid sequence that are highly conserved between these proteins, such as those that exceed the overall homology value of 66%, may be responsible for the known shared functions of these proteins, inhibition of the activated collagenase family of enzymes. Areas of low homology are likely to be responsible for those functions which are unique for individual TIMP molecules. Thus, the regions of low homology between residues 45 and 70 and the carboxyl terminus of TIMP-2 may be responsible for the binding of TIMP-2 to the latent form of the 72-kDa type IV collagenase. Such regions must exist and account for the failure of TIMP-1 antibodies to detect TIMP-2, as previously reported (Goldberg et al., 1989), as well as the failure of TIMP-2 antibodies to detect TIMP-1 as demonstrated in the current report (Fig. 3).

Comparison of the cDNA sequences of human timp-2 with human timp-1 shows little homology considering that seen at the amino acid level (Fig. 7B). This result implies that these genes diverged early in the evolution of this gene family. The lack of homology at the cDNA level may also explain why timp-2 mRNA transcripts are not detected in Northern blot analyses using timp-1 probes and also why screening cDNA libraries with timp-1 probes fails to yield timp-2 clones.

Northern blot analyses of oligo(dT)-selected poly(A) RNA as well as total cytoplasmic RNA from human tumor cell lines detect the presence of two transcripts when probed with the full-length timp-2 cDNA clone pT2-MO1. The origin of these
cells studied by other investigators. We have previously demonstrated that TGF-β1 induces the 72 kDa type IV collagenase mRNA and protein levels in the same tumor cell lines studied in the present report. We now demonstrate that TGF-β1 induces TIMP-2 mRNA transcript levels. Thus, TGF-β1 treatment has an opposite effect on TIMP-2 compared with the 72-kDa type IV collagenase transcript levels in human tumor cells. This suggests that TGF-β1 treatment may result in augmented type IV collagenolytic activity due to up-regulation of the enzyme coupled with down-regulation of an associated inhibitor, TIMP-2. This could result in an enhanced invasive phenotype of tumor cells treated with TGF-β1, although TGF-β1 does induce an increase in timp-1 transcript levels. These observations demonstrate the complex multilevel regulation of type IV collagenolytic activity. However, it is clearly evident that the transcriptional regulation of timp-2 is independent of timp-1.

Northern blot analyses of human colorectal tumor and adjacent normal tissues again demonstrated two mRNA transcripts when probed with the timp-2 cDNA clone. However, in the RNA from these tissue samples the 3.5-kb transcript clearly predominates, with only trace amounts of the 1.0-kb message detectable in the normal tissues. There was no correlation of timp-2 transcript levels and adenocarcinoma tissues. However, transcript levels for timp-1 show a distinct correlation with the malignant tumor samples. All adenocarcinoma tissue samples showed elevated timp-1 levels compared with adjacent normal colonic mucosa. This result was highly unexpected in light of the malignant nature of the tumor tissues examined and the report of Khokha et al. (1989) which demonstrated an inverse correlation between timp-1 mRNA levels and the invasive phenotype. Immunohistochemical studies of the distribution of TIMP-1 protein in these tumor samples will be helpful in developing an understanding of these observations.

In summary, we have cloned and sequenced a full-length cDNA which encodes the pro-TIMP-2 protein. At the protein level there is a marked homology between members of the TIMP family. However, at the nucleotide level this homology is notably less, suggesting early divergence of these genes. Finally, the data presented clearly demonstrate that timp-2 is regulated independently of timp-1, both in cell culture as evidenced by studies using TPA and TGF-β1 and in vivo as evidenced by comparison of transcript levels for these inhibitors in human colon adenocarcinoma tissue and adjacent normal colonic mucosa.

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Note Added in Proof—The cDNA sequence reported is identical to the human metalloproteinase inhibitor (MI) sequence reported by Boone et al. (Boone, T. C., Johnson, M. J., DeClerck, Y. A., and Langley, K. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2800–2804.)

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