Abstract. Background: Matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), have been implicated in invasion and metastasis. The distribution of MMPs and TIMPs in the invasion front of liver metastases from colorectal cancer were investigated in order to understand their potential role in invasiveness. Materials and Methods: Freshly frozen material of colorectal metastases of the liver was microdissected into four separate compartments, namely pure liver, liver invasion, tumour invasion and pure tumour. RNA was isolated and analyzed on Affymetrix microarrays. Expression of TIMP-1 was confirmed by quantitative polymerase chain reaction in 10 colorectal liver metastases. Cellular localisation of TIMP-1 was examined by immunohistochemistry. Results: Affymetrix microarray data revealed that several MMP and TIMP genes including MMP-2, -3, -7, -9, -11, -12, -14, -15, -16, -19 and -24, and TIMP-1, -2 and -3 were generally up-regulated in both invasion front compartments. Among these genes, TIMP-1 showed the highest expression. The qPCR results indicated an average 15-fold up-regulation of TIMP-1 in the liver invasive front and an average 13-fold up-regulation in the tumor invasive front, each compared to normal liver tissue. Immunohistochemistry revealed expression of TIMP-1 in tumour epithelia as well as in host tissue cells, including fibroblastic cells. Conclusion: Our data indicate that tumour invasion in colorectal liver metastasis is associated with increased TIMP-1 RNA and protein levels in both tumour and host cells.

Besides unrestricted proliferation and reduced apoptosis, unbalanced invasive behaviour is the third major prerequisite of malignancy. Invasion by tumour cells is dependent on a permissive host environment at the primary invasive site as well as at the site of metastasis.

Excessive degradation and remodelling of the extracellular matrix (ECM) is one of the hallmarks of cancer progression at nearly every step of the metastatic cascade. Proteases contribute to each step from the first breakdown of the basal membrane of the primary tumour up to the extended growth of established metastases (1). Matrix metalloproteinases (MMPs) are a family of 24 enzymes which play an important role in this process. Naturally occurring tissue inhibitors of metalloproteinases (TIMPs 1 to 4) normally regulate and counterbalance the proteolytic activity of MMPs by binding to both the latent and active forms of MMPs in a 1:1 stoichiometry (2, 3). Overexpression of TIMPs by means of gene transfer (4-6) as well as application of synthetic MMP inhibitors (6) has shown marked antitumor activity in various animal models. However, a tumour-promoting effect of TIMPs 1 to 3 has been reported in vitro which occasionally translated into promotion of cell growth and metastasis in vivo (6). In addition, clinical trials using synthetic MMP inhibitors were of limited success (7). These conflicting data indicate that a deeper understanding of the MMP/TIMP interplay and of potential additional functions is required. Colorectal carcinoma is the second most frequent cancer disease in both genders (8). For patients with this type of cancer, liver metastases are the main cause of death. They often remain the only manifestation of the disease once the primary tumour has been surgically removed (9, 10). We have shown that adenoviral gene transfer of TIMP-1 (5) or TIMP-2 (4) into the unaffected liver tissue of mice can inhibit the growth of colorectal liver metastases. This argues for a role of TIMPs particularly at the tumour/host interface. As part of ongoing studies to correlate preclinical efficacy data with a more basic understanding of the biology of the invasive front, we recently reported global gene expression profiles of the invasive front in these preclinical animal models. In order to put this into a clinical context, here we analyzed MMPs and TIMPs in clinical samples. Analysis was performed in a compartment specific fashion by dividing the tissue into a) liver tissue distant from the invasive front,
liver tissue at the invasive front, tumour tissue at the invasive front and tumour distant from the invasive front.

Materials and Methods

**Clinical specimens.** Tissue collection was approved by the University of Heidelberg Ethics Committee. Tissue was collected with informed consent of 20 patients (10 female, 10 male, average age: 62.4 years) who underwent resection of liver metastases from colorectal cancer in the Department of Surgery, University Hospital Heidelberg, Germany. Pure liver tissue was obtained at least 5 cm away from the liver metastases. One portion of each collected specimen was immediately embedded in Tissue Tek® (Sakura Finetek, Europe), snap frozen in precooled (−80°C) 2-methyl-1-propanol and stored at −80°C. Another portion was immediately fixed in 4% neutral buffered formalin, incubated for 24 h and the paraffin embedded.

**Tissue preparation and laser microdissection (LMD).** From frozen tissue blocks, 16 μm-sections were cut using a cryostat (Leica, Wetzlar, Germany) and stained using cresyl violet according to the Ambion (Austin, TX, USA) LCM staining kit protocol. Four distinct cell populations were separately microdissected with LCM equipment (Molecular Machines & Industries, Eching, Germany or PALM, Bernried, Germany): a) pure liver tissue at least 5 cm away from the invasive front, b) liver invasive front tissue extending up to 10 cell layers into the liver, c) tumour invasive front tissue extending up to 10 cell layers into the tumour and d) pure tumour tissue at least 100 cell layers away from the invasive front (Figure 1).

**RNA amplification.** Total RNA from microdissected samples was extracted (RNeasy Mini Kit, Qiagen, Hilden, Germany) and its quality was evaluated using an Agilent (Waldbrohn, Germany) 2100 Bioanalyzer. From five clinical specimens, equal amounts of totalRNA from identical compartments were pooled to 250 ng totalRNA, amplified (MessageAmp Biotion Kit, Ambion, Austin, TX, USA) and probed on the Human Genome U133 set (A+B) (Affymetrix, Santa Clara, CA, USA).

**Microarray analysis.** The scanned images from the chips were processed using Affymetrix GCOS and Excel (Microsoft, Seattle, WA, USA) software.

**Relative quantitative real-time PCR.** Microdissection and RNA isolation for relative qPCR were essentially performed as for hybridization experiments. Three nanograms of total RNA were used for quantification. Reverse transcription, qPCR, normalization (against 18S RNA) and efficiency correction (against 18S RNA) were performed essentially as described elsewhere (11). Oligonucleotides for qPCR were designed using the Primer3 software (Whitehead Institute, Cambridge, MA, USA). The sequences for 18S RNA were: forward primer: 5'-AA CGG CTA CCA CAT CCA AG-3', reverse primer: 5'-CCT CCA ATG GAT CCT CGT TA-3'; those for TIMP-1 were: 5'-TGGAG AAGGGTGGAAAACCGAG-3', reverse primer: 5'-TCTCCTCTTT CCCAGGAATT-3. TIMP-1 expression was quantified as described (12) and expression data were evaluated statistically by the Wilcoxon signed rank test.

**Immunohistochemistry.** One micrometer sections from archival paraffin embedded tissue blocks were placed on capillary gap microscope slides (DakoCytomation, Glostrup, Denmark) and baked overnight at 37°C. Sections were deparaffinized by xylene, rehydrated in graded concentrations of ethanol and then pretreated with protease K (10 μg/ml) for 20 minutes. Endogenous peroxidase activity of the tissue was quenched by treatment with 4% H2O2 for 20 minutes. Non-specific binding sites were blocked in 1 mol/L phosphate-buffered saline (PBS) with 10% normal rabbit serum and an Avidin/Biotin Blocking Kit (Vector Laboratories, Burlington, CA, USA). Sections were incubated with sheep polyclonal antibodies at a final concentration of 16 μg/ml against TIMP-1 at 4°C. The antibodies were raised by immunization with TIMP-1 purified from human dermal fibroblasts and specifically bind to both free and MMP-complexed human TIMP-1 (13). After washing with 0.05% Triton-X 100/PBS, sections were incubated with biotinylated rabbit-anti-sheep IgG (1:200, PK 4006; Vector Laboratories) for 1 h. Subsequently, the IgG antibodies were detected with avidin-coupled horseradish immunoperoxidase (Vector Laboratories) and visualized using diaminobenzidine (Peroxidase Substrate Kit DAB, E108; Vector Laboratories). Finally, sections were counterstained with hematoxylin, dehydrated in graded concentrations of ethanol and mounted. To exclude unspecific staining, negative controls of each specimen were prepared by running the immunohistochemical procedure as described above but by omitting the polyclonal sheep antibody. Hence, immunostaining of tissue slides was independently analysed by two clinical pathologists.

Results

**Differential gene expression of MMPs and TIMPs in the invasive front of human colorectal liver metastases.** We started our analysis by obtaining a global overview of the gene expression levels of all MMPs and TIMPs represented on Affymetrix U133 A+B chips covering the whole human transcriptome. Tissue from clinical samples was used and separately microdissected after distinction into four compartments (Figure 1). Prior to RNA amplification and further processing, RNA of 5 clinical specimens was pooled. The overview of gene expression shows that roughly 50% of MMPs (11/24) and 75% (3/4) of TIMPs were expressed at least in one of the compartments (Figure 2). Ten out of 11 present MMPs displayed slight to marked up-regulation in at least one of the two invasive front compartments as compared to pure liver or tumour. With respect to substrate specificity, these MMPs represent all gelatinases (MMP-2 and MMP-9), all transmembrane membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-24), 2 out of 3 stromelysins (MMP-3, MMP-11), 1 out of 2 matrixins (MMP-7) and 2 MMPs (MMP-12, MMP-19). None of the MMPs from the group of collagenases or the glycosylphosphatidylinositol (GPI) anchored protein membrane-type MMPs showed overexpression. Regarding the inhibitors of MMPs, TIMP-1 exhibited a marked up-regulation in both invasive front compartments and was most prominent in the liver part of the invasion front. Expression of TIMP-2 was more prominently increased in the tumour part of the invasive front compared to the liver part of the invasive front but was absent from tumour tissue. Conversely,
TIMP-3 expression was fairly homogenously distributed in all four compartments. TIMP-4 was not detected by the Affymetrix chips U133 A+B in any compartment. Quantification of TIMP-1 gene expression in individual clinical specimens. In order to further substantiate TIMP-1 up-regulation and to evaluate the variability of TIMP-1 gene
expression in the invasive front, mRNA levels were determined in 10 individual samples by semi-quantitative real-time PCR. As shown in Figure 3A, in all cases, the liver compartment displayed the lowest values. With the exception of 2 cases, the tumour compartments showed the next lowest values. The highest values were seen either in the liver invasion (6/10 specimen) or the tumour invasion (3/10 specimen) compartments. These values ranged between a 4-fold (sample 2) and a 38-fold (sample 4) up-regulation in the liver invasive front as compared to liver. In the tumour invasive front, the values ranged from approximately 3-fold (sample 10) to a 44-fold (sample 7) up-regulation, and in the tumour from a 1.5-fold (sample 1) to a 9-fold (sample 11) up-regulation compared to the liver. According to Figure 3B, the liver invasion compartment displayed the highest mean value of TIMP-1 expression of with an average of approximately 15-fold up-regulation as compared to the liver ($p=0.002$, Wilcoxon signed rank test). On average, the expression of TIMP-1 in the tumour invasive front was 13-fold ($p=0.002$, Wilcoxon signed rank test), and the tumour 4-fold higher compared to the expression in the liver. These results confirm the prominent up-regulation of TIMP-1 RNA in the invasive front of colorectal liver metastases in the 10 examined clinical samples in good correlation with the data obtained by Microarray hybridisation.

Localisation of TIMP-1. In order to determine the cellular origin of TIMP-1 up-regulation we performed immunohistochemical analyses in 20 clinical specimens. In 13 samples, TIMP-1 was detectable, all of which showed a preponderance at the invasive front. In two samples, TIMP-1 was nearly exclusively localized in tumour cells. TIMP-1 was most prominently localized to small groups of tumour cells at the very extreme front of the invasive margin (Figure 4 A, B). In 8 samples TIMP-1 was almost exclusively expressed in host cells (Figure 4 C, D), which were most abundant in the liver part of the invasive front. Host cells displaying TIMP-1 protein were spindle-shaped like fibroblasts or myofibroblasts, or had a microvessel-like appearance. In 3 samples, both tumour and host cells displayed TIMP-1 protein in the invasive front with a similar intensity (data not shown).
Figure 4. Immunohistochemistry for TIMP-1 in the invasive front of liver metastases from colorectal cancer. Positive immunoreactivity (red-brown colour) against TIMP-1 was present in 13 out of 18 specimens. In 2 out of 13 samples (Figure A, B), TIMP-1 was nearly exclusively present in tumor epithelia (arrows in B) with strongest reactivity in the invasive margin (arrows in A). In 8 out of 13 samples, TIMP-1 was nearly exclusively detected in fibroblast-like host cells (arrows in D), which are mainly localised in the invasive margin of the liver (arrows in C). A, D: bar=1000 μm, B, C: bar=125 μm. L, normal liver; LI, liver part of the invasive front; TI, tumor part of the invasive front; T, tumor.
Discussion

Our screening results using gene expression arrays show an abundant occurrence of several MMPs and TIMPs in the liver invasive front and in the tumor invasive front. Ten out of 11 MMPs (MMP-2, -3, -7, -9, -11, -12, -14, -15, -16, and -19) and 2 out of 3 TIMPs (TIMP-1 and -2), displayed a slight to marked up-regulation in at least one of the two invasive front compartments as compared to pure liver or tumor. It is tempting to speculate about their possible relevance of invasion front-specific overexpression. MMP-2, -3, -7, -9, -12, -14 and -16 are related to tumor invasion and metastasis due to their capacity of promotion of angiogenesis (14, 15) or degradation of ECM (14, 16-18). This might explain their up-regulation in the invasive compartments in order to promote tumor growth into normal liver tissue. Their distribution in the different tissue compartments is in good accordance with previous reports about their cellular localisation in colorectal cancer. MMP-2, -9 and -14 were found to be most prominently up-regulated in the liver invasive front, indicating stromal cells as their main source. These findings are consistent with previous localisation studies of MMPs in primary colorectal cancer (19-21). In analogy, MMP-7 was most abundant in the tumor invasive front, implying colon cancer cells to be its principal origin, as described before (22). TIMP-2 displayed an increased expression in the tumour invasive front and liver invasive front compared to normal liver tissue. These findings suggest tumour cells and stromal cells to produce TIMP-2 in colorectal metastases, similar to findings in primary colorectal cancer (23). However, dependent on its concentration, TIMP-2 can enhance or constrain tumour cell invasion by interacting with MMP-2 (24). These conflicting observations make it difficult to hypothesise, whether the overexpression of TIMP-2 in both invasive compartments supports tumour invasion or not and further investigation of the role of TIMP-2 in liver metastases from colorectal cancer is needed to clarify this situation. Our main finding was a pronounced overexpression of TIMP-1 in both the liver invasion and the tumor invasive compartments. Immunohistochemistry revealed positive TIMP-1 staining in host cells as well as in tumour epithelia. These results are somewhat different from an earlier study in which expression of TIMP-1 mRNA was reported throughout the colorectal liver metastasis with no particular enrichment in the invasive front (25). In addition, only stromal fibroblastic cells, but not tumour epithelia, displayed TIMP-1 gene expression (25). Since Zeng et al. localized TIMP-1 with in situ hybridisation (25), whereas we performed qPCR and immunohistochemistry, methodological differences may explain the conflicting results. An overexpression of TIMP-1 in the invasive front has, however, been reported in the invasive margin of colorectal primary tumours (26) as compared to the inner parts of the tumour. In colorectal primary tumours, TIMP-1 expression has been reported in stromal cells (25, 26), and in both, stromal and epithelial cells (27-29). From a mechanistic point of view, increased levels of TIMP-1 in the invasive front can be interpreted in at least two ways: i) TIMP-1 may be up-regulated subsequent to increased MMP activity to limit proteolytic activity at is necessary for an ordered and successful process of tumour invasion. This concept is underscored by a variety of reports which find a positive correlation of elevated TIMP-1 levels in serum and tumour tissue of tumour patients with unfavourable prognosis (6) or increased tumour aggressiveness (30). Within this reasoning elevated TIMP-1 levels may be rather a sign of elevated proteolytic activity, usually associated with a negative prognostic value. However, a relative preponderance of TIMPs versus MMPs may shift the balance towards proteolysis inhibition and make them a positive prognostic sign. In fact, an increase of the TIMP/MMP ratio has been found to be associated with lower tumour grade (31). Whether the very prominent up-regulation of TIMP-1 as compared to the rather moderate increases of MMPs in our samples (i.e. low MMP/TIMP ratio) does indeed indicate a good prognostic sign requires further evaluation with a higher number of samples. However, within this concept any (therapeutic) increase of TIMP-1, which will increase the TIMP/MMP ratio would be beneficial for the host. ii) From a different conceptual view, the rise of TIMP-1 expression may be unequivocally tumour-promoting due to its function as a growth promoter and apoptosis inhibitor (6). These roles would be compatible with both the observed expression of TIMP-1 by tumour cells and by host cells because tumour growth would profit from increased survival and proliferation as well as from increased angiogenesis and stromal support. The benefit of the therapeutic use of TIMPs or synthetic protease inhibitors will probably depend on which of the above mechanisms is more dominant. If a particular tumour depends on the fine balance of proteolytic and antiproteolytic activity, a moderate rise in TIMPs may be sufficient for an antitumor effect. In contrast, this may be fatal if the growth promoting effects prevail. We have, however, some indications that unnaturally high levels of e.g. adenovirally administered TIMP-1, or as expressed by strong viral promoters can apparently restrict MMP activity thereby preventing invasion irrespective of, or overriding any growth promoting effects (5). Regarding the therapeutic use of TIMPs, or other MMP inhibitors, it will be important to examine at which level of TIMP activity the switch from tumour promotion to tumour prevention takes place.

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References


