Secreted metalloproteinases (MPs) and their specific inhibitors (TIMPs, tissue inhibitors of MPs) are important mediators of extracellular matrix metabolism. Previous studies have linked either excessive MP release or reduced TIMP-1 production to the invasive and metastatic phenotypes of cancer cells. In the present study we investigated the relationship between the expression of TIMP-1 and the clinical behavior of 28 non-Hodgkin’s lymphomas. Northern blot analysis showed that levels of TIMP-1 mRNAs correlated directly with clinical aggressiveness: tumors in the high-grade category contained the highest levels of TIMP-1 transcripts approaching those found in maximally growth factor-stimulated fibroblasts in vitro. In situ hybridization localized the TIMP-1 expression to stromal cells of endothelial and fibroblastic origin. In contrast, transcripts hybridizing with metalloproteinase gene probes (interstitial collagenase and 72-Kd type IV collagenase) were expressed at very low levels in malignant lymphomas and their expression was not coordinately regulated with that of TIMP-1. The majority of tumors expressed either interstitial collagenase or 72-Kd type IV collagenase, and only a small number expressed both. Interstitial collagenase transcripts were only detected in high-grade tumors. The relative levels of TIMP-1 expression did not correlate with the degree of fibrosis of the tumors. Our data suggest the importance of tumor-stromal interactions in non-Hodgkin’s lymphomas, and moreover, our results indicate a possible relationship between high-level, localized expression of TIMP-1 and the malignant phenotype of high-grade advanced-stage lymphomas.

Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) RNA Is Expressed at Elevated Levels in Malignant Non-Hodgkin’s Lymphomas

By Anna E. Kossakowska, Stefan J. Urbanski, and Dylan R. Edwards

SECRETED PROTEASES and their specific inhibitors are important activities in connective tissue homeostasis. In adult organisms extracellular matrix (ECM) turnover is low, reflecting tight, coordinated control over the expression of protease zymogens and inhibitors, and over the proteolytic activation of the former to yield active enzymes. During embryonic development or in wound healing responses in adults, extensive remodeling of the ECM occurs through the rapid, localized, and transient induction of proteases and inhibitors under the influence of a diverse array of stimuli that includes growth factors, hormones, and cytokines. Loss of control of the extracellular proteolytic balance leading to an excess of degradative enzymes over inhibitory activities is associated with aberrant and excessive ECM destruction that has clear links to the invasive character of tumor cells.

Key players in collagenolysis are the members of the metalloproteinase (MP) family: specific (or interstitial) collagenase, stromelysin, and gelatinase (type IV collagenases [92 Kd and 72 Kd]). The activities of these enzymes are held in check by the tissue inhibitors of MPs (TIMPs). To date, two TIMPs encoded by distinct genes have been identified. The protein known as TIMP-1 is a 28-Kd secreted glycoprotein expressed by a wide range of cell types in vivo and in vitro. A second protein of 21 Kd termed TIMP-2 has recently been isolated, indicating that the MP inhibitors are a gene family whose members likely have specialized functions. TIMP-1 forms stoichiometric complexes with MPs, rendering them inactive. Through the use of model systems such as the human amnion invasion assay, it has been possible to demonstrate that MPs and TIMPs are important factors in the invasive phenotype of tumor cells: elevated production of MPs facilitates cellular invasion whereas exogenous TIMP-1 blocks the process. Furthermore, two recent lines of experimentation have indicated that TIMP-1 can act in vivo as a tumor suppressor. Firstly, cell lines derived from normal murine fibroblasts in which TIMP-1 expression was downregulated by antisense RNA display both tumorigenic and metastatic behavior. Also, injection of recombinant human TIMP-1 protein into mice significantly reduced lung colonization by B16-F10 melanoma cells, and by a highly metastatic Ha-ras transformed rat embryo cell line.

TIMP-1 has also been characterized as an erythroid-potentiating activity (EPA) that stimulates the growth of both early and late erythroid progenitors. The relationship of this activity to its more clearly understood role as an MP inhibitor remains unclear. TIMP-1/EPA may play a role in hematologic malignancies because differentiation of the human leukemia cell line K562 induced by exposure to phorbol esters has been shown to elicit its expression at elevated levels, and also exogenous TIMP-1 can promote the growth of these cells. However, its role in lymphoid malignancies has not been explored.

The previous observations prompted us to investigate whether aberrant TIMP-1 expression might be a useful correlate of malignancy in human tumors. We have analyzed a series of malignant non-Hodgkin’s lymphomas (NHLs) that vary in their biologic behavior and clinical aggressiveness. They are classified into separate groups according to the Working Formulation Classification, which is based on clinico-pathologic correlations.

The low-grade lymphomas characterized by a prolonged clinical course tend to grow expansively while the high-grade tumors show local aggressiveness and an invasive phenotype of tumor cells: elevated production of MPs facilitates cellular invasion whereas exogenous TIMP-1 blocks the process. Furthermore, two recent lines of experimentation have indicated that TIMP-1 can act in vivo as a tumor suppressor. Firstly, cell lines derived from normal murine fibroblasts in which TIMP-1 expression was downregulated by antisense RNA display both tumorigenic and metastatic behavior. Also, injection of recombinant human TIMP-1 protein into mice significantly reduced lung colonization by B16-F10 melanoma cells, and by a highly metastatic Ha-ras transformed rat embryo cell line.

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with high clinical aggressiveness. The high-grade group is composed of heterogeneous subgroups that vary morphologically, immunologically, and genotypically. In addition, there are substantial differences as to the clinical aggressiveness in this group of lymphomas. Many analyses done in the last decade sought with limited success to identify which aspects of the phenotype of malignant NHLs are associated with high clinical aggressiveness.

The differences in growth characteristics between low- and high-grade malignant NHLs suggested to us that altered ECM metabolism may be involved in the establishment of particular types of clinical behavior. Consequently, we undertook a study of the expression of TIMP-1 and MPs in these tumors. Our data demonstrate that the highest levels of TIMP-1 transcripts are found in the most aggressive tumors, although expression is confined to stromal cells. These findings are discussed with regard to the roles of tumor-stromal interactions and aberrant ECM metabolism in malignancy.

MATERIALS AND METHODS

Tissue processing. Freshly resected tissue of NHLs was placed in sterile, plastic vials and frozen in liquid nitrogen. It was subsequently stored at -70°C. A portion of tissue was examined by quick section to assure the quality of the material. The rest was examined histopathologically, phenotypically by flow cytometry and immunocytochemistry, and genotyped by Southern blot analysis of Ig and T-cell receptor gene rearrangements.

Histopathology. Sections were stained with hematoxylin-eosin, reticulin stain, and Masson trichrome. All cases were classified according to the Working Formulation and degree of fibrosis was assessed independently by two pathologists. Fibrosis was assessed as minimal (+), moderate (+), or extensive (+++) as previously described.

RNA isolation. RNA was isolated from the tissue homogenized in quanidinium isothiocyanate and processed according to methods described previously.

DNA and RNA probes. A full-length human TIMP-1 gene probe was isolated by polymerase chain reaction (PCR) amplification from cDNA prepared from human MRCS cells that had been stimulated for 12 hours with basic fibroblast growth factor. The PCR product was generated using oligonucleotides that introduced BamHI and HindIII sites 5' and 3' of the coding region of human TIMP-1. Restriction digested product was subcloned into pGEM2 (Promega, Madison, WI), sequenced, and used for the preparation of specific probes. The human interstitial collagenase cDNA probe was described previously, and the 72-Kd type IV collagenase cDNA was a 210-bp fragment of C-terminal and 3' noncoding sequences generously provided by Dr A. Docherty (Celltech, Slough, UK). For Northern blot analysis, DNA probes were labeled with 32P by nick-translation. Sense and antisense TIMP-1 RNA probes were synthesized with the use of SP6 and T7 RNA polymerases after digestion of the template with HindIII and BamHI, respectively. The RNA probes for in situ hybridization were labeled with biotinylated UTP as described.

Northern blot analysis. Total cellular RNA (10 μg) from each case was electrophoresed in formaldehyde containing 1.1% agarose gels. The RNA was transferred to Hybond-N membranes (Amersham, UK) in 20X SSC buffer and fixed by baking at 80°C for 2 hours. The blots were probed with nick-translated 32P-labeled probes (specific activity > 107 cpm/μg) and autoradiographed using Kodak XAR-5 film (Eastman Kodak, Rochester, NY). Subsequently, blots were reprobed with a murine 18S rRNA clone to confirm equivalence of loading. The amount of hybridized RNA was measured densitometrically with the aid of a Pharmacia Ultrascan XL laser densitometer, using autoradiograms that had signals within the linear response range of the x-ray film.

In situ hybridization. In situ hybridization was performed essentially according to previously described methods. Briefly, 5-μm thick sections were placed on slides coated with 2% aminopropyltriethoxysilane and fixed with paraformaldehyde. The sections were then hydrated in ethanol series and incubated with proteinase K (1 μg/mL) for 30 minutes. After washing and acetylation, the hybridization mixture (50% formamide, 10% wt/vol dextran sulfate, 2X SSC, pH 7.0, 0.1X Denhardt’s solution, and 100 μg/mL yeast tRNA), with biotin-labeled sense or antisense RNA probes, was applied directly to the sections and covered with siliconized, autoclaved coverslips and parafilm. The slides were hybridized at 47°C in a moist chamber containing 4X SSC for 16 to 20 hours. After washing at an appropriate stringency the sections were incubated with Streptavidin-alkaline phosphatase conjugate and the color development was achieved with the use of BCIP (5-bromo-4-chloro-3-indolylphosphate) and NBT (nitroblue tetrazolium) mixture. The slides were mounted in an aqueous solution of glycerine jelly.

RESULTS

Among 28 cases classified according to the Working Formulation and examined for TIMP-1 expression, 11 belonged to the low-grade group (A through C), five to the intermediate (D through G), and 12 to the high-grade category (H). All cases in which no degradation of RNA was observed showed expression of TIMP-1, with the exception of one case of small lymphocytic lymphoma (grade A). TIMP-1 expression was low in the group of low-grade lymphomas and higher but variable in the high-grade category (Fig 1). All 12 cases in the high-grade group belonged to the category of immunoblastic lymphomas (grade H in Working Formulation). In one case significant degradation of RNA was observed, most likely related to extensive necrosis. Among the remaining 11 cases, six showed marked over-expression of TIMP-1 (3.3 to 7.5 x average level in the low-grade group) (Fig 1). TIMP-1 expression in these cases approached that observed in maximally growth factor-stimulated human MRC-5 fibroblasts, which displayed a relative value of 9.5 on the same scale (data not shown). Two of the immunoblastic lymphomas that showed over-expression of TIMP-1 presented as primary extranodal lymphomas involving lung and breast. Morphologically, all of these tumors showed considerable pleomorphism and had variable phenotypes. Genotypically, three of the cases were of B-cell origin, two showed T-cell lineage, and in one case no Ig or T-cell receptor gene rearrangements were detected (Table 1).

We examined whether there was any correlation between degree of fibrosis and TIMP-1 expression. Although it is well recognized that fibrosis within lymph nodes may vary depending on their anatomic location and malignant lymphoma type, it seemed appropriate to exclude the possibility that high TIMP-1 levels are influenced by or are associated with extensive fibrosis. Among these 11 cases of immunoblastic lymphomas one showed minimal degree of fibrosis (nodal), seven moderate fibrosis (five nodal, two extra-nodal), and three extensive (two nodal and one nodal).
TIMP-1 RNA IN NON-HODGKIN’S LYMPHOMAS

Fig 1. TIMP-1 expression in malignant NHLs. (a) Northern blot analysis of TIMP-1 RNA levels in total RNA from NHLs. Blots were hybridized with nick-translated human TIMP cDNA as described in Materials and Methods and were subsequently stripped and reprobed with the 18S rRNA gene probe.7 The results show representative data from 19 of the tumor samples analyzed. (b) Quantitative determination of relative TIMP-1 RNA levels by densitometry. Densitometric estimates of TIMP-1 transcripts are expressed relative to the signals obtained with the 18S rRNA probe. To allow comparisons between separate Northern blots, at least two common samples were included on each blot and TIMP signals were normalized to the values obtained with these RNA samples. Numbers in parentheses indicate the numbers of tumors represented in the B- and H-grade samples.

Extra-nodal). High TIMP-1 expression did not correlate with the degree of fibrosis seen on histologic sections. Levels of TIMP-1 expression, phenotype, genotype, degree of fibrosis, and clinical data on all patients with immunoblastic lymphomas are summarized in Table 1.

Degree of fibrosis among the low-grade lymphomas also differed. In that group, among 11 cases, two showed minimal fibrosis, seven moderate, and two extensive. However, as shown in Fig 1, levels of TIMP-1 expression were fairly uniform in all but the case of small lymphocytic

<table>
<thead>
<tr>
<th>TIMP Expression*</th>
<th>Fibrosis/ Anatomic Location</th>
<th>Phenotype</th>
<th>Genotype</th>
<th>Stage</th>
<th>Patient Age (y)</th>
<th>Sex</th>
<th>Length of Survival (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.49</td>
<td>+ Scalene LN</td>
<td>T cell</td>
<td>T cell</td>
<td>III</td>
<td>34/F</td>
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<td>60A</td>
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<td>B cell</td>
<td>IV E</td>
<td>45/F</td>
<td></td>
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<td>B cell</td>
<td>IV E</td>
<td>36/F</td>
<td></td>
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</tr>
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<td>+ Axillary LN</td>
<td>B cell</td>
<td>B cell</td>
<td>IV</td>
<td>50/M</td>
<td></td>
<td>8D</td>
</tr>
<tr>
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<td>± Inguinal LN</td>
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<td>ND</td>
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<td>85/F</td>
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<td>T cell</td>
<td>II</td>
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<td>B cell</td>
<td>II</td>
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<tr>
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<td>B cell</td>
<td>IV</td>
<td>36/M</td>
<td></td>
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<td>B cell</td>
<td>III</td>
<td>17/M</td>
<td></td>
<td>34A</td>
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Abbreviations: ND, not determined; E, extra-nodal; A, alive; D, deceased; LN, lymph node.

*Relative levels of TIMP expression are the values used in Fig 1.
†+, Minimal fibrosis; +, moderate; ++, extensive.
lymphoma, which showed no detectable TIMP transcripts and moderate fibrosis.

We also analyzed TIMP-1 expression in immunoblastic lymphomas by in situ hybridization. Figure 2 displays a set of results from two of six tumors analyzed to date. Consistently, we detected hybridization of antisense TIMP-1 RNA probes exclusively to tumor stromal cells, with the tumor cells themselves showing no staining. Endothelial cells lining the sinusoids and capillaries in the tumors were also strongly positive for TIMP-1 RNAs.

The same malignant lymphomas were also tested for the expression of metalloproteinase mRNAs by Northern blot analysis. Representative results are shown in Fig 3. All metalloproteinases were expressed at low levels. Interstitial collagenase was only found in high-grade lymphomas, but its expression did not correlate with the levels of TIMP-1 mRNAs (Table 2). A novel transcript of about 1 kb (identified by a dotted arrow in Fig 3) was also observed. At present its origin is uncertain. 72-Kd type IV collagenase was found in a few cases in both low- and high-grade categories. Again its presence did not correlate with the levels of TIMP-1 expression (Table 2). Also, interstitial collagenase and 72-Kd type IV collagenase were expressed independently.

DISCUSSION

We approached this study with the notion that if TIMP-1 has a tumor suppressor activity, its expression should inversely correlate with the clinical aggressiveness of a defined set of human tumors. Our results indicate that this simple direct relationship cannot hold true at least in the context of malignant NHLs. For categories B (low grade) and H (high grade) that were taken to exemplify extremes of the Working Formulation classification, a consistent pattern emerged. All of the B-grade tumors expressed TIMP-1 to a similar moderate level, whereas the high-grade set showed a much greater range of relative levels of expression, with TIMP-1 transcript abundances ranging from those typical for the low-grade tumors up to values at least sevenfold higher. Moreover, patient survival within the H grade showed another striking trend: with one exception, long-term survival past 1 year was restricted to those individuals whose tumors contained relatively less TIMP-1 mRNAs. The significance of this finding is not
Fig 3. Interstitial collagenase and 72-kD type IV collagenase expression in malignant NHLs. Northern blot analyses of interstitial collagenase and 72-kD type IV collagenase in total RNA derived from the NHLs as presented in Fig 1. Blots were hybridized with nick-translated probes as described in Materials and Methods. The panel on the left also contains two lanes of RNA derived from growth factor-stimulated human MRC-5 fibroblasts. Some variable nonspecific hybridization to a region of the blot corresponding to 28S rRNA is apparent with both MP probes. Only tumors of category H are shown in the left panel because interstitial collagenase transcripts were not detected in lower grade tumors. The expected transcripts are pointed out with an arrow. The ~1-kb transcript seen on the blot hybridized with interstitial collagenase is pointed out by a dotted arrow.

Table 2. Expression of TIMP-1, Interstitial Collagenase, and 72-kD Type IV Collagenase

<table>
<thead>
<tr>
<th>H TIMP-1*</th>
<th>Interstitial Collagenase</th>
<th>72-kD Type IV Collagenase</th>
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<tbody>
<tr>
<td></td>
<td>2.5-kb Transcript</td>
<td>~1.0-kb Transcript</td>
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<tr>
<td>7.49</td>
<td>–</td>
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<td>3.23</td>
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<tr>
<td>1.93</td>
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<td>1.81</td>
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<td>1.47</td>
<td>–</td>
<td>+</td>
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<tr>
<td>0.71</td>
<td>+</td>
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Abbreviation: ND, not done.
*Data from Table 1.

Certain because of five deceased patients four were in stage IV, whereas among the patients who are alive three were at stage II, two in stage III, and one in stage IV. However, the immediate message of these studies remains that elevated TIMP-1 expression appears to be associated with a more aggressive tumor phenotype because TIMP-1 transcripts are present at higher levels in advanced-stage malignant NHLs.

The in situ hybridization studies shown in Fig 2 demonstrate that TIMP-1 transcripts are not localized to the malignant lymphocytes, but instead are present within stromal cells, including cells of fibroblastic origin and endothelial cells lining sinusoids and capillaries. Owing to the relative insensitivity of the in situ hybridization technique, we cannot rule out the possibility that the tumor cells themselves express TIMP-1 at a low level. However, any such expression would be considerably less than that of their stromal neighbors. In addition, Northern blot analysis of cultured lymphoma cells showed no detectable TIMP-1 transcripts (data not shown), further supporting the observation that TIMP-1 in malignant lymphomas is mainly if not exclusively produced by stromal cells. The absolute level of expression by the stromal cells is worthy of consideration. These cells account for only approximately 20% of the total cells in each specimen because the tissues for Northern blot analyses were taken from the center of the tumors to minimize the presence of non-neoplastic tissue. The signals obtained in the Northern blot analyses indicate that the average level of TIMP-1 transcripts in H-grade tumors is one third of that observed in growth factor-stimulated
human MRC-5 fibroblasts in vitro (data not shown), where TIMP-1 mRNA accumulates to between 0.1% and 0.2% of the total mRNA population. This indicates that positively staining stromal cells in the tumors contain large quantities of TIMP-1 RNA. It must be emphasized that such high levels of expression are untypical of normal connective tissue stroma: previous in situ studies of TIMP-1 in the developing mouse embryo showed that transcripts are essentially undetectable by this technique except in regions of extensive ECM remodelling, such as sites of osteogenesis.6

On the other hand, the expression of MPs was very low and detectable only in some of the cases. Although interstitial collagenase was only detected in high-grade lymphomas, 72-Kd type IV collagenase was expressed in both high- and low-grade tumors and there was no correlation between the levels of TIMP-1 and the MPs (Table 2). Thus, TIMP-1, interstitial collagenase, and 72-Kd type IV collagenase are independently regulated in this in vivo context. These data support recent in vitro studies demonstrating distinct patterns of expression of interstitial collagenase and 72-Kd type IV collagenase in both normal fibroblastic cells and tumorigenic cell lines.3,4

To analyze a possible association between degree of fibrosis and TIMP-1 expression, we independently assessed fibrosis in tissue sections and TIMP-1 expression from the same surgical specimen. It is of note that the former had been implicated as a prognostic factor in both low- and high-grade malignant lymphomas.26 While extensive fibrosis seen in 2 of 10 cases of immunoblastic lymphomas was associated with better prognosis in accordance with previous observations,26 the tumors with extensive fibrosis were not characterized by high TIMP-1 expression. Therefore, it follows that fibrosis seen on histologic sections is independent of high TIMP-1 expression. It appears also that extra-nodal location does not influence TIMP-1 expression (Table 1).

At first sight there seems to be a paradox between what we know about the function of TIMP-1 as an agent that blocks excessive ECM destruction and cellular invasiveness, and our observation that it is expressed at the highest levels in malignant lymphomas in the advanced stages. One possible explanation of these data is that although TIMP-1 expression is high in these tumors, it may be inadequate to cope with a larger localized production of MPs. Thus, the extracellular proteolytic balance may still tilt in favor of ECM destruction and invasive behavior. The restriction of interstitial collagenase transcripts to high-grade tumors offers some support to this notion. However, it must be pointed out that these RNAs were present at much lower levels than those observed for TIMP-1. Thus, unless as yet unrecognized translational controls limit the production of TIMP-1 in the tumor stromal cells, it seems likely that there would be excess TIMP-1 protein over interstitial collagenase in the malignant lymphomas. Also, it should be noted that there is no correlation between tumor biologic behavior as judged by long-term survival of patients and expression of either interstitial collagenase or 72-Kd type IV collagenase (Tables 1 and 2). Preliminary data show that stromelysin-1 and 92-Kd type IV collagenase expression are also regulated independently of TIMP-1 expression. To address more fully the question of whether an imbalance of MPs:TIMP exists in these tumors, further studies using an expanded pool of MP gene probes, coupled to detailed biochemical, morphologic, and in situ hybridization data will be necessary.

It is also worth noting that TIMP-1 has been shown to have an erythroid-potentiating activity,14,19 and therefore one of its actions is that of a hematopoietic growth factor. Although it has been reported to be erythroid specific,20,31 its role as a cytokine or growth factor in malignant lymphoid tumors has not been explored. It has been shown that phorbol ester (TPA) treatment of the neoplastic human leukemia cell lines K562 and HL-60 stimulates expression of TIMP-1.18,26 This in turn has been associated with an autocrine action of TIMP-1 as EPA that stimulates K562 cells.32 One possible interpretation of our findings is that elevated TIMP-1 production may contribute to the biologic behavior of NHLs by acting as a growth stimulus to the tumor cells. The complex relations between the different functions of TIMP-1 during cellular differentiation and neoplastic transformation have to be explored further, particularly in the context of hematologic malignancies.

In vitro studies have shown that TIMP-1 gene expression is strongly influenced by growth factors such as basic fibroblast growth factor and transforming growth factor β,27 by cytokines such as interleukin-1,17 and by steroid hormones.38 These agents induce transcriptional activation after 3 to 4 hours of exposure of quiescent, unstimulated cells.32 Therefore, it is possible that the high levels of TIMP-1 transcripts detected in stromal cells in Fig 2 are attributable to locally acting, tumor-derived factors.

This report documents that malignant NHLs show differences in molecular mechanisms involved in ECM metabolism and, furthermore, that these changes can be related to the clinical behavior of the tumors. Elevated TIMP-1 expression was strongly correlated with tumor aggressiveness. These observations may prove useful in the formulation of therapeutic decisions and may ultimately open new avenues in the treatment of these malignancies.

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Tissue inhibitor of metalloproteinases-1 (TIMP-1) RNA is expressed at elevated levels in malignant non-Hodgkin’s lymphomas

AE Kossakowska, SJ Urbanski and DR Edwards