**TIMP1** and Adverse Prognosis in Non-Small Cell Lung Cancer

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ABSTRACT

The tumor inhibitor of metalloproteinase (TIMP) family is a natural inhibitor of several matrix metalloproteinase enzymes which are involved in the process of tumor cell invasion through the extracellular matrix. The aim of this study was to examine TIMP1 RNA expression levels in relation to the clinicopathological features in resected primary non-small cell lung cancer (NSCLC). Total cellular RNA, obtained from 45 cases of NSCLC and adjacent normal lung tissue, was examined using Northern blot analysis. TIMP1 RNA expression levels were heterogeneous in NSCLC but was significantly higher in the adenocarcinoma compared to the squamous cell carcinoma subtype. Although the TIMP1 RNA levels did not correlate with sex, smoking, tumor, node, or TNM stage, there was a statistically significant survival disadvantage for cases with relatively high TIMP1 RNA expression, suggesting a role for TIMP1 in determining the prognosis of resected NSCLC.

INTRODUCTION

An essential step in the process of tissue invasion and metastasis of tumor cells involves the degradation of tissue barriers in the extracellular matrix, particularly the basement membrane. This process is regulated by proteolytic enzymes of the matrix metalloproteinase family (1, 2), including the type IV collagenases which are comprised of two types, 72 and 92 kDa. These collagenases, either secreted by tumor cells or by neighboring stromal cells, specifically degrade type IV collagen which forms an important component of basement membranes (3). There is considerable evidence which indicates that type IV collagenase activity correlates with the process of tumor cell invasion (4–9).

In lung cancer specifically, similar data suggest an important role for the metalloproteinases in the process of invasion and metastasis. For instance, in oncogene-immortalized human bronchial epithelial cell lines, invasiveness and metastatic capacity correlated negatively with the expression of type IV procollagen as well as positively with the expression of type IV collagenases, indicating that both decreased collagen production and increased matrix degradation are important in determining metastasis (10). In addition, others have shown that the levels of the 72- and 92-kDa type IV collagenases in NSCLC cell lines were higher in lines with the most metastatic, invasive, and tumorigenic potential (11). Studies of primary tumors have also shown increased RNA levels of the 72-kDa type IV collagenase, whereas the expression levels of the 92-kDa type IV collagenase appear to be more variable in SCCs (12, 13). Furthermore, the levels of the activated 72-kDa type IV collagenase enzyme correlated with tumor spread in primary NSCLC tumors (8). Moreover, abnormalities of the basement membrane were found to be associated with elevated type IV collagenase levels (12, 14). Finally, elevated serum levels of the 72-kDa type IV collagenase have been demonstrated in lung cancer patients, which correlated with the development of metastasis (15).

The collagenases are inhibited by their natural inhibitors, the TIMP family comprised of three members, TIMP1, TIMP2, and TIMP3 (1, 16–18). TIMPs have been proposed to be important inhibitors of tumor invasion of the extracellular matrix based on *in vitro* and animal studies (19–22). However, the recent correlation of elevated TIMP1 RNA levels with advanced colorectal cancer (23), backed up by experimental evidence of coexistent growth-promoting activity (24, 25), has led to the speculation that TIMP1 may alternatively promote tumor growth.

To investigate which of these opposing functions (metastasis inhibition versus growth promotion) may be operating in lung cancer, we examined the levels of TIMP1 RNA expression specifically in relation to clinicopathological features. A cohort of surgically staged resected primary NSCLC cases was studied to provide a clearly defined homogenous sample set to avoid confounding by other variables.

MATERIALS AND METHODS

Sample Collection. Surgically resected tumors and corresponding normal lung tissue were obtained from patients enrolled in this study at The Prince Charles Hospital after informed consent, was snap frozen in liquid nitrogen, and was stored at −80°C. Total RNA was obtained by a modification of the guanidinium isocyanate method (26). RNA integrity was assessed by denaturing agarose gel electrophoresis and quantified by spectrophotometry.

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4 The abbreviations used are: NSCLC, non-small cell lung cancer; TIMP, tumor inhibitor of metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SCC, squamous cell carcinoma; LC, lung cancer tissue; NL, normal lung tissue.
**Northern Blot Hybridization.** Northern blot analysis was used to determine the level of TIMP1 RNA expression in each sample. Twenty μg of total RNA/sample were size fractionated by denaturing 12% agarose gel electrophoresis and transferred to a Hybond-N membrane (Amersham) by capillary blotting in 20× SSPE for 16 h. Prior to hybridization, filters were prehybridized for 4 to 6 h at 65°C in 10 ml/filter of 6× SSPE, 5× Denhardt’s solution, 2.5 mM sodium PP, 0.5% SDS, and 100 μg/ml sheared salmon sperm DNA. The TIMP1 and GAPDH (internal loading control) probes were labeled with [α-32P]dCTP by random hexamer priming (27, 28). Hybridization was performed at 65°C for 16 h followed by washing twice in 2× SSPE and 0.1% SDS and once in 0.5× SSPE and 0.1% SDS. Filters were then blotted dry, wrapped in plastic film, and exposed to Kodak XAR film in intensifying cassettes (DuPont) at 80°C.

**Densitometry.** To quantitate the extent of hybridization and to control for loading differences, laser densitometry was used (LKB Bromma, Uppsala, Sweden) to analyze the signal from the TIMP1 probe in comparison to the control GAPDH (29) probe. Autoradiograph exposures were chosen for scanning such that the signals had not saturated the film, and where possible, multiple exposures of the same filter were scanned in some cases. The relative expression of TIMP1 RNA was calculated after normalizing for GAPDH RNA expression:

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\text{LC:NL ratio} = \frac{(\text{LC}_{\text{TIMP1}}/\text{LC}_{\text{GAPDH}})}{(\text{NL}_{\text{TIMP1}}/\text{NL}_{\text{GAPDH}})}
\]

**Statistical Analyses.** The significance of possible differences between the means of interval data was examined using the Mann-Whitney U test for nonparametric data. To analyze the univariate survival differences between groups, Kaplan-Meier actuarial survival curves and log rank analysis were used (SPSS for Windows V6.1). The date of histological diagnosis was taken as the first day of follow-up. The survival data were censored on November 1, 1994, with all cases assessed as being deceased or alive at their longest duration of follow-up. All P values were derived from two-tailed tests.

**RESULTS**

Forty-five cases of resected primary NSCLC tumors and corresponding normal lung tissue were examined for the levels of TIMP1 RNA expression using Northern blot analysis. The mean age of the patients was 59.8 years, and the range was 39–81 years. There were 14 SCCs, 23 adenocarcinomas, 2 large cell carcinomas, 5 adenosquamous carcinomas, and 1 atypical carcinoid. Other clinicopathological features are summarized in Table 1. Representative examples of different levels of TIMP1 RNA expression are shown in Fig. 1. The relative level of TIMP1 RNA expression for each tumor is calculated as the ratio of TIMP1 RNA expression in LC compared to NL, i.e., LC:NL TIMP1 RNA ratio. In other words, a LC:NL TIMP1 ratio of 1 indicates equivalent TIMP1 RNA expression in the tumor and normal tissue, a ratio of 2 indicates tumor TIMP1 RNA overexpression of twice the normal lung tissue levels, and a ratio of 0.5 indicates tumor TIMP1 RNA underexpression of half the normal lung tissue levels.

The LC:NL TIMP1 RNA expression ratio varied considerably between cases, with a range of 0.12–3.78 as depicted in Fig. 2. The distribution of LC:NL ratios was skewed to the right, with the mean and median TIMP1 RNA expression ratio being 1.18 and 1.01, respectively. Most NSCLC tumors thus expressed TIMP1 RNA at about the same level as that expressed in NL. In addition, most of the tumors which overexpressed TIMP1 RNA, did so at a modest level, usually less than about 2-fold that of NL tissue.

There was no significant difference in the levels of TIMP1 RNA expression associated with sex, smoking history, tumor, node, or TNM stages (Table 1). The TIMP1 RNA expression levels between the different histological subtypes demonstrated considerable overlap, although small numbers of the less common subtypes were available. Nonetheless, in the major NSCLC subtypes, the mean LC:NL TIMP1 RNA ratio was significantly higher in adenocarcinomas (mean, 1.33) compared to the SCCs (mean, 0.97; P = 0.017, Mann-Whitney U test). In view of this difference, TIMP1 RNA expression was also compared to lymph node involvement in the SCC and the adenocarcinoma subtypes individually. In the SCC subtype, eight cases had N0 disease compared to six cases with N1 or N2 disease, but there was no difference in the level of TIMP1 RNA expression (P = 0.755, Mann-Whitney U test). Similarly, there was no difference between the levels of TIMP1 RNA expression in the 15 adenocarcinomas without lymph node involvement compared to the 8 with nodal involvement (P = 0.925, Mann-Whitney U test).

In considering the prognostic value of TIMP1, the tumors were classified into three subsets based on the level of TIMP1 RNA expression. These were a subset with relatively low expression (LC:NL ratio ≤ 0.5), approximately normal expression (0.5 < LC:NL ratio < 2), and relatively high expression (LC:NL ratio ≥ 2.0). Using this arbitrary classification, 12 NSCLC cases had low expression, 27 had normal expression, and 6 had high expression.
epithelial tumor. For instance, ELISA-measured TIMP1 enzyme levels were comparatively low, whereas TIMP2 levels were higher in one study of NSCLC cell lines (11). Moreover, TIMP1 mRNA was almost undetectable using Northern blot analysis in about 50% of various human tumor cell lines, including some derived from NSCLC (7).

On the other hand, the overall TIMP1 RNA levels in resected NSCLC samples could reflect production by tumor cells as well as by stromal cells. It is controversial whether TIMP1 is mainly secreted by and localized to the stromal cells, or both stroma and tumor because of conflicting in situ hybridization results (12, 13). In any case, this study demonstrates a marked difference between TIMP1 RNA expression between the two major histological subtypes of NSCLC, with higher levels of TIMP1 RNA in adenocarcinomas compared to SCCs. Because adenocarcinomas are much more likely to metastasize than SCCs (30), the higher levels of TIMP1 RNA in adenocarcinomas suggest that TIMP1 may be associated with more aggressive behavior, in keeping with a possible growth-promoting role for TIMP1. There was however no survival difference between the adenocarcinomas and SCCs in this series (P = 0.53, log rank test).

Alternatively, another explanation is surgical bias, in that resectable adenocarcinomas may comprise a selected subset of adenocarcinomas without tumor dissemination, perhaps due to intrinsically higher TIMP1 RNA levels. If so, one may expect lower TIMP1 RNA levels in adenocarcinomas which have disseminated compared to localized disease. However, in this study, there was no association of TIMP1 RNA levels with the nodal stage in adenocarcinomas (nor in the SCCs or overall NSCLC group). Indeed, these findings confirm and expand on the data from a smaller study of 14 SCC cases where there was no correlation of TIMP1 RNA expression levels with tumor stage or differentiation (12).

This study has demonstrated higher levels of TIMP1 RNA in adenocarcinomas, a relatively aggressive subset of NSCLC, as well as a striking association of these high levels with an adverse outcome. This association, albeit in a relatively small patient subset, is consistent with the hypothesis that TIMP1 may, instead of inhibiting metastasis, have a role in determining survival in...
NSCLC, possibly by growth-promoting or other activity. However, it should be noted that invasion and metastasis of lung cancer cells is likely to be complex, requiring other factors besides the metalloproteinase cascade [e.g., a human anaplastic lung carcinoma cell line with a mutated KRAS gene was rendered less tumorigenic by transfection with the KREVI transformation suppressor gene, despite the transfected line having higher type IV collagenase and lower TIMP1 and TIMP2 levels compared to the parent cell line (11)]. Consequently, the data indicate the need for larger, multivariate prospective studies to confirm these results and to assess the independent prognostic value of measuring TIMP1 RNA levels and other metastatic factors for optimizing the management of resectable primary NSCLC.

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