Abstract. Background: Tissue Inhibitors of Metalloproteinases (TIMPs) play a critical role in extracellular matrix remodeling, which is involved in tumor growth and metastasis. Elevated TIMP levels are reported in association with cancer progression. In particular, it has been shown that TIMP-1 and -2 levels are increased in cervical cancer patients. We analyzed, for the first time, TIMP-4 expression in cervical tumor samples. Materials and Methods: Semiquantitative RT-PCR was performed in 26 tumor and 6 normal cervical samples. Results: The study included 32 samples, 7 IB samples, 9 IIB samples, 10 IIIB samples and a control group (n=6) of normal cervical squamous epithelial tissues. Whereas none of the control samples expressed TIMP-4, 24 (88%) of the 26 cervical cancer samples expressed the inhibitor. Higher TIMP-4 levels were found in advanced stage disease (p=0.016, Chi-square test). Conclusion: TIMP-4 is expressed de novo in cervical cancer. Higher inhibitor expression levels were found in stages II and III.

Extracellular matrix (ECM) remodeling is an essential process during cancer invasion, angiogenesis and metastasis. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that degrade all ECM components and play a key role in these processes (1). The expression of MMPs is highly regulated at transcription, translation and latent enzyme secretion, pro-enzyme activation and inactivation levels. The tissue inhibitors of MMPs (TIMPs), a multifunctional four member protein family, regulate MMPs after their secretion to the extracellular environment. Thus, net MMPs activity is a result of the balance between enzymes levels and their physiological inhibitors (2). To date, four types of TIMPs have been identified. TIMP-1, -2 and -4 are secreted in soluble forms, whereas TIMP-3 is bound to ECM proteins (3).

Increased MMP expression and ECM proteolytic degradation have been detected in a wide range of cancers and correlate with primary tumor invasion and metastasis (1). Consistently, tumor cell invasion and metastasis can be inhibited by up-regulation of TIMPs expression (4). It has been demonstrated that in vivo injection of TIMPs inhibits tumor cell invasion and metastasis in animal models (4). In addition, several studies have demonstrated that over-expression of TIMPs inhibits primary tumor growth in some cell types (5). However, the TIMPs are a family of proteins that may affect a broad spectrum of cellular behaviors. In fact, TIMPs regulate apoptosis and angiogenesis, present growth stimulatory activity in several cell lines and promote carcinogenesis in murine models (6).

TIMP-4 was the last identified and cloned tissue inhibitor (7). Unlike other TIMPs, tissue-specific expression of TIMP-4 is limited to the heart, pancreas, kidney and brain in adult humans (7). The role of TIMP-4 in some cancers has been suggested in some investigations. It has been shown that TIMP-4 mRNA and protein are expressed in human mammary carcinoma in contrast to healthy tissue samples (8). In endometrial carcinoma, TIMP-4 is over-expressed and this elevated expression correlates with myometrial invasion (9). On the other hand, TIMP-4 mRNA expression was found in human glioma tumor cells samples and shows negative correlation with malignancy (10). These paradoxical effects underlie the complex role of these inhibitors in cancer.

Cervical cancer is the second most common female malignancy worldwide. This disease is curable when detected early, but conventional cytological and histological techniques are insufficient to predict the course of the disease. Therefore, it is necessary to understand and evaluate several factors contributing to cervix tumor progression. Nair et al. have shown basement membrane dissolution as the disease progresses from low-grade squamous epithelial lesion to high-grade squamous epithelial lesion (11). Consistently, several studies have shown an increase in MMP-2, MMP-9 and MMP-14

Key Words: TIMP-4, cervical cancer.

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expression in samples, but also augmented TIMP-1 and -2 levels (12). These results suggest that ECM proteins may participate in cervical carcinogenesis. Since the changes in ECM turnover seem to be a factor contributing to cervical cancer progression, we sought to analyze the TIMP-4 mRNA expression in cervical carcinoma samples.

Materials and Methods

Cell culture. The cervical cancer cell lines HeLa, CasKi and SiHa were obtained from ATCC (MD, USA) and the CaLo cell line was a kind gift from Dr. Alejandro Garcia. Cells were cultured as a monolayer in Dulbecco’s modified Eagle medium (DMEM) containing 10% (V/V) fetal bovine serum (GIBCO, Bethesda, MD, USA) and incubated at 37°C in a humidified atmosphere of 5% (V/V) CO₂, as reported previously (13).

Clinical samples. Cervical tissue was collected from patients undergoing hysterectomy for malignant and various non-malignant diseases of the cervix at the Instituto Nacional de Cancerologia (Mexico). Written consent was obtained from patients before the samples were collected. Cervical samples were histopathologically graded by a pathologist.

Table I. TIMP-4 positivity in cervical cancer tumor samples.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. Patients</th>
<th>TIMP-4 Positive (n=23)</th>
<th>TIMP-4 Negative (n=3)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 53.45 (32-82)</td>
<td>58.82(32-82)</td>
<td>50 (37-66)</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IB 7</td>
<td>6</td>
<td>1</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>IIB 9</td>
<td>7</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIB 10</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology of tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell 22</td>
<td>19</td>
<td>3</td>
<td>0.48</td>
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</tr>
<tr>
<td>Adenocarcinoma 2</td>
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<td>2</td>
<td></td>
<td></td>
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<tr>
<td>Adenosquamous 2</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menopause 7</td>
<td>6</td>
<td>1</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Pre-menopause 3</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-menopause 16</td>
<td>15</td>
<td>1</td>
<td></td>
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<tr>
<td>Current status a</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Disease-free 11</td>
<td>9</td>
<td>2</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Persistent disease 13</td>
<td>12</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dead 2</td>
<td></td>
<td>2</td>
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<td></td>
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<tr>
<td>Tumor size n=19</td>
<td>n=16</td>
<td>n=3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5 cm 6</td>
<td>5</td>
<td>1</td>
<td>0.69</td>
<td></td>
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<tr>
<td>&gt;5-&lt;10 cm 10</td>
<td>8</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10-&lt;15 cm 1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;15 cm 2</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Median time follow-up 15.3 months

Figure 1. TIMP-4 mRNA expression in cervical cancer cell lines. Upper panel: RT-PCR analysis of HeLa, CasKi, SiHa and CaLo cervical cancer cell lines. To the left molecular weight marker (100 bp ladder, Invitrogen). Lower panel: RT-PCR of GAPDH, used as a mRNA load control.
products were normalized to those obtained from GAPDH (mRNA) amplification, used as internal reference gene. Gene expression measurements were repeated at least two times. TIMP-4 relative levels were qualified as low if included in the 25th percentile, and high when the values exceeded those values. Percentiles were obtained from the distribution values of the TIMP-4 expression.

Statistical analysis. TIMP-4 expression values were compared with pathological tumor parameters in order to detect any correlation. Differences were tested for statistical significance using ANOVA’s (age and tumor size) and Chi-square tests (FIGO stage, histology of tumors, menopause and current status). The statistical package Intercooled Stata ver. 7.0 (TX, USA) was used for analyses and statistical significance was indicated when the p value was less than 0.05.

Results

First, we performed semiquantitative RT-PCR analyses of TIMP-4 expression in a panel of cervical cancer cell lines, including the widely employed SiHa, Caski and HeLa cells. As shown in Figure 1, all the cell lines tested expressed the transcript for TIMP-4, although different levels were observed in each line.

Next, we analyzed TIMP-4 mRNA levels using the same approach in 26 tumoral and 6 normal cervical samples. In order to provide accurate determinations, we normalized the relative expression in each sample to a cervical cancer cell line control. To verify equal RNA input, GAPDH mRNA was amplified simultaneously. Figure 2 shows a representative panel of results, which are presented in Table I.

As expected from the restricted expression of TIMP-4 reported (7, 16), normal cervical samples do not express mRNA for TIMP-4. In contrast, 24 (88%) of the 26 cervical cancer biopsies had detectable levels of this transcript. There was no correlation between TIMP-4 positivity and histology, menopause, tumor size, FIGO stage or disease status (Table I). Survival times were similar for both groups (p=0.23 log rank test). Next, we establish arbitrary cut-off points based on the 25th percentile, derived from the TIMP-4 expression distribution in our samples. These cut-offs separated patients with low (94 arbitrary units, 6 patients) and high (172 arbitrary units, 20 patients) TIMP-4 expression. There was no correlation with histology, menopause, tumor size or disease status (Table II). Survival times were similar for groups with low or high TIMP-4 expression (p=0.64, p=0.39, respectively, log rank test). Nevertheless, a clear correlation was found between FIGO cancer staging and TIMP-4 expression. Samples from patients with cervical cancer in stages II and III presented higher TIMP-4 levels (p=0.016 Chi-square test) (Table II).

Discussion

Tissue inhibitors of matrix metalloproteinases (TIMPs) are multifunctional proteins with both matrix metalloproteinase (MMP) inhibitory effects and growth-regulatory activity. Due to these multiple functions, TIMPs have complex roles in cancer.

Recently, the presence of TIMP-1 and-2 mRNA has been shown in cervical cancer cell lines (17) and cancer samples (11, 18). Immunoreactivity for TIMP-1 and-2 increases during cervical cancer progression, in particular for TIMP-2 (11, 18). The TIMP-2 levels in these tumors correlate with the stage of the menstrual cycle (19) and it is a poor prognosis factor (20). There are no reports in cervical cancer concerning the other two members of this family.

TIMP-4, the latest cloned member of the Tissue Inhibitor of Metalloproteinases family, presents the most restricted pattern of tissue expression, including only kidney, placenta, colon and testes (7). Nevertheless, it has been demonstrated that this inhibitor is up-regulated in gliomas (10), endometrial (9) and breast cancer (21, 22), thus showing a possible participation in the carcinogenic process. In vitro and in vivo
evidence have shown that TIMP-4 inhibits invasion, metastasis and tumor growth of breast cancer cells (23), but only when its cDNA is transfected directly to the cells. Contrary to that, systemic delivery of TIMP-4 by electroporation-mediated intramuscular injection of naked TIMP-4 DNA stimulates tumorigenesis of breast cancer cells in nude mice (8). These opposing results may be derived from the anti-apoptotic properties of TIMP-4 which are, at least partially, derived from the up-regulation of Bcl-2 and Bcl-X(L) proteins, from clonal differences in the cells used or for TIMP-4 inactivation in blood, allowing for lower levels of the inhibitor in the tumor. Recently, Jian et al. (6) proposed a biphasic role of TIMPs in cancer. In early stages TIMPs could support carcinogenesis by means of stimulating growth and protecting cells from apoptosis, as reported by various authors (24-27). In later stages, when the cells have acquired enough mutations to obviate the need for these functions, the anti-metalloproteinase activity may play a major inhibitory role in cancer progression (28, 29). Thus, over-expression in a particular cancer could reflect early expression changes of TIMP and not necessarily correlate with progression. In this paper, we present evidence that TIMP-4 is expressed de novo in cervical cancer patients. Higher TIMP-4 levels were found in advanced stage disease. Nevertheless, we could not find statistical support for TIMP-4 as a prognostic factor, when a log rank test was used. As mentioned before, this could be due to an expressional change in early stages that is conserved and enhanced during cancer progression. Analyzing early samples of intraepithelial carcinomas or even cervical dysplasias could help to clarify this point. On the other hand, the limited number of samples could be hampering the statistical analysis. We are currently increasing sampling and following time in order to exclude this possibility. More interestingly, this report supports the possibility of using TIMP-4 as a diagnostic marker in cervical cancer. As in the previous case, analysis of samples from intracervical neoplasia and dysplasia samples should be performed to address this question.

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