Expression of Thrombomodulin in Esophageal Squamous Cell Carcinoma and Its Relationship to Lymph Node Metastasis

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ABSTRACT
Thrombomodulin (TM) is a thrombin receptor that was identified originally on the endothelium and acts as a natural anticoagulant. However, we reported previously that TM was also expressed in the squamous epithelium mainly at the intercellular bridges. In this study, we examined TM expression in the primary lesions of 106 patients with esophageal squamous cell carcinomas and in the lymph node metastatic lesions of 59 patients using immunohistochemical methods. The carcinoma tissues expressed TM mainly at the cell-cell boundaries and in the cytoplasm. When TM expression was compared between the primary and metastatic lesions in the 59 patients who had lymph node metastasis, 41 (69%) showed decreased TM expression, 18 (31%) showed no change, and none (0%) showed an increase in the metastatic lesions. Wilcoxon's signed-rank test indicated that tumor cells that were positive for TM expression were significantly rarer in the metastatic lesions than in the primary tumors (P < 0.0001). This result indicates that the decrease in TM expression is associated with metastasis of the carcinoma cells. This phenomenon is very similar to that of E-cadherin, although the structures of both molecules are quite different. The reduction of TM expression seems to play an important role in the metastatic process of esophageal cancer.

INTRODUCTION
Esophageal SCC is characterized by extensive local growth and lymph node metastasis. The status of lymph nodes is of crucial prognostic importance in esophageal SCCs (1, 2). Elucidating the manner of lymph node metastasis seems to be useful in improving the prognosis of the patients. TM is a receptor for thrombin and plays an essential role as a cofactor in thrombin-catalyzed activation of protein C. Activated protein C acts as a natural anticoagulant through proteolytic degenaration of coagulation factors Va and VIIIa (3, 4). Previously, we isolated (4) and characterized (5–7) TM from the endothelium and acts as a natural anticoagulant. However, we reported previously that TM was also expressed in the squamous epithelium mainly at the intercellular bridges. In this study, we examined TM expression in the primary lesions of 106 patients with esophageal squamous cell carcinomas and in the lymph node metastatic lesions of 59 patients using immunohistochemical methods. The carcinoma tissues expressed TM mainly at the cell-cell boundaries and in the cytoplasm. When TM expression was compared between the primary and metastatic lesions in the 59 patients who had lymph node metastasis, 41 (69%) showed decreased TM expression, 18 (31%) showed no change, and none (0%) showed an increase in the metastatic lesions. Wilcoxon’s signed-rank test indicated that tumor cells that were positive for TM expression were significantly rarer in the metastatic lesions than in the primary tumors (P < 0.0001). This result indicates that the decrease in TM expression is associated with metastasis of the carcinoma cells. This phenomenon is very similar to that of E-cadherin, although the structures of both molecules are quite different. The reduction of TM expression seems to play an important role in the metastatic process of esophageal cancer.

MATERIALS AND METHODS
Tissue Samples. Surgical specimens were obtained from 106 patients (93 men and 13 women) with primary esophageal SCCs, who underwent subtotal esophagectomy with lymph node dissection in the Department of Surgery I, Kagoshima University School of Medicine, Kagoshima City, Japan, from 1980–1992. The ages of the patients ranged from 36–82 years old with an average of 63 years old. The locations of the primary lesions were as follows: cervical, 2; upper thoracic, 17; midthoracic, 57; and lower thoracic, 30 patients. The degrees of tumor invasion classified by the new TNM classification (19) were as follows: T1, 56; T2, 29; and T3 and T4, 21 patients. The degrees of stage were as follows: stage I, 38; stage IIa, 9; stage IIb, 24; stage III, 11; and stage IV, 24 patients. All 106 patients were diagnosed histopathologically as to SCC differentiation (well differentiated, 36; moderately differentiated, 55; and poorly differentiated, 15 patients). Of the 106 patients, 59 had metastatic lesions in the lymph nodes (139 lesions in total). TM expression in the primary tumors and in the lymph node metastatic lesions was examined immunohistochemically.

Antibodies. Rabbit antiserum against TM was obtained as described previously (8). The IgG fraction of the antiserum was used for immunostaining. The antiserum was isolated from rabbit serum using protein A-Sepharose and was monospecific, as determined by Western blotting. Biotinylated affinity-purified goat antirabbit IgG and ABC were purchased from Vector Laboratories (Burlingame, CA) as the Vectastain Elite ABC kit.

Staining Procedures. Staining for TM was performed on formalin-fixed, paraffin-embedded tissue sections by an immunoperoxidase method using ABC (20), as described previously (18, 21), with some modifications. In brief, the paraffin blocks were cut into 4-μm-thick serial sections and deparaffinized with xylene. Endogenous peroxidase was blocked by incubating the sections in 0.3% hydrogen peroxide in absolute methanol at room temperature for 30 min. After rehydration through a series of decreasing concentrations of ethanol in water, the sections were washed in 0.01 M PBS (pH 7.4).

Sections were incubated with rabbit anti-TM serum for detection of TM at a concentration of 5 μg/ml diluted in PBS containing 1% BSA for 16 h at 4°C. The sections were washed in PBS and incubated with biotinylated goat antirabbit IgG at the recommended concentration for 30 min at room temperature. After being washed in PBS, the sections were incubated with ABC at the recommended concentration for 30 min at room temperature. After the sections had been washed again in PBS, they were reacted with diaminobenzidine

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The abbreviations used are: SCC, squamous cell carcinoma; TM, thrombomodulin; IgG, immunoglobulin G; ABC, avidin-biotinylated horseradish peroxidase complex; FM, femtomolar.

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substrate (20 mg/dl) for visualization, rinsed with tap water, counterstained with hematoxylin, and mounted.

The specificity of the immunostaining for TM was determined by preabsorption of the antiserum by the antigen and by replacing the rabbit antiserum with normal rabbit serum. Immunostaining was abolished completely after absorption of the antibody with antigen and was always negative in the control sections stained using normal rabbit serum.

**Evaluation of the Results by Scoring.** Staining results were evaluated as follows: We observed the whole areas of neoplasms by sequentially examining at less powerful (×10) optical fields the sections stained by the antibody; and we calculated the approximate percentages of positively stained neoplastic cells. When we could observe multiple metastatic lesions, the averages of the percentages were adopted. The results were graded as follows: 0, completely negative; 1+, under 5% of neoplastic cells stained; 2+, 5-50% of neoplastic cells stained; and 3+, over 50% of the neoplastic cells stained.

**RESULTS**

**TM Expression Pattern in Normal Squamous Epithelium and Esophageal SCCs.** In all of the cases examined, TM expression was seen at cell-cell boundaries of normal stratified squamous epithelium mainly in the lower layer, except in the basal layer cells (Fig. 1). In the carcinoma tissues showing positive TM expression, TM was expressed mainly at the cell-cell boundaries surrounding the keratinized region, except for the outermost layer in well-differentiated lesions (Fig. 2A), and was also seen occasionally in the cytoplasm in poorly differentiated types (Fig. 3A).

**Comparison of TM Expression in the Primary Tumors between the Group Negative for Metastasis and the Group Positive for Metastasis.** There was no significant difference in TM expression between the primary tumors of the group that was negative for metastasis (47 patients) and those of the group that was positive for metastasis (59 patients; Fig. 4).

**Comparison of TM Expression between Primary and Metastatic Lesions of Lymph Nodes.** We compared TM expression between the primary and metastatic lesions in 59 patients who had lymph node metastasis. The matrix of Fig. 5 represents the correlation between the degree of TM expression in primary esophageal carcinoma and in lymph node metastasis from the same patient. In the matrix, the abscissa represents the degree of TM expression at the primary sites, and the ordinate represents the degree of TM expression at the corresponding metastatic lesions. The circles in this matrix represent individual patients.

In the matrix, the patients tended to be distributed heavily in the right lower field; this distribution pattern indicated that the tumor cells that were positive for TM expression were far fewer in the metastatic lesions than in the primary tumors (Wilcoxon's signed-rank test, \( P < 0.0001 \)). None (0%) of the patients showed increased TM expression in the metastatic lesions. In the 59 patients who had lymph node metastases, 41 (69%) showed decreased TM expression in the metastatic lesions; of the 41, 19 (32%) became negative, and 18 (31%) showed no change of TM expression in the metastatic lesions.

Fig. 2 shows TM expression in the case of a well-differentiated SCC in the primary tumor (A) and in the lymph node metastatic lesion (B) from the same individual. The primary tumor showed positive TM expression mainly at the cell-cell boundaries (Fig. 2A), whereas the metastatic lesion showed no TM expression (B).

Fig. 3 shows TM expression in the case of a poorly differentiated SCC in the primary tumor (A) and in the lymph node metastatic lesion (B). The primary tumor showed positive TM expression at the cell-cell boundaries and in the cytoplasm (Fig. 3A), whereas the metastatic lesion showed no TM expression (B).

**DISCUSSION**

To our knowledge, this is the first report of a study demonstrating TM expression and an inverse correlation of TM expression with the lymph node metastasis in esophageal SCC. As shown in Fig. 4, the degree of TM expression in the primary tumors does not seem to be related to metastatic potential of the carcinoma cells. Therefore, factors other than TM expression in the primary tumors should be taken into consideration to explain the mechanism of SCC metastasis. However, TM expression was decreased significantly in the lymph node metastatic lesions in 69% of the cases, as shown in Fig. 5. Tumor metastasis is a complex series of multiple processes, and two steps seem to be important: detachment of cancer cells; and adhesion of such cancer cells to endothelial cells (22, 23). Even in the cases showing dominant TM expression in the primary tumors, the lesions had some SCC cells with negative or low TM expression; these may have high metastatic potential related to the following two possibilities: (a) TM is a new cell-cell adhesion molecule similar to E-cadherin, and SCC cells that are negative for TM expression may have weak cell-cell adhesion, which induces easy detachment of cancer cells; and (b) SCC cells that are negative for TM expression may have low anticoagulant activity, which may induce easy adhesion of cancer cells to endothelial cells.

Imada et al. (24) showed by protein and DNA sequencing and by functional assays that FM, a marker protein of fetal development (25), was identical to TM. It has been proposed that the amino-terminal region of human FM/TM contains 12 amino acids in a spatial align-
Fig. 2. TM expression in the case of a well-differentiated SCC in the primary lesion (A) and in the metastatic lymph node (B). In the carcinoma tissues showing positive TM expression, TM was expressed mainly at the cell-cell boundaries surrounding the keratinized region, except for the outermost layer. TM expression in the primary carcinoma was positive, whereas the metastatic lesion was negative in this area; X 200.

ment compatible with the consensus animal lectin fold (24). Because some investigations have shown the existence of a new mechanism of cell-cell adhesion via animal lectin domains, Imada et al. (24) suggested that TM may be a member of these adhesive molecules with lectin-like activity. TM expression at the cell-cell boundaries of esophageal squamous epithelium may be consistent with the nature of TM as a member of the adhesive molecules with lectin-like activity. Expression of the epithelium-specific cell adhesion molecule E-cadherin (26) is inversely correlated with lymph node metastasis in SCCs of the head and neck region (27). The reduction of E-cadherin expression seems to play an important role in invasion and metastasis of human breast cancers (28), gastric cancers (29, 30), prostatic cancers (31), and esophageal cancer (32). In addition, a human esophageal cancer cell line, loss or dysfunction of E-cadherin has been shown to diminish intercellular adhesion and result in the acquisition of invasive capability (33). Our observations in this study indicated that loss of TM expression is associated with metastasis of the SCC cells. This phenomenon is very similar to that of E-cadherin, although the molecular structure of TM (9) is quite different from that of E-cadherin (26). Our preliminary study using immunocytochemistry, immuno-blotting, and Northern blotting demonstrated that esophageal SCC cell lines produced the same TM molecule as do endothelial cell lines (human umbilical vein endothelial cells; data not shown). Additional experimental investigations using SCC cell lines should clarify whether TM functions as a cell-cell adhesion molecule or whether reduction of TM expression results in cell dissociation and acquisition of cell motility.

We are also very interested in whether SCC cells expressing TM have an anticoagulant activity, because blood coagulation has been shown to play an important role in the metastatic process. It has been reported that warfarin, which prevents the synthesis of procoagulant (factors II, VII, IX, and X), lowered the numbers of tumor metastases significantly (34, 35). Thrombin plays an important role in the adhesion of cancer cells to endothelial cells through fibrin formation and platelet activation (36, 37). In addition, on the basis of evidence of experimental metastasis, several investigators have reported the relationship between coagulation activity and tumor metastasis (38–43). Because TM is a natural anticoagulant (3, 4), TM expressed in SCC cells may inhibit the adhesion of the cancer cells to endothelium. In the human body, SCC cells that are positive for TM expression may have high anticoagulant activity, resulting in loose adhesion of the cancer cells to the endothelium. In contrast, SCC cells that are negative for TM expression may have low anticoagulant activity, thereby inducing easy adhesion of the cancer cells to the endothelium, which, in turn, facilitates metastasis. Therefore, SCC cells that are negative for TM expression may tend to form metastatic lesions as a result of the low anticoagulant activity, resulting in decreased TM expression in the lymph node metastatic lesions.

Recently, O’Reilly et al. (44) identified a novel angiogenesis in-
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Fig. 3. TM expression in the case of a poorly differentiated SCC in the primary lesion (A) and in the metastatic lymph node (B). TM was also seen occasionally both at the cell-cell boundaries and in the cytoplasm. TM expression in the primary carcinoma was positive, whereas the metastatic lesion was negative; × 170.

Fig. 4. Comparison of TM expression in the primary lesions between groups with LN(+) and without LN(-) metastasis. TM expression was not significantly different between groups.

Another possible explanation for the decreased TM expression in lymph node metastatic lesions is that SCC cells that are positive for TM expression are transformed to SCC cells that are negative for or have lower TM expression after implantation in lymph nodes. To

hibitor named angiostatin, which mediates the suppression of metastasis. This molecule, having a molecular mass of 38 kilodaltons, was purified from serum and urine from tumor-bearing mice and identified as a plasminogen fragment (44). Activated protein C can bind with tissue plasminogen activator inhibitor type 1 and enhance fibrinolysis (45). However, at present, the precise mechanism of the formation pathway of this newly identified metastasis inhibitor, angiostatin, is not clear. Therefore, whether our observation showing the decreased TM expression in metastatic lesion is related to angiostatin, and whether the TM-protein C pathway is involved in formation of angiostatin from plasminogen, remain to be elucidated.

Fig. 5. Matrix representing correlation between the degree of TM expression in primary esophageal carcinoma and in metastatic lesions from the same patients based on immunohistochemical staining. Ovals, individual patients. The degree of TM expression was classified by the percentage of tumor cells stained positively. In the matrix, the patients tended to be distributed in the right lower field; this distribution pattern indicates that the tumor cells that were positive for TM expression were far fewer in the metastatic lesions than in the primary tumors (Wilcoxon's signed-rank test, \( P < 0.0001 \)).
verify this possibility, additional experimental studies using esophageal SCC cell lines under various culture conditions or in animal models are necessary.

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