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Expression and localization of thrombomodulin in preneoplastic bronchial lesions and in lung cancer

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Abstract Thrombomodulin (TM) is an endothelial surface glycoprotein that acts as a natural anticoagulant. It inhibits thrombin and accelerates the activation of the anticoagulant protein C. TM has been detected in dermal keratinocytes, where it is associated with terminal differentiation. It can also be detected in various types of squamous malignant neoplasms and in malignancies of endothelial and mesothelial origin, such as Kaposi's sarcoma or malignant mesothelioma, but is absent in pulmonary adenocarcinomas (AC). Seventy-two lung tumour specimens [33 squamous cell carcinomas (SQCC), 23 AC, 1 large cell carcinoma, 8 small cell lung cancers (SCLC) and 7 multidifferentiated tumours (MT)] were analysed immunohistochemically by staining with an anti-TM antibody in order to assess TM expression. All of the SQCC stained positively for TM. In contrast, only 9 AC and 4 MT and none of the SCLC showed positive anti-TM staining. Seven hyperplastic bronchial epithelial specimens and eight preneoplastic bronchial lesions (five cases of moderate dysplasia, two cases of severe dysplasia and one case of carcinoma in situ) were used as controls.

Normal or hyperplastic areas of bronchial epithelium revealed no positive reaction. However, a distinct positive anti-TM staining pattern related to the degree of keratiniziation of dysplastic lesions was seen. The present results suggest that anti-TM immunostaining is a useful marker for squamous cell carcinoma in the differential diagnosis of pulmonary carcinoma, also indicating keratinocyte differentiation in dysplastic bronchial epithelium.

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Introduction

Thrombomodulin (TM) is a 75,000 kDa endothelial surface glycoprotein localized on the luminal endothelial surface of human blood and lymphatic vessels that forms a 1:1 complex with thrombin. Binding of thrombin to this high affinity receptor alters its specificity toward several substrates and the complex activates protein C approximately 1,000 times faster than thrombin alone [6]. TM is also expressed on the syncytiotrophoblasts of human placental tissue [11], various human blood cells (neutrophils, monocytes and platelets) [3, 12, 19], megakaryocytes [14], alveolar and synovial tissue lining macrophages and on surface mesothelium of body cavities, such as the pleural cavity [12]. TM contains six epidermal growth factor (EGF)-like repeats, three of which are required for thrombin binding. The endothelial glycoprotein plays a major role in the regulation of intravascular coagulation, neutralizing thrombin clotting activity by forming a complex with thrombin. This complex enhances the degradation of factor Va and factor VIIIa by activating protein C [5, 6]. TM is thought to be an antithrombotic agent on endothelial and mesothelial surfaces, and its expression indicates endothelial cell damage [1]. This conclusion is underlined by the fact that increased plasma thrombomodulin levels are found in patients suffering from disseminated intravascular coagulation, pulmonary thromboembolism, adult respiratory distress syndrome, chronic renal failure and acute hepatic failure [20].

The physiological functions of thrombin (platelet activation or fibrinogen clotting) may also be of major importance in metastatic spread by stimulating the adhesion of tumour cells to vascular endothelial cells [13]. Complex formation with TM inhibits all of these thrombin related activities. Thus, TM may also serve as an inhibitor

of haematogenous metastases. Imada et al. also suggest a lectin-like activity of TM [8], which seems to be independent of the thrombin-binding receptor regions of the glycoprotein [16].

TM-negative hepatic carcinomas have been reported to have a better clinical outcome but similar correlations were not found in squamous cell carcinoma (SQCC) of the oesophagus [18, 21]. In various malignant neoplasms increased TM plasma levels have been described and in patients with carcinoma of the pancreas elevated TM plasma levels are correlated with a more aggressive course of disease [10].

The physiological function of extravascular TM is not yet fully understood. Yonezawa et al. describe extravascular TM-expression localized in the suprabasal layer of keratinocytes in human epidermis [22]. Negative TM-staining in basal cell carcinoma and enhanced staining in more differentiated SQCC of the skin has been interpreted as indicating that TM expression is an early stage of keratinocyte differentiation [17]. A recent study reported positive TM immunostaining in 21 of 26 SQCC of various origins, as well as in squamous metaplasias and squamous carcinoma in situ [9]. In comparative studies investigating TM expression in malignant mesotheliomas and pulmonary adenocarcinomas, it has been suggested that TM may be a specific marker for neoplasms of mesothelial origin [2]. Although there are some reports regarding the expression of TM in some tumours or tumour cell lines [15, 22], there are no detailed data on the expression and localization of TM in pulmonary neoplasms or in hyperplastic and preneoplastic lesions of the lung.

Material and methods

Eighty-seven biopsy specimens were obtained through diagnostic bronchofibreoscopy from patients with clinically or radiologically suspected malignant pulmonary lesions. Specimens were formalin-fixed and embedded in paraffin. Histomorphological examination of haematoxylin and eosin and elastica van Gieson stained sections revealed 7 hyperplastic epithelial lesions, 5 severe dysplasias, 1 carcinoma in situ and 72 primary lung tumours. Tumours were classified according to the WHO classification of lung tumours [23] and included 33 SQCC, 23 adenocarcinomas (AC), 1 large cell carcinoma, 8 small cell carcinomas (SCLC) and 7 multidifferentiated tumours (MT).

Immunostaining was performed using a mouse monoclonal antibody against the sixth repeated EGF-domain of human thrombomodulin (Dako, Carpinteria, Calif., USA). Four micrometre sections were cut consecutively from formalin-fixed and paraffin-embedded tissue specimens and mounted on poly-L-lysine coated slides. After deparaffinization the slides were rinsed in TRIS-buffered saline (TBS). Endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide for 30 min at room temperature. Sections were incubated with the monoclonal mouse anti-thrombomodulin antibody (diluted 1:30) overnight. On the next day the slides were rinsed with TBS, incubated with an alkaline phosphatase conjugated rabbit-anti-mouse IgG for 30 min at room temperature, rinsed with TBS and incubated with an alkaline phosphatase-mouse-anti-alkaline-phosphatase (APAAP)-complex (Dakopatts, Glostrup, Denmark) for 45 min at room temperature. Finally, the slides were incubated with a chromogen alkaline-phosphatase-substrate for 30 min at room temperature and counterstained with Mayer's haematoxylin.

In negative controls, all reagents except the primary antibody were used. Blood vessels served as internal positive control. Intensity of TM-staining was evaluated semiquantitatively [absent (0), slight (1), moderate (2) and strong immunoreactivity (3)].

Results

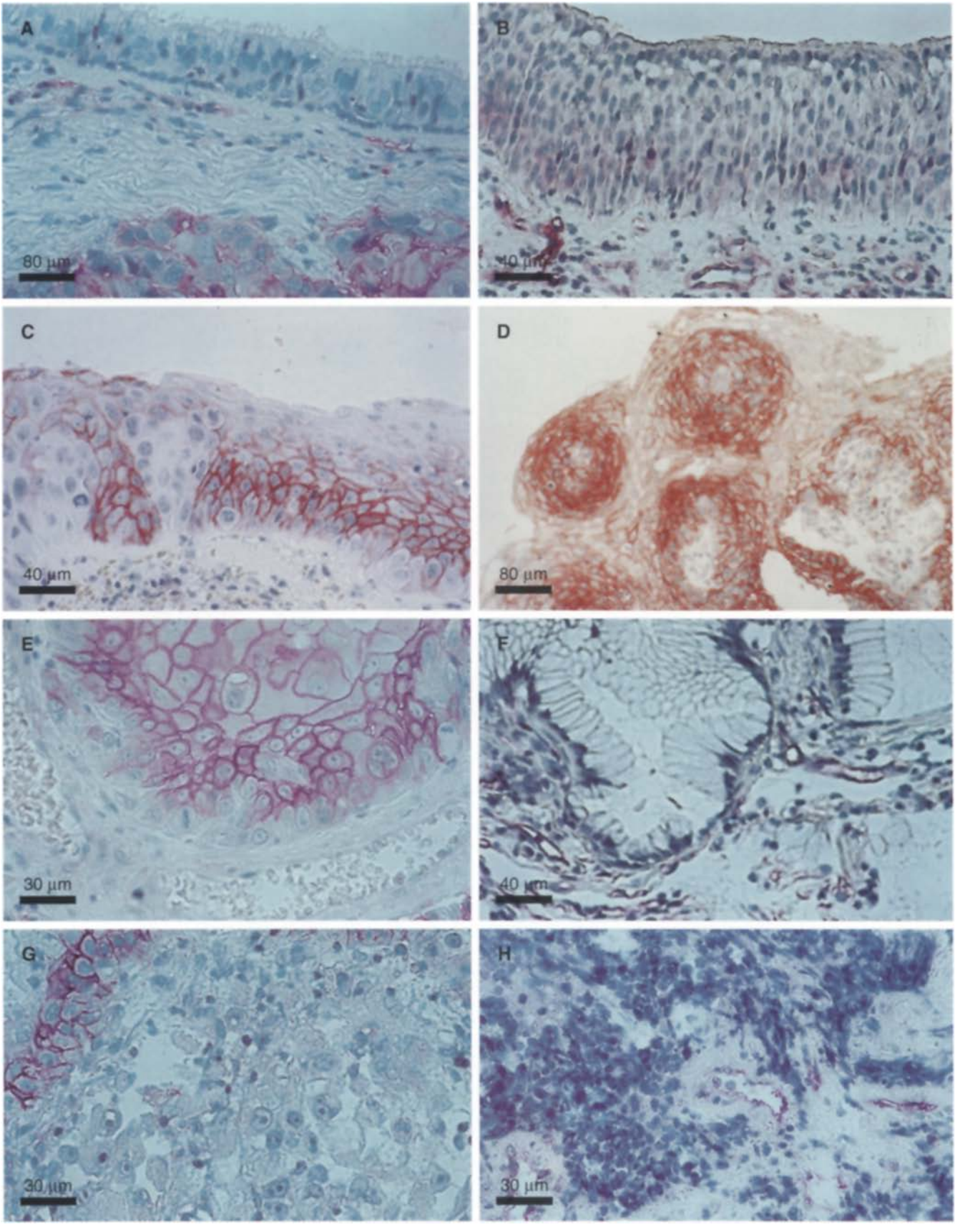
TM expression was established by the dark purple reaction product obtained by the APAAP-technique [4]. Normal bronchial epithelium (Fig. 1A), mucous glandular epithelium and pneumocytes exhibited no TM staining. In contrast, endothelial cells of the blood vessels and endothelial surface of alveoli stained positive. Hyperplastic bronchial lesions were TM-negative (Fig. 1B).

All squamous dysplastic lesions investigated showed positive TM-immunostaining. However, the intensity and the extent were variable, depending on the grade of keratinization of the lesion. In relation to the cornification of the stratified structure, TM was found to be expressed in the epithelium of dysplastic lesions, except for basal cells and surface covering cells (Fig. 1C). A heterogeneous staining pattern was found in the case of a carcinoma in situ: invasive malignant cells were negative (Fig. 1C). All SQCC expressed TM in their tumour cells: 15 tumours showed strong, 11 moderate and 7 weak positive staining. TM was localized mainly on the cell surfaces, at cell-cell boundaries surrounding the keratinized region (Fig. 1E). In the central region of keratin pearl formation TM expression was weak or absent (Fig.1D).

Only 1 of 23 cases of adenocarcinoma stained strongly positive for TM. Staining was localized in the cytoplasm of tumour cells. Two AC were moderately positive, 6 were weak and 14 cases were negative for anti-TM immunostaining. All three bronchioloalveolar carcinomas were negative (Fig. 1F). Only the endothelial surface of the alveoli was TM positive in these tumours.

Three MT revealed TM positivity in the tumour components with squamous differentiation (one strong, two moderate), while the adenoid component was TM negative. No detectable immunostaining product was found in the lesions of patients suffering from SCLC (Fig. 1H) or large cell carcinoma (Fig. 1G).

Fig. 1A–H Immunohistochemical localization of thrombomodulin (TM) in normal human bronchial epithelium, in dysplastic lesions of bronchi and in lung tumours. No TM-immunostaining was seen in normal bronchial epithelium (**A**) and hyperplastic bronchial lesions (**B**). In the transitional area of squamous dysplasia and carcinoma in situ (**C**) TM was expressed in the whole area of the dysplastic lesions, except in some of the basal and surface covering cells. Loss of immunostaining was detected in the invading cells of carcinoma in situ. TM staining was weak or absent in the central areas of keratin pearl formation (**D**), but was easily detectable on the cell surfaces of squamous cell lung cancer cells, markedly highlighting the cell-cell boundaries (**E**). Bronchioloalveolar carcinoma cells (**F**) were negative for anti-TM immunostaining. Only the alveolar endothelium stained positively. No detectable immunostaining in the tumour cells of the large cell carcinoma (**G**) and in the small cell carcinoma (**H**) ▶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Discussion

Our results demonstrating enhanced expression of thrombomodulin in squamous dysplastic lesions and SQCC of the lung are in accordance with other reports of thrombomodulin expression in squamous metaplastic and malignant lesions. The characteristic positive staining pattern of intercellular bridges suggests a mutual relationship between the glycoprotein TM and the cornification process of epithelial cells. The consequent positive staining pattern of lung SQCC may allow the use of anti-TM-immunostaining as an additional marker for squamous differentiation in the differential diagnosis of malignant pulmonary neoplasms. The focal weak staining observed in seven cases may be explained by poor fixation with subsequent loss of the antigen.

Considering the correlation between TM expression and keratinocyte differentiation of dysplastic bronchial epithelial lesions, TM immunostaining may be regarded as evidence indicating that squamous epithelial neoplasms develop preferentially in preneoplastic lesions. However, the obvious difference between TM-expression in SQCC and other types of pulmonary neoplasms suggests a different cellular origin of these malignant lesions since bronchioloalveolar tumour cells were negative for TM. These findings coincide with the hypothesis that this type of lung cancer arises from transformed pneumocytes [7].

The observed correlation between TM-negativity and an increased metastatic capacity in hepatic cell carcinoma [18] points to a possible inhibitory effect of TM in tumour cell – endothelial cell adhesion. Interestingly, the invading cells of carcinoma in situ (Fig. 1C) and SQCC were also negative for anti-TM immunostaining (Fig. 1E), sustaining this theory. The individual clinical course of different histomorphological types of non-SCLC, for example the higher haematogenous metastatic potential of pulmonary AC versus the preference for locally destructive growth of SQCC, may be caused in part by TM expression in these tumours. Even the fact that pulmonary AC are often associated with recurrent thrombosis can be interpreted as the result of impaired thrombin-binding caused by relatively low TM-levels. Further investigations are necessary in order to assess the important function of TM in the haematogenous metastatic spread of malignant pulmonary neoplasms.

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