Thrombomodulin activity and localization

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ABSTRACT An overview on the properties, actions and localization of thrombomodulin (TM) in situations of tissue injury and in selected tumors is presented. The localization and activity of TM after injury to vascular endothelium shows that following balloon catheter denudation of the endothelium of the rabbit aorta, the activity and immunohistochemical staining is markedly reduced. The functional and antigenic levels approach the control levels approximately one week after the initial injury. The results suggest that the neointimal smooth muscle cells express TM. This phenotypic plasticity of the neointimal smooth muscle cells may be important in conferring thrombo-resistance to the lumenal lining cells of vessels after injury. Studies are also reviewed on the use of soluble recombinant TM to prevent thrombosis after ligature of vessels in an experimental model. Further characterization on the immunohistochemical distribution of TM in normal tissues and tumors shows that staining with a monoclonal anti TM antibody can be very useful in separating mesotheliomas from pulmonary adenocarcinomas. These studies may lead to insights concerning the role of TM in tissue-injury-repair and tissue differentiation.

KEY WORDS: *thrombomodulin, vascular injury, mesothelioma*

Introduction

This review will describe some of the characteristics of thrombomodulin (TM). We will characterize TM activity and distribution during experimental vascular injury in the rabbit aorta. Also, we will further describe the distribution of TM in specific human tumors.

There are several mechanisms which seem to be important in maintaining a thrombo-resistant surface on endothelial cells. These include the presence of heparin-like molecules which interact with antithrombin III to inactivate the serine proteases of the coagulation pathways, the synthesis of prostacyclin (PGI_2) which prevents platelet adhesion, the synthesis of nitric oxide (NO) or Endothelial Derived Relaxation Factor (EDRF) which inhibits platelet function, the production of ADPase which inhibits platelet activation, the production and release of tissue plasminogen activator (TPA) which promotes fibrinolysis, and the synthesis of thrombomodulin (TM). (Brady and Warren, 1990). Thrombomodulin is a transmembrane protein which can accelerate the activation of protein C. Activated protein C (APC) acts as an anticoagulant by combining with protein S to inactivate Factors Va and VIIIa of the blood coagulation pathway and by binding thrombin (Esmon, 1989). The bound thrombin is not capable of cleaving fibrinogen to fibrin (Preissner *et al.*, 1990). The APC also has fibrinolytic enhancing activity because it seems to inactivate the inhibitor of tissue plasminogen activator (TPA) (van Hinsbergh *et al.*, 1985). The anticoagulant and profibrinolytic functions of PC which are triggered by the interaction of thrombin and TM and formation of APC are important in preventing thrombosis from occurring on endothelial surfaces.

Human thrombomodulin contains 559 amino acid residues and has similarities to the LDL receptor. The molecule contains both O-glycosylation and N-glycosylation sites as well as having 1-2 molecules of chondroitin sulfate bound to it. There are six repeated Epidermal Growth Factor (EGF) homologous domains and a region coding for a hydrophobic transmembrane sequence of 23 amino acids. The amino terminal domain has homology to lectin-like proteins. Studies using site directed mutagenesis have delineated which regions of the molecule are necessary for thrombin binding and protein C activation (Hayashi *et al.*, 1990). The DNA sequences including the promoter sequences for human, bovine and mouse TM have been ascertained. There is extensive interspecies homology of the TM gene. There do not appear to be any introns (Dittman and Majerus, 1990). There are several factors which have been reported to regulate TM expression. These include the down regulation of TM by the cytokine interleukin I, tumor necrosis factor and endotoxin (Naworth and Stern, 1986; Naworth *et al.*, 1986; Moore *et al.*, 1987; Conway and Rosenberg, 1988; Moore *et al.*, 1989; Giddings,

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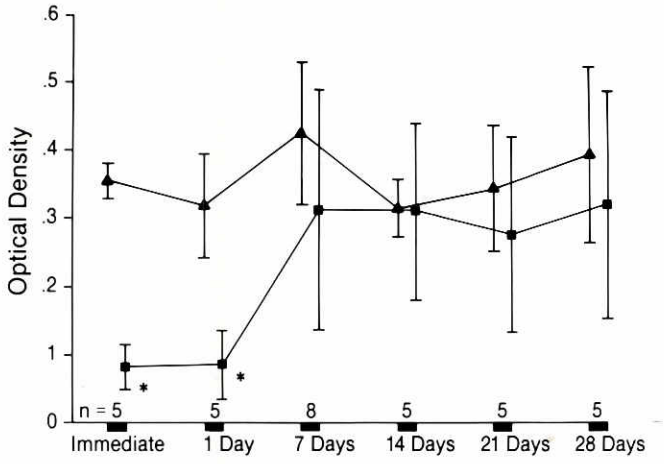

Fig. 1. Thrombomodulin activity on rabbit aorta after denudation of the endothelium. *Control intact aortas* (▲); *the denuded aortas* (■); * *denotes p <0.01.*

1990). Agents which increase cAMP such as forskolin have been reported to up-regulate TM activity in endothelial cells in culture (Hirokawa and Aoki, 1990; Imada *et al.*, 1990b; Ishii *et al.*, 1990; Maruyama *et al.*, 1991; Soff *et al.*, 1991).

Distribution of TM has been studied in normal tissues. In addition to the lining cells of arteries, veins, capillaries and lymphatics, TM has been found in several other types of cells. Thrombomodulin has been found in mesothelial cells, meningeal lining cells, synovial cells, syncytiotrophoblasts, megakaryocytes, platelets and squamous cell carcinoma cells (Maruyama *et al.*, 1985; DeBault *et al.*, 1986a,b; Boffa *et al.*, 1987a,b; Yonezawa *et al.*, 1987; Yasunaga *et al.*, 1989; Ogura *et al.*, 1990). Thrombomodulin has been used to immunochemically stain a variety of vascular tumors and choriocarcinomas (Yonezawa *et al.*, 1988; Maruyama *et al.*, 1989). We have recently reported the presence of TM on mesotheliomas and have found that TM is absent from almost all adenocarcinomas of the lung. This difference in TM content is useful in discriminating mesotheliomas, particularly those with an adenomatous pattern of growth from true adenocarcinomas. A review of these studies will be further discussed. We have also found that human mesothelioma cells in culture produce TM. These cells can be shown to have mRNA for TM and they can be induced to markedly increase TM expression by treatment with tetradecanoylphorbol acetate (PMA) (Collins *et al.*, 1992).

Early reports suggested that there was no TM in the vessels of the human brain (Maruyama *et al.*, 1985). However, other reports show detectable staining of the blood vessels of the central nervous system of rabbits (DeBault *et al.*, 1986a). We have found a subset of cells in the islets of the pancreas which are labelled by a monoclonal Ab directed against TM. We have also found staining with a monoclonal anti-TM antigen on peripheral nerves (Fink, unpublished data).

Fetomodulin (FM), which has been identified in differentiated F9 mouse embryonal carcinoma cells, has been found to be the same as TM (Imada *et al.*, 1990a). The expression of fetomodulin increases after treatment with various agents such as dibutyryl cAMP which induces parietal endodermal differentiation. Thrombomodulin appears early in the development of the mouse embryo. In the developing mouse embryo FM was found in vessels, the lung bed and in neural ectoderm (Imada *et al.*, 1990a,b).

Little is known about the synthesis and distribution of TM in an animal model of vascular tissue injury and repair. The following sections will present some of our recent work on TM activity and localization after endothelial denudation, the use of TM as a marker in the analysis of mesotheliomas, and some comments about the use of recombinant TM as an anticoagulant.

Thrombomodulin activity and localization after balloon angioplasty of the rabbit aorta

Our work on the synthesis and localization of thrombomodulin (TM) in rabbit aortas after injury to the vessel wall using a balloon catheter is reviewed. This model for injury-repair has been well described and shows that the intimal proliferation occurring after denudation of the endothelium is derived from altered smooth muscle cells (Schwartz *et al.*, 1975; Spaet *et al.*, 1975; Reidy, 1988). These smooth muscle cells appear to change their phenotype as they become part of the proliferating intimal repair reaction and form some of the luminal lining cells (Prescott *et al.*, 1991; Fuster *et al.*, 1992). After de-endothelialization the newly exposed surface becomes relatively thrombo-resistant (Groves *et al.*, 1979; Eldor *et al.*, 1981). Because TM has been implicated as one component of endothelial cells which may be responsible for their resistance to thrombus formation, we have studied TM activity and disposition after injury to the aorta.

Thirty-three New Zealand white rabbits underwent balloon catheter stripping of the abdominal aorta. A 4-French balloon thromboembolectomy catheter (Baxter Healthcare Corp., Memphis, TN) was passed proximally through a small arteriotomy in the femoral artery. After passage to 30 cm, demonstrated by prior measurement to be in the proximal descending thoracic aorta, the balloon was inflated with 0.4 ml of saline and the catheter withdrawn to the aortic bifurcation. Five such passages insured endothelial denudation as evidenced by Evans Blue staining. The proximal thoracic aorta was left intact so that each rabbit served as its own control.

On postoperative days 0, 1, 7, 14, 21, and 28, the aortas of 5 animals were excised and the animals sacrificed by exsanguination. Five additional rabbits were sacrificed without aortic stripping so that TM activity in undisturbed thoracic and abdominal aortic endothelium could be compared. One additional animal at each time point was given 75 cc of 0.05% Evans Blue dye as an intravenous bolus and sacrificed 30 minutes after injection.

The aorta was harvested, segments were cut longitudinally and placed in an acrylic resin template with eight reaction wells 6 mm in diameter in contact with the luminal surface (modified from Stern *et al.*, 1984). The method of measuring TM included incubation of the vessel with thrombin, inactivation of unbound thrombin with hirudin, subsequent incubation with recombinant human protein C. An aliquot was removed and incubated with the chromogenic substrate for protein C (Cook *et al.*, 1991). Thrombomodulin activity on the intimal surface, as evidenced by generation of activated protein C, was determined spectrophotometrically as change in optical density (OD) at 480 nm.

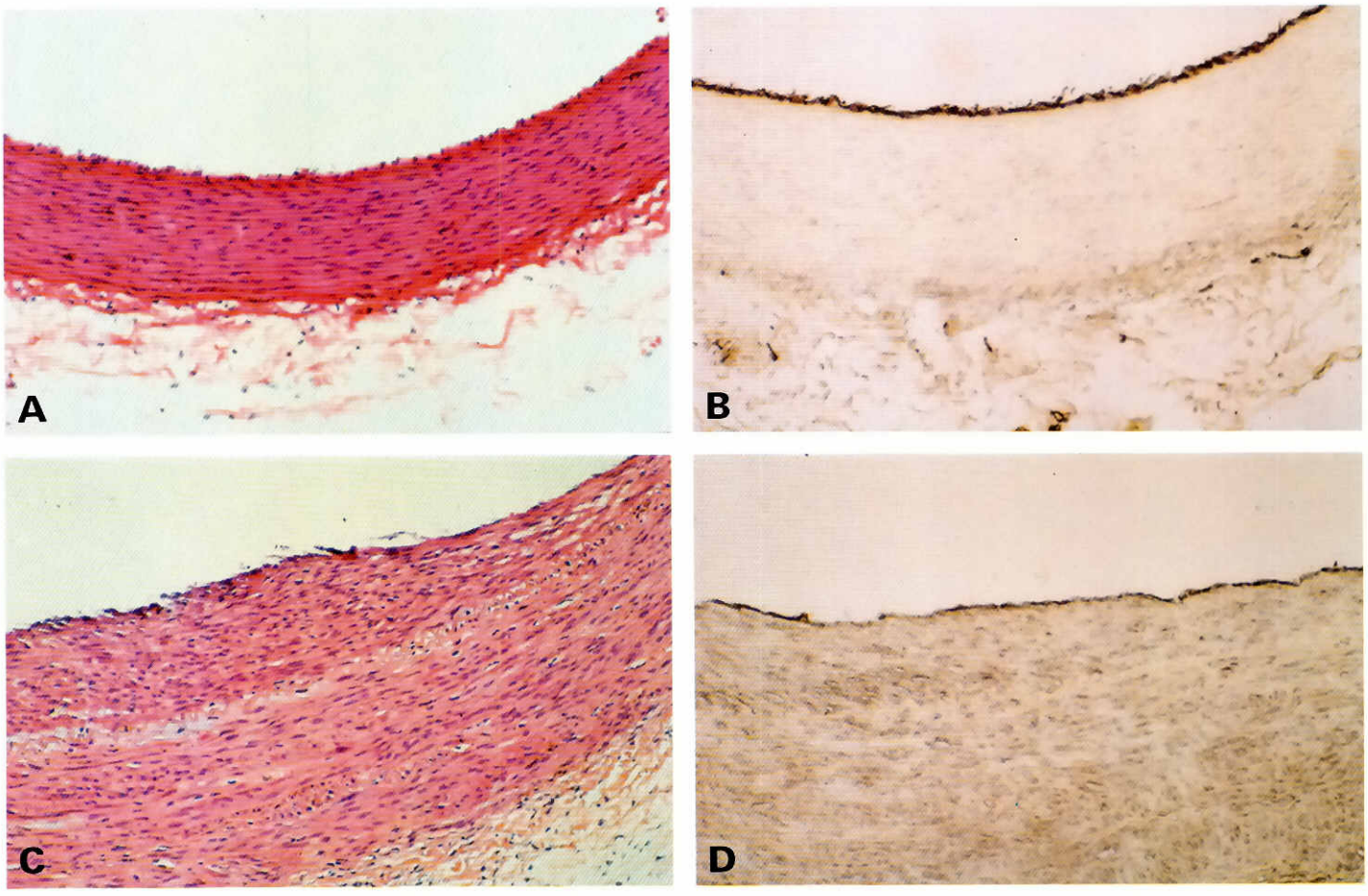

Fig. 2. Cross section of the aortas of normal and fourteen-day post-denudation rabbits (A) *H&E of control aorta;* **(B)** *anti-TM staining of control aorta;* **(C)** *H&E of treated aorta with neointimal proliferation;* **(D)** *anti-TM staining of treated rabbit aorta.*

Background OD from wells without a vessel were subtracted from those in which intima was exposed. Control wells not containing thrombin and/or protein C were run on each segment. One-way analysis of variance (ANOVA) was utilized to determine differences between intact and denuded aorta over the entire period.

TM activity in undisturbed thoracic (0.8588±0.12) and abdominal (0.873±0.086) aorta was not statistically different. Immediately after balloon catheter stripping TM activity decreased to 0.086, or 23.5% of intact values ($p<0.01$). This was similar to *in vitro* results when inhibitory anti-TM antibodies were added to the assay (29.1%). By 7 days the mean activity had increased to 0.314±0.177 (73.9% of intact), which was not statistically different from the unballooned aortic segment. Examination at 14, 21 and 28 days also showed no difference in TM functional activity between ballooned and intact aorta. ANOVA revealed no difference in TM activity in the intact aorta between groups undergoing balloon catheter endothelial stripping; values in undisturbed aortas were inexplicably greater. In the denuded aortic segment, TM activity was significantly less immediately after stripping and on postoperative day 1 than on days 7, 14, 21, and 28 (Fig. 1). The TM functional activity returned to control levels approximately one week after the endothelial denudation.

Paraffin sections of formalin-fixed rabbit aorta were prepared. The 6μ sections were stained with a mouse monoclonal antibody prepared against human TM which reacts with the EGF4, 5, 6 domains of human TM (a generous gift from J. Morser, Berlex, Inc., South San Francisco, CA) using the avidin-biotin complex (ABC) immunoperoxidase technique (Hsu *et al.*, 1981). Adjacent sections were stained with hematoxylin and eosin.

Immunohistochemical staining revealed TM antigen loss immediately after denudation. As the neointima formed, TM was expressed on cells at the luminal surface; other cells in the neointima were weakly stained with anti-TM, indicating the presence of a TM-like substance in these cells. Examination of aortas stained with Evans Blue dye showed loss of endothelium immediately after denudation, with lack of complete reendothelialization at 7 days.

In these studies there was extensive endothelial denudation. The injury by a balloon catheter has been shown to lead to a neointima which is predominantly composed of smooth muscle cells. The repair processes after balloon angioplasty in the rabbit and other animal models have been described (Campbell and Campbell, 1985). In our studies, as well as those of others, the lack of complete reendothelialization over the neointima was substantiated by the increased permeability to Evans Blue even three weeks after the injury (Eldor *et al.*, 1981). It is generally accepted that when there is extensive endothelial denudation the neointima produces a «false» endothelial lining composed of modified smooth muscle

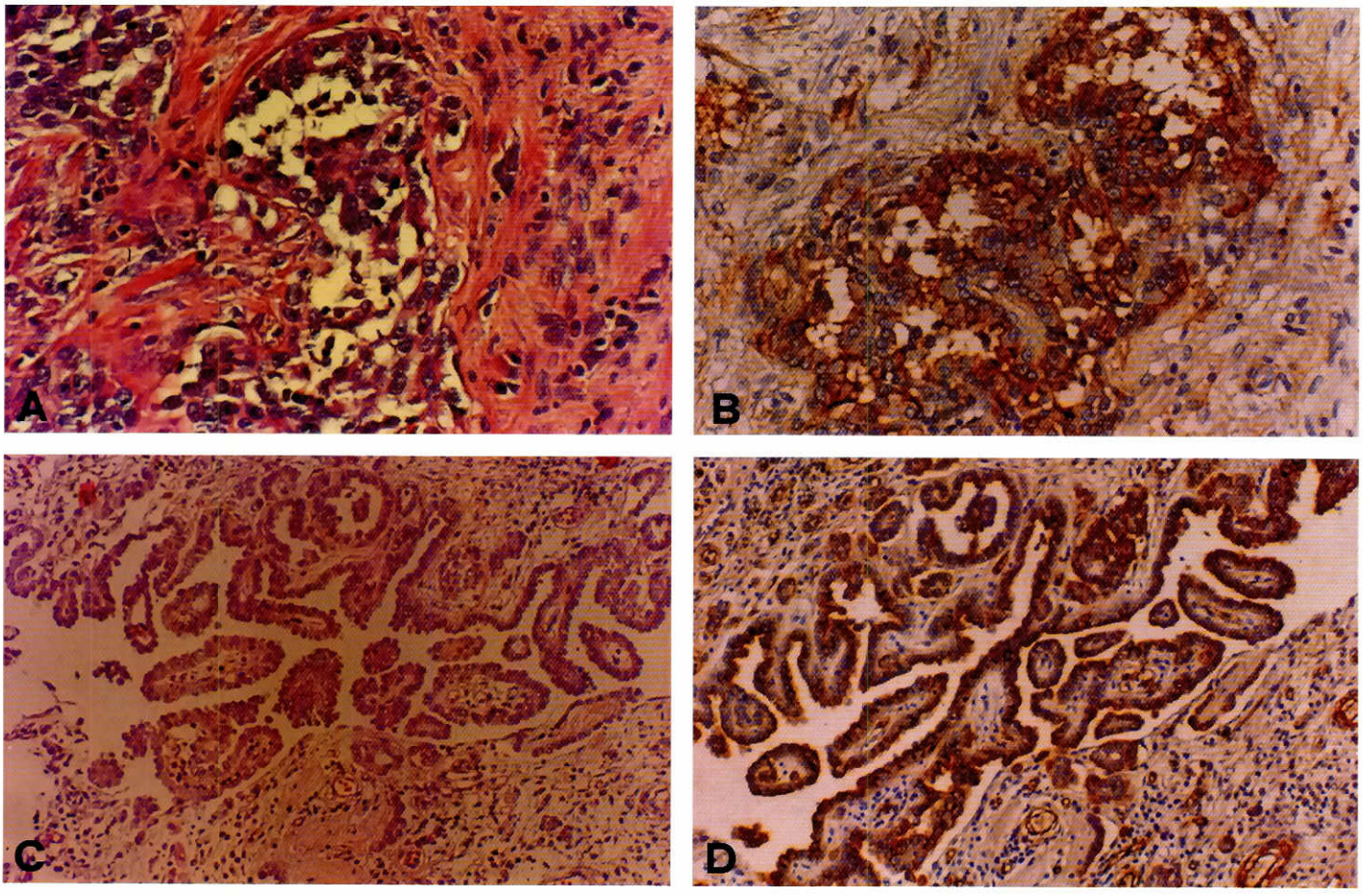

Fig. 3. Sections of human mesotheliomas. (A,C) *H&E of mesotheliomas;* **(B,D)** *anti-TM staining of mesotheliomas. The staining of the endothelium on the vessels acts as an internal positive control.*

cells. The smooth muscle cells which form the luminal lining cell or the «false» intimal lining cells are cells with some characteristics of smooth muscle cells but an antithrombogenic surface which does not allow extensive platelet adhesion or fibrin formation.

This repair reaction is another manifestation of the phenotypic plasticity of the smooth muscle cell (Campbell and Campbell, 1985; Kocher *et al.*, 1991; Edwards and Wagner, 1992). The altered smooth muscle has profound alterations in its synthesis of extracellular matrix proteins and proteoglycans (Tan *et al.*, 1991). Another characteristic of EC is the synthesis of prostacyclin (PGI_2) which contributes to the thrombo-resistant character of EC by preventing platelet adhesion. In studies on rabbit aortas after de-endothelialization with a balloon catheter there was marked decrease in PGI_2 during a period where there was only a neointima of smooth muscle cells and relatively little reendothelialization. Studies on smooth muscle cells examined *in vitro* show that they can markedly increase their synthesis of TM and that they also increase their synthesis of PGI_2 (Eldor *et al.*, 1981).

The studies described in this report show that the modified smooth muscle cells up-regulate their synthesis of TM and accumulate relatively large amounts of TM on the luminal lining cell. Fig. 2 shows the increased immunohistochemical staining detected by the use of a monoclonal antibody directed against human TM. The increase of TM in the smooth muscle in the wall of a rabbit aorta suggests that removal of intimal smooth muscle cells from their normal positional and quiescent state of growth leads to marked induction of TM synthesis. The induction of phenotypic changes in smooth muscle cells may be related to enhanced access to a different nutrient milieu, alteration in cytokine stimulation, changes in the intercellular junctions and matrix constraints, or a change in control because of the stimulation to proliferate and migrate.

The induction of TM and subsequent generation of protein C may play a role in reestablishing a normal, non-thrombogenic surface during the first several days after vascular injury. A better understanding of the injury-repair processes in blood vessels and the relationship of this process to the events occurring during hemostasis and coagulation will be important in evaluating and designing therapies for vascular disease.

The expression of thrombomodulin in pleural mesotheliomas and mesothelioma cells in culture

Thrombomodulin has been shown to be present on the surfaces of a variety of human tumors. It has been used to immuno-

histochemically stain vascular tumors and choriocarcinomas (see above), the staining is variable on meningiomas and is negative on synovial sarcomas (L. Fink, unpublished data). However, because we know that normal mesothelial cells stained positively for TM, we have examined whether TM could be used to discriminate between adenocarcinomas of the pleural cavity and mesotheliomas.

In these studies, the tumors were formalin fixed, paraffin embedded, sectioned and studied for their TM, Leu M1 and CEA staining using the ABC method of immunohistochemistry (Hsu *et al.*, 1988; Warnock *et al.*, 1988; Ordonez, 1989; Wick *et al.*, 1990) whenever possible for the presence or absence of microvilli using electron microscopy.

We have found that TM was present on normal endothelial cells and mesothelial cells as well as a subpopulation of macrophages in the lung. The bronchial epithelium, mucous glandular cells, and pneumocytes did not stain with TM. Thirty-one mesotheliomas and two mesothelial cell lines stained with anti TM. Forty-four of 48 primary pulmonary adenocarcinomas stained negatively with anti-TM (Collins *et al.*, 1992). Fig. 3 shows the anti TM staining pattern of a mesothelioma and of a typical pleural adenocarcinoma.

The mesothelial cells lines were established and characterized (Hsu *et al.*, 1988), with approximately 20% of the cells staining positively with the anti TM antibody. Treatment with PMA caused a marked increase in the number of cells producing TM. We have not been able to relate the change in TM production to any changes in the distribution of cells in the cell cycle. This is similar to the observations on cultured rabbit endothelial cells (DeBault *et al.*, 1984). The presence of TM messenger RNA in the mesothelioma cells in culture was demonstrated by *in situ* hybridization with an antisense 26 mer probe (Collins *et al.*, 1992).

These studies demonstrate the utility of differential staining with anti TM in elucidating the lineage of certain tumors. The differential staining of mesotheliomas and adenocarcinomas will assist in discriminating between these tumor types when they are morphologically indistinguishable with conventional light microscopy.

Summary

We have presented an overview of the structure and function of TM. We have reviewed and given a description of some of our studies which mark the reappearance of TM after denudation of the rabbit aortic endothelium. The functional and antigenic levels of TM approach the control levels approximately one week after the endothelial denudation. Endothelial regeneration is incomplete at this time. These studies suggest that smooth muscle cells comprising the neointima express TM or TM-like substance. This is another example of the phenotypic plasticity of smooth muscle cells. We suggest that this phenotypic alteration is at least partially responsible for the thrombo-resistance of the neointima. In other experiments where recombinant soluble TM was injected into rats prior to ligature of the vena cava we have been able to prevent the thrombosis which normally ensues (Solis *et al.*, 1991).

We have summarized studies on the expression of TM in various tissues including various tumors. Our observations further characterize the normal range of tissues expressing TM. We have shown that staining with anti TM can be extremely helpful in separating mesotheliomas from adenocarcinomas. Additional studies are being pursued to define whether TM has a physiological role in preventing thrombus formation or modulating inflammatory reactions in cavities lined by mesothelial cells or synovial cells. The localization of TM may also lead to an understanding of the role of TM in the processes of tissue injury-repair and tissue differentiation.

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