Thrombomodulin expression by human keratinocytes. Induction of cofactor activity during epidermal differentiation.

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Research Article

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Thrombomodulin Expression by Human Keratinocytes
Induction of Cofactor Activity during Epidermal Differentiation

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Abstract

Thrombomodulin is an endothelial cell surface glycoprotein that inhibits the procoagulant activities of thrombin and accelerates activation of the anticoagulant protein C. Because protein C deficiency is associated with cutaneous thrombosis, we investigated the expression of thrombomodulin in human skin. Thrombomodulin was detected by immunohistochemical staining both in dermal endothelial cells and in epidermal keratinocytes. Within the epidermis, thrombomodulin staining was limited to keratinocytes of the spinous layer, suggesting that thrombomodulin is induced when basal keratinocytes begin to terminally differentiate. Thrombomodulin expression also correlated with squamous differentiation in epidermal malignancies; little or no thrombomodulin staining was seen in five basal cell carcinomas, whereas strong thrombomodulin staining was observed in each of five squamous cell carcinomas. Human foreskin keratinocytes cultured in medium containing 0.07 mM calcium chloride synthesized functional thrombomodulin with cofactor activity comparable to thrombomodulin in human umbilical vein endothelial cells. Stimulation of keratinocyte differentiation with 1.4 mM calcium chloride for 48 h produced 3.5-, 3.2-, and 5.6-fold increases in thrombomodulin cofactor activity, antigen, and mRNA, respectively. These observations suggest that thrombin is regulated by keratinocyte thrombomodulin at sites of cutaneous injury, and indicate a potential role for thrombomodulin in epidermal differentiation. (*J. Clin. Invest.* 1994. 93:1846–1851.) Key words: differentiation • keratinocyte • protein C • thrombin • thrombomodulin

Introduction

Thrombomodulin is a cell surface glycoprotein that functions as an anticoagulant by altering the substrate specificity of the serine protease thrombin. Binding to thrombomodulin inhibits the procoagulant activities of thrombin, and markedly enhances its ability to activate protein C (1–3). Activated protein C inhibits coagulation by proteolytically inactivating coagulation factors Va and VIIIa, and by promoting fibrinolysis (4).

Thrombomodulin was initially identified as an endothelial cell protein (5), and immunohistochemical studies have demonstrated it to be present on endothelial cells throughout the vasculature (6, 7). Constitutive expression of thrombomodulin on the luminal surface of blood vessels localizes coagulation to sites of vascular injury.

Thrombomodulin has been proposed to be a specific marker for cells of endothelial origin (8). However, thrombomodulin is also synthesized in varying amounts by other types of cells, including syncytiotrophoblasts (6), platelets (9), megakaryocytes (10), monocytes (11), neutrophils (12), and synovial lining cells (11, 13). Murine thrombomodulin has been shown to be identical to a differentiation antigen, fetomodulin, that is transiently expressed in nonvascular tissue during embryonic development (14). Therefore, thrombomodulin may have both vascular and extravascular functions.

The association of cutaneous thrombosis with severe protein C deficiency (15–17) suggests that the thrombomodulin/protein C pathway is particularly important in skin. Thrombomodulin is known to be expressed by endothelial cells of dermal vessels (7). Interestingly, thrombomodulin or an immunologically similar protein has also been reported to be present in human epidermis, a nonvascular tissue (8). In the rabbit, however, thrombomodulin was not detected in epidermis (13), and no confirmatory or functional studies of human epidermal thrombomodulin have been reported. It therefore remains uncertain whether functional thrombomodulin is synthesized by epidermal cells.

To improve our understanding of the function of thrombomodulin in skin, we investigated the expression of thrombomodulin in human epidermis and cultured human keratinocytes. Our results confirm that both normal and neoplastic keratinocytes express substantial quantities of thrombomodulin. Moreover, keratinocyte thrombomodulin stimulates activation of protein C by thrombin, and its expression strongly correlates with epidermal differentiation.

Methods

Materials. Thrombin and protein C were purified from human plasma as described previously (18). Human antithrombin III was a gift of Dr. Douglas Tollefsen (Washington University, St. Louis, MO). Restriction enzymes MluI and BglII, and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Nuclease S1 was obtained from U.S. Biochem. Corp. (Cleveland, OH), and calf intestinal alkaline phosphatase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). γ-[^{32}P]ATP was purchased from Amersham Corp. (Arlington Heights, Illinois). Triton X-100 was purchased from Fisher Scientific (Pittsburgh, PA), S-2366 was obtained from Kabi Pharmacia Hepar, Inc. (Franklin, OH), and porcine intestinal heparin

was obtained from Elkins-Sinn, Inc. (Cherry Hill, NJ). Biotinylated horse anti–mouse IgG was purchased from Vector Laboratories (Burlingame, CA) and 3,3' diaminobenzidine tetrahydrochloride dihydrate was purchased from Aldrich Chemical Company (Milwaukee, WI).

Immunohistochemistry. Immunohistochemical staining was performed with a mouse monoclonal antibody that recognizes an epitope within the fifth epidermal growth factor–like domain of human thrombomodulin (19). This antibody has been demonstrated to bind specifically to both natural (20) and recombinant (19) human thrombomodulin. Formalin-fixed, paraffin-embedded sections of normal skin, invasive squamous cell carcinoma, and basal cell carcinoma of the skin were deparaffinized in xylene, rehydrated in graded alcohols to water, and rinsed in PBS. Endogenous peroxidase activity was blocked with 0.3% (vol/vol) H_2O_2 for 30 min at room temperature. Nonspecific background staining was prevented by application of normal horse serum (Vector Laboratories). Sections were then incubated with mouse anti–human thrombomodulin IgG for 2 h at room temperature, rinsed with PBS, incubated with biotinylated horse anti–mouse IgG for 30 min at room temperature, rinsed with PBS, and covered with avidin-biotin-peroxidase complex (Vector Laboratories). After 30 min at room temperature, the sections were rinsed with PBS, and peroxidase staining was demonstrated by incubation with 0.05% (wt/vol) 3,3' diaminobenzidine tetrahydrochloride dihydrate. A counterstain of 10% Harris hematoxylin was applied before coverslipping. Negative control slides were prepared by substituting preimmune mouse serum for the primary antibody. Staining intensity was scored as (−) negative, (+) weakly positive, (++) moderately positive, or (+++) strongly positive. Identically treated sections of lung and placenta were used as positive controls for thrombomodulin expression.

Cell culture. Human keratinocytes were isolated from neonatal foreskins after overnight incubation in 0.25% trypsin/0.1% sucrose solution at 4°C. Cells were plated at a density of $\sim 4 \times 10^4$ cells/cm^2 (one foreskin/60-mm plate) and cultured for three to five passages in serum-free keratinocyte growth medium (KGM)[1] (Clonetics Corp., San Diego, CA) containing 0.07 mM calcium chloride (22). After reaching 80% confluency during the terminal passage, keratinocytes were incubated in KGM containing 1.4 mM calcium chloride to induce squamous differentiation. This "calcium switch" method has been demonstrated to promote keratinocyte differentiation by both morphologic and biochemical criteria (22–24). Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics Corp. and cultured for three to seven passages in endothelial cell growth medium (21) as described previously (25).

Thrombomodulin cofactor activity. Adherent cultures of keratinocytes or HUVEC were washed with PBS, and cells were collected by scraping. Cell suspensions were centrifuged at 1,000 g for 5 min, and the pellets were resuspended in 100 μl of 20 mM Tris-HCl, pH 8.0, 0.6% (vol/vol) Triton X-100, 100 mM NaCl, 3 mM $CaCl_2$. After incubation for 5 min at room temperature, nuclei and cellular debris were removed by centrifugation. Thrombomodulin cofactor activity was measured by a modification of a two-stage protein C activation assay described previously (18). In the first stage, cell lysates were incubated for 30 min at 37°C in assay buffer (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1% bovine serum albumin) containing 2.6 nM human thrombin, 0.84 μM human protein C, and 2.4 mM $CaCl_2$. The reaction was stopped by addition of 25 μg/ml antithrombin III and 25 U/ml heparin. In the second stage, the amidolytic activity of activated protein C was measured by determining the rate of hydrolysis of the chromogenic substrate S-2366 (L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroaniline hydrochloride). Cofactor activity, defined as the rate of thrombomodulin-dependent formation of activated protein C, was calculated from a standard curve generated with human activated protein C (Haematologic Technologies Inc., Essex, VT). The total protein concentration of cell lysates was determined by a modified Bradford protein assay (Bio-Rad Laboratories, Richmond, CA).

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1. *Abbreviations used in this paper:* HUVEC, human umbilical vein endothelial cells; KGM, keratinocyte growth medium.

Thrombomodulin antigen assay. The concentration of thrombomodulin antigen in cell lysates was measured by a sandwich ELISA that uses two mouse monoclonal anti–human thrombomodulin antibodies with nonoverlapping epitopes (Diagnostica Stago, Franconville, France).

Nuclease S1 protection analysis. Plasmids containing cDNA inserts for human thrombomodulin (pUC19TM12 [26]) and human γ actin (pHFγA-1, provided by Dr. L. Kedes, University of Southern California, Pasadena, CA [27]) were linearized by digestion with MluI and BglII, respectively. The linearized plasmids were treated with calf intestinal alkaline phosphatase and end labeled with γ-[^{32}P]ATP and T4 polynucleotide kinase. Total cellular RNA was isolated from cultured cells by acid guanidinium thiocyanate-phenol-chloroform extraction (28). The end-labeled thrombomodulin and actin plasmids were hybridized overnight at 55°C with 50 or 5 μg of total cellular RNA, respectively. Hybridization conditions, nuclease S1 digestion, and analysis on denaturing PAGE were performed as described previously (26). Gels were analyzed by autoradiography, and the thrombomodulin and actin fragments were quantitated by direct radioanalytic imaging (Ambis Radioanalytic Systems, San Diego, CA).

Results

Thrombomodulin expression in normal human epidermis. To examine the expression of thrombomodulin in epidermis, immunohistochemical staining was performed on 13 biopsy specimens containing normal human skin. In all specimens examined, specific thrombomodulin staining was observed in a consistent pattern: no staining was detected in the basal layer of the epidermis, but strong staining was observed in keratinocytes of the suprabasal spinous layer. Thrombomodulin staining decreased in intensity progressively in the granular layer of keratinocytes, and was not detected in the cornified layer (Fig. 1 *A*). Staining was concentrated at the periphery of cells, suggesting that keratinocyte thrombomodulin is expressed primarily on the cell surface. As expected, endothelial cells of dermal capillaries also stained for thrombomodulin, although the intensity of staining was generally less than that of keratinocytes. No staining was observed when preimmune mouse serum was substituted for the antithrombomodulin antibody (Fig. 1 *B*). These results confirm that thrombomodulin is present in human epidermis, and suggest that thrombomodulin is selectively expressed early in keratinocyte differentiation.

Thrombomodulin expression in epidermal malignancies. To determine if thrombomodulin is also expressed by malignant human keratinocytes, immunohistochemical staining was performed on five cases each of invasive squamous cell carcinoma and basal cell carcinoma of the skin. Based on the degree of keratinization, three cases of squamous cell carcinoma were classified as well differentiated, one as moderately differentiated, and one as poorly differentiated (Table I). In all cases of squamous cell carcinoma, specific thrombomodulin staining was observed in both the neoplastic cells and in the adjacent normal epidermis. Within invasive nests of tumor cells, nonkeratinized peripheral cells stained strongly while central, more highly keratinized cells stained weakly or were negative (Fig. 1, *C* and *D*). Interestingly, a diffuse cytoplasmic pattern of staining was seen in the poorly differentiated squamous cell carcinoma, while the more differentiated neoplasms stained primarily in a membrane-specific pattern similar to that of normal epidermis (Fig. 1 *E*). In four cases of basal cell carcinoma, no thrombomodulin staining of neoplastic cells was seen, although specific thrombomodulin staining of vascular endothelial cells was observed (Fig. 1 *F*). In one case of
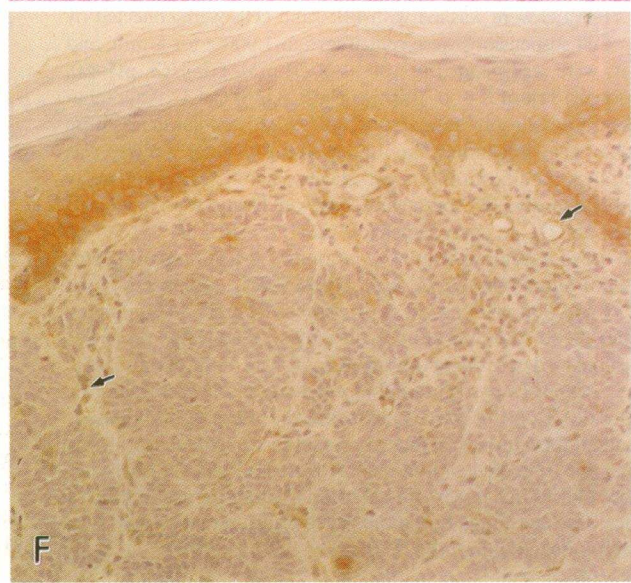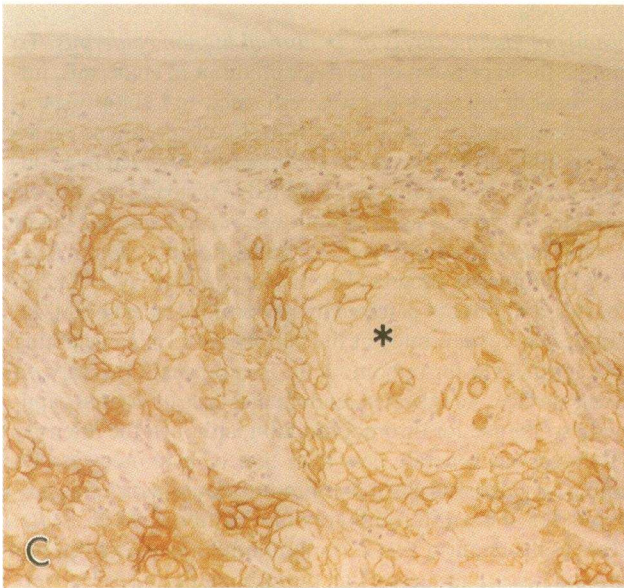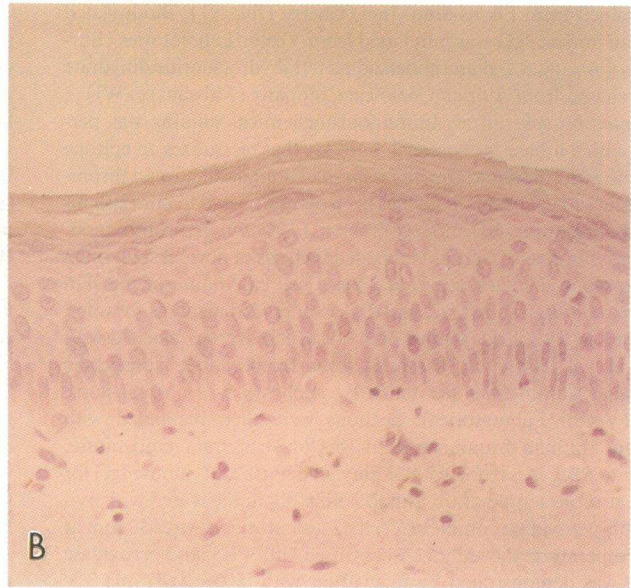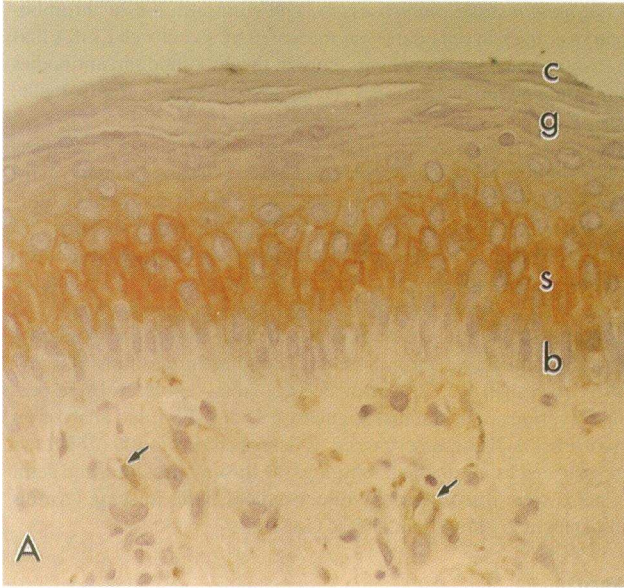
Table I. Immunohistochemical Staining
of Thrombomodulin in Squamous Cell Carcinomas (SCC)
and Basal Cell Carcinomas (BCC) of the Skin

Case	Diagnosis	Site	Keratinization	Thrombomodulin staining
			%	
1	SCC	Chest	90	+++
2	SCC	Temple	5	++
3	SCC	Submental	80	+++
4	SCC	Ear	30	+++
5	SCC	Neck	90	+++
6	BCC	Ear	0	−
7	BCC	Face	0	−
8	BCC	Preauricular	5	−*
9	BCC	Chin	0	−
10	BCC	Nose	0	−

* Weak (+) thrombomodulin staining was seen in areas of focal squamous differentiation.

Table II. Thrombomodulin Antigen
and Cofactor Activity in Cell Lysates

Lysate	Antigen*	Cofactor activity[‡]	Specific activity[§]
	μg/mg	pmol/h per mg	mol/h per mol
Keratinocytes	0.14±0.04	48±13	21±4
HUVEC	0.42±0.11	95±16	18±6

* Thrombomodulin antigen was measured by ELISA, and is expressed as micrograms of thrombomodulin per milligram of total protein. [‡] Thrombomodulin cofactor activity was measured in a protein C activation assay, and is expressed as picomoles of activated protein C generated per hour per milligram of total protein. [§] Specific activity is expressed as moles of activated protein C generated per hour per mole of thrombomodulin. Values represent the mean ± SD of five determinations for keratinocytes, and six determinations for HUVEC.

basal cell carcinoma, weak thrombomodulin staining of tumor cells was seen in areas of focal squamous differentiation (Table I). These results demonstrate that thrombomodulin expression correlates with squamous differentiation in both normal and neoplastic keratinocytes.

Thrombomodulin expression in cultured keratinocytes. To determine if the relationship between thrombomodulin expression and epidermal differentiation observed in vivo is reproduced in vitro, we measured the activity of thrombomodulin in primary cultures of human foreskin keratinocytes. Our initial studies were performed with keratinocytes cultured in serum-free medium containing 0.07 mM calcium chloride, a condition that inhibits squamous differentiation (22–24). The concentration of thrombomodulin antigen in keratinocyte lysates was determined by ELISA, and thrombomodulin cofactor activity was measured in a two-stage protein C activation assay. Compared with HUVEC lysates, keratinocyte lysates contained ∼ 50% lower amounts of both thrombomodulin antigen and cofactor activity (Table II). The specific activities of keratinocyte and HUVEC lysates were similar, however, indicating that thrombomodulin synthesized by proliferating human keratinocytes is comparable to endothelial cell thrombomodulin in its ability to promote protein C activation by thrombin.

We next measured thrombomodulin activity in differentiating keratinocytes. After keratinocytes were cultured to 80% confluency in the presence of 0.07 mM calcium chloride, the calcium chloride concentration of the medium was increased to 1.4 mM. This concentration of calcium has been shown to induce both structural and biochemical features of terminal differentiation in cultured keratinocytes (22–24). After incubation for 48 h in the presence of 1.4 mM calcium chloride, thrombomodulin cofactor activity increased by 3.5-fold (Fig. 2 A) and thrombomodulin antigen increased by 3.2-fold (Fig. 2 B). These results demonstrate that the correlation between thrombomodulin expression and squamous differentiation seen by immunohistochemistry in vivo is partially reproduced in calcium-treated keratinocytes.

To determine if the increase in cofactor activity observed during keratinocyte differentiation is associated with an increase in thrombomodulin mRNA, nuclease S1 protection assays were performed. Total cellular RNA was hybridized to radiolabeled human thrombomodulin and actin probes, digested with nuclease S1, and subjected to denaturing PAGE and autoradiography (Fig. 3). Thrombomodulin mRNA was readily detected in keratinocytes cultured in the presence of 0.07 mM calcium chloride (lane 2), and increased progressively after incubation for 24 and 48 h in medium containing 1.4 mM calcium chloride (lanes 3 and 4). These incubation conditions did not affect the quantity of actin mRNA. No protected fragments were seen when hybridizations were performed in the absence of RNA (lane 1). Quantitation by direct radioanalytic imaging revealed that thrombomodulin mRNA, normalized to actin mRNA, increased by 2.2- and 5.6-fold after keratinocytes were incubated with 1.4 mM calcium chloride for 24 and 48 h, respectively. This suggests that the increase in thrombomodulin in differentiating keratinocytes is mediated by an increase in thrombomodulin mRNA.

Discussion

The epidermis is a stratified squamous epithelium that differentiates continuously, replacing itself every 12–24 d (29). It is

Figure 1. Immunohistochemical localization of thrombomodulin in normal epidermis and epidermal malignancies. (A) Normal skin stained with mouse antithrombomodulin IgG (×250). Strong staining is seen in the spinous layer of epidermis (s), and in dermal capillary endothelial cells (arrows). No staining is seen in the basal layer (b), weak staining is observed in the granular layer (g), and no staining is seen in the cornified layer (c). (B) Normal skin stained with preimmune mouse serum (×250). No specific staining is observed. (C) Invasive squamous cell carcinoma stained for thrombomodulin (×150). Within invasive foci, strong staining of peripheral cells and weak or absent staining of central, highly keratinized cells is seen (*). (D) Invasive squamous cell carcinoma stained with hematoxylin and eosin (×150) showing focus of highly keratinized cells (*). (E) Higher magnification view of invasive squamous cell carcinoma stained for thrombomodulin reveals peripheral staining of individual cells (×450). (F) Basal cell carcinoma stained for thrombomodulin (×150). No staining of neoplastic cells is seen, but staining is observed in the spinous layer of overlying normal epidermis, and in vascular endothelial cells (arrows).
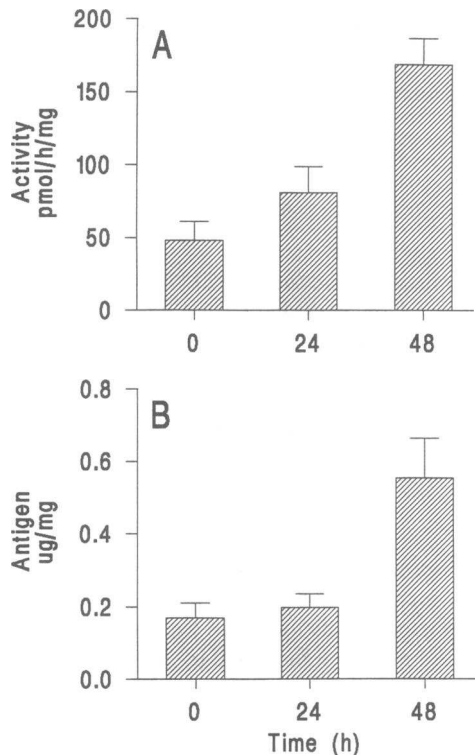
Figure 3. Induction of thrombomodulin mRNA in cultured keratinocytes. Nuclease S1 protection analysis of thrombomodulin (*TM*) and actin mRNA was performed with total cellular RNA isolated from keratinocytes incubated in KGM containing 1.4 mM calcium chloride for 0 (lane *2*), 24 (lane *3*), or 48 (lane *4*) h. In lane 1, digestion with nuclease S1 was performed in the absence of RNA.

Figure 2. Induction of thrombomodulin activity and antigen in cultured human keratinocytes. Keratinocytes were incubated for the indicated times in KGM containing 1.4 mM calcium chloride, and cell lysates were prepared as described in Methods. (*A*) The thrombomodulin cofactor activity of cell lysates was measured in a protein C activation assay, and is expressed as picomoles of activated protein C generated per hour per milligram of total protein. (*B*) Thrombomodulin antigen was measured by ELISA, and is expressed as micrograms of thrombomodulin per milligram of total protein. Values represent the mean±SD of five determinations. The values for thrombomodulin activity and antigen at 48 h are significantly increased compared with the corresponding values at 0 h (*P* < 0.01 by two-tailed Student's *t* test).

composed of a basal layer of mitotically active cells, and several suprabasal layers of postmitotic keratinocytes (29, 30). This study demonstrates that thrombomodulin is selectively expressed by keratinocytes of the spinous layer of epidermis, indicating that thrombomodulin expression is transiently induced when basal keratinocytes begin to terminally differentiate. Several other keratinocyte proteins, including the cytoskeletal keratins K1 and K10, are also selectively expressed by spinous cells (30).

The markedly stronger thrombomodulin staining of cutaneous squamous cell carcinomas compared with basal cell carcinomas indicates that thrombomodulin expression correlates with differentiation in malignant as well as normal keratinocytes. We have also detected thrombomodulin staining in stratified squamous epithelia of other tissues, including vagina, uterine ectocervix, esophagus, and oral mucosa, and in squamous metaplasia of nonstratified epithelia (data not shown). Therefore, thrombomodulin expression appears to be a consistent feature of squamous differentiation.

A correlation between thrombomodulin expression and keratinocyte differentiation was also observed in cultured keratinocytes. Stimulation of terminal differentiation by incuba-
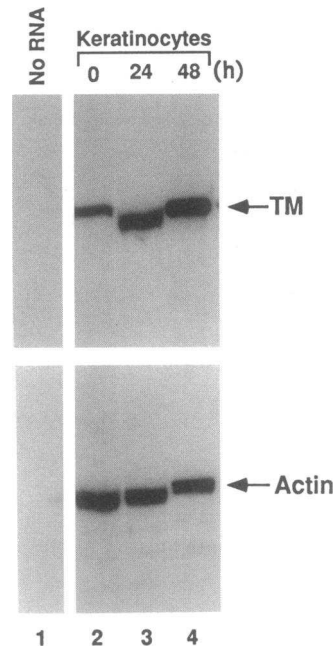
tion with 1.4 mM calcium chloride for 48 h produced proportionate increases in thrombomodulin cofactor activity and antigen, indicating that the specific activity of thrombomodulin did not change substantially during differentiation. A parallel increase in thrombomodulin mRNA was observed, suggesting that, in this system, the activity of keratinocyte thrombomodulin is regulated primarily by the level of thrombomodulin mRNA. Because the induction of thrombomodulin in vitro mimics the morphological pattern of thrombomodulin expression in vivo, this culture system should prove to be useful for future studies of the mechanisms of regulation of keratinocyte thrombomodulin.

Although the function of thrombomodulin within the epidermis is unknown, its ability to stimulate protein C activation by thrombin suggests that keratinocyte thrombomodulin may regulate wound healing. After cutaneous injury, primary hemostasis is achieved through formation of a platelet/fibrin plug. Subsequently, granulation tissue is produced, and an expansion of the basal layer of keratinocytes leads to reepithelialization of the wound (31). Thrombin has been proposed to enhance granulation tissue formation by stimulating mitogenic responses in fibroblasts, macrophages, and endothelial cells (32). Thrombin has also been reported to stimulate phosphoinositide hydrolysis in keratinocytes (33), and may promote keratinocyte proliferation during wound reepithelialization (32). Thrombomodulin produced by differentiating keratinocytes could regulate these cellular effects of thrombin by: (*a*) promoting protein C–mediated inhibition of thrombin production, and (*b*) directly competing for thrombin binding to signal-transducing thrombin receptors (34, 35). Regulation of thrombin activity by thrombomodulin within the epidermis may also modulate the function of cell-associated plasminogen activators (36), which have been proposed to facilitate keratinocyte migration during wound healing (37).

It is also possible that keratinocyte thrombomodulin functions independently of thrombin. Thrombomodulin is identical to the murine differentiation antigen fetomodulin, which may function in cellular differentiation during embryonic de-

velopment (14). In mouse embryos, this antigen is expressed by both vascular and nonvascular cells (14, 38). The extracellular sequence of thrombomodulin contains an amino-terminal domain that is homologous to the lectin-like domains of many cell adhesion molecules (2). This domain is not required for thrombomodulin cofactor activity (3), and its function remains obscure. It is tempting to speculate that this domain may mediate cellular interactions important in epidermal differentiation.

Acknowledgments

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