Human recombinant tissue inhibitor of metalloproteinases (rTIMP) at 0.2 – 4.6 μM was found to stimulate the growth of normal human keratinocytes, in primary cultures on a plastic support, and to markedly increase their growth on a tridimensional culture system, the skin equivalent, as shown by histology, DNA measurements, and planimetry. In contrast, rTIMP had no effect on the growth of normal human fibroblasts.

The growth of keratinocytes on extracellular matrix components produced by keratinocytes cultured in the presence or absence of rTIMP was similar, suggesting that rTIMP does not stimulate keratinocyte growth by modifying either the quantity or the composition of the extracellular matrix deposited.

rTIMP was labeled with 125I-iodine in order to study its interaction with keratinocytes in culture. Binding of 125I-rTIMP to keratinocytes was found to be temperature and time dependent. Under steady-state conditions at 22°C, one class of specific rTIMP binding sites was identified with a Kd of 8.7 nM and 135,000 sites/cell. Such findings are in keeping with the known potentiatating effect of TIMP on erythroid precursors, and indicate that this protein has at least two distinct activities. J Invest Dermatol 97:679–685, 1991

Evidence now available supports the view that proteinases and proteinase inhibitors are involved in the modulation of cell proliferation [1]. In a preliminary investigation [2], we examined the effect of proteinase inhibitors on epidermal cell proliferation and showed that several proteinase inhibitors, such as soy bean trypsin inhibitor and tosyllycine chloromethyl ketone, were without effect. In contrast, tissue inhibitor of metalloproteinases (TIMP) appeared to enhance keratinocyte growth both on a plastic support and on skin equivalents.

TIMP is a ubiquitous glycoprotein (Mr 28,500) that inhibits a number of extracellular matrix (ECM) metalloproteinase including interstitial collagenase, several type IV collagenases (gelatinases), and stromelysin [3 – 9]. Although keratinocytes produce ECM metalloproteinases (as do a variety of other cell types) they primarily synthesize interstitial collagenase [10,11] and the 92-kDa type IV collagenase (gelatinase) [8]. The ECM metalloproteinase gene family consists of a structurally related group of secreted enzymes that contain a putative zinc binding site [8]. When activated, these enzymes degrade the collagenous and non-collagenous connective tissue components. The interaction between TIMP and interstitial collagenase has been studied most extensively. Inhibition of active collagenase was shown to be stoichiometric, with a 1:1 molar ratio of inhibitor to enzyme being required for complete inhibition of enzyme activity [3]. Inhibition occurs by the formation of a very high affinity (Kd < 10^-9 M) noncovalent enzyme-inhibitor complex [6].

The ability of TIMP to regulate the activity of ECM metalloproteinases suggested that the physiologic function of this inhibitor involves the control of ECM degradation. It has now also been shown that a protein having erythroid potentiating activity [12] is identical in its amino acid sequence to TIMP [13,14]. This finding, together with the above-mentioned effect of TIMP on epidermal cell growth, prompted us to investigate further its general influence on human skin cell proliferation.

In the present investigation, we demonstrate that TIMP stimulates keratinocyte growth under various culture conditions, but that it has no significant effect on the proliferation of human skin fibroblasts. Its proliferative effect would appear to result from an interaction with the keratinocyte membrane and appears unrelated to its function as a metalloproteinase inhibitor.

MATERIALS AND METHODS

Cell Cultures on Plastic

Keratinocyte Cultures: For keratinocyte growth assays, human keratinocytes were obtained from breast plastic surgery. Primary cultures were established according to the usual procedure. Briefly, skin was cleaned of excess deep dermis and subcutaneous fat, cut into thin pieces, rinsed in calcium free phosphate-buffered saline (PBS), and incubated in 0.1% trypsin (Boehringer Mannheim) in calcium-free PBS overnight at 4°C. The epidermis was then separated from the dermis with forceps and incubated for 15 min in...
McKeehan solution (Eurobio Laboratories) at 37°C. The undisguised pieces of epidermis were discarded and epidermal cells were maintained in Earle's Modified Eagle's medium (EMEM) (Flow Laboratories) supplemented with antibiotics and 10% fetal calf serum (FCS), and incubated at a seed density of 1.6 × 10⁶ cells per cm² on Petri dishes (35 mm) or on 12-well culture plates (Costar). After 24 h, the medium was changed to EMEM supplemented with antibiotics, and 10% FCS, 20 ng/ml EGF, 0.4 μg/ml hydrocortisone, and 8.4 ng/ml cholera toxin, with or without 0.5 – 100 μg/ml rTIMP. Culture medium was changed three times weekly and replaced by fresh culture medium with or without rTIMP, as appropriate. The cultures were studied for growth parameters just before the stationary phase was reached, i.e., at pre-confluence, which occurred between days 5 and 12, depending on the keratinocyte donor.

**Fibroblast Cultures:** For fibroblast growth assays, cells obtained from adult breast skin were used between the fifth and ninth passages and seeded at a density of 5 × 10⁵ cells/cm². Twenty-four hours after plating, the medium was changed to EMEM supplemented with antibiotics and 10% FCS, with or without 0.5 to 100 μg/ml rTIMP. The cultures were studied for growth parameters just before the stationary phase was reached, i.e., at pre-confluence, which occurred between days 3 and 5, according to the fibroblast strain.

**Cell Growth Evaluation** Cell growth was determined by removing the medium and detecting the cells with 0.05% trypsin solution in calcium-free PBS. The viable cells were counted by trypan blue exclusion on a hemocytometer.

**Skin Equivalent Cultures**

**Dermal Equivalent:** Normal human breast tissue was obtained from breast plastic surgery and used to establish skin fibroblast cultures. These cells were used in preparing dermal equivalents [15]. Fibroblasts from confluent or subconfluent culture were removed by trypsin treatment and suspended at 4 × 10⁶ cells/ml in EMEM, containing 10% FCS, 100 units of penicillin and 100 µl of streptomycin per ml (Flow Laboratories), and 2.5 µg/ml amphotericin B (Squibb). The dermal equivalents were prepared as previously described [16,17]. Briefly, 6.2 ml of concentrated EMEM with antibiotics, 1.8 ml of FCS, 1.0 ml of human placental collagen solution at 3 mg/ml in distilled water (kindly provided by Merieux), and 2.0 ml of fibroblast suspension were mixed together and quickly poured into 90-mm bacteriologic Petri dishes, then placed in a 95% air:5% CO₂ atmosphere at 37°C. A gel formed immediately.

**Epidermalization:** Two-milliliter punch biopsies were taken from breast tissue, and each biopsy inserted epidermis up into the center of a freshly cast dermal equivalent. Gel contraction keeps the biopsy firmly in place. After 3 d, culture medium containing an equal volume of EMEM supplemented with antibiotics, and 10% FCS, with 20 ng/ml of EGF (Collaborative Research), 0.4 μg/ml of hydrocortisone (Roussel), 8.4 ng/ml of cholera toxin (Sigma) with or without rTIMP at a final concentration of 1 or 5 μg/ml was added.

The recombinant TIMP used in these experiments was purified from *Escherichia coli* to homogeneity as described [4,18] and yielded a single band of approximately 20 kDa, on sodium dodecyl sulfate polyacrylamide gel electrophoresis, characteristic of unglycosylated rTIMP. Monospecific anti-rTIMP antibody produced a single band on Western blot analysis using either recombinant or native TIMP (not shown). Recombinant TIMP, although unglycosylated (Mr 21,500), is fully active as a metalloproteinase inhibitor, functioning in a manner identical to the pure native protein from human skin fibroblasts [13,14,18].

All assays were carried out in triplicate. The culture medium was changed twice a week. At 8 d, the dermal equivalents were placed on stainless steel grids to maintain the surface of the skin equivalent at the air-medium interface. On day 17, the skin equivalents were separated into two groups; one was examined histologically and the other processed for evaluation of epidermal growth.

**Histology:** Semi-thin sections were prepared following fixation in Karnovsky's medium, post fixed, dehydrated, embedded in epon, and sectioned as previously described [19]. Cellular parameters were studied on microscopic slides by automated image analysis [20].

**Measurement of Epidermal Outgrowth Area:** To visualize the area of keratinocyte outgrowth over the surface of the dermal equivalent, the cultures were stained with Nile Blue sulfate 1:10,000 (Sigma) for 30 min, and rinsed for 15 min in a 0.5% NaCl solution. The area of epidermal outgrowth was measured on photographs with a Nackert NS 1000 image analyzer.

**Measurement of Epidermal DNA Content:** After Nile Blue sulfate staining, the punch biopsy was discarded and the epidermal outgrowths were gently removed from the dermal equivalent with forceps. Each epidermal outgrowth was hydrolyzed in 500 µl of a solution composed of 2% Triton X-100 and 4 N NH₄OH (v/v) for 1 h at 100°C. DNA content was assayed using a fluorimetric procedure [21]. Purified fetal calf thymus DNA (Sigma) was used as a standard and hydrolyzed Nile Blue sulfate solution as a control.

**Preparation of Keratinocyte Extracellular Matrix (ECM) proteins:** ECM proteins were elaborated by keratinocytes cultured in the presence or absence of rTIMP. These ECM proteins adhered to the culture wells (kECM). Further characterization of the ECM proteins produced by keratinocytes was not undertaken here because it has been shown previously [22,23] that keratinocyte ECM contains laminin, fibronectin, glycoprotein, and type IV collagen. The keratinocytes were removed with 0.5% Triton x-100, followed by 0.025 N NH₄OH as described [24,25] and a fresh primary culture of keratinocytes was seeded (1.6 × 10⁶ cells/cm²) on the coated wells. Controls were primary cultures of keratinocytes seeded on plastic wells. All assays were performed in triplicate. Just prior to reaching confluence, these cultures were stopped and the keratinocyte growth evaluated by cell count.

**Statistical Analysis** For each culture condition the experiments, performed in triplicate, were compared by Student t test for unpaired values.

**Binding of (125I)rTIMP to Human Skin Keratinocytes** The rTIMP was iodinated using the lactoperoxidase method ([125]I from Amersham, France) as described [26] to a specific activity of 2–3.7 × 10⁶ cpm/µg. Binding assays were performed in 24-well culture plates with fibroblasts or keratinocytes grown to confluence as previously described [27,28]. Primary cultures of keratinocytes were obtained, seeded, and fed in epidermal culture medium as outlined above. All experiments were performed 2–4 d after the last feeding. Just prior to binding assays, culture medium was aspirated and cells were washed twice with EMEM containing 0.1% bovine serum albumin (BSA). Labeled and unlabeled rTIMP were diluted in the same medium. Cell number was determined in duplicate wells by Coulter counter and confirmed by hemocytometer.

To determine non-specific binding, excess unlabeled rTIMP (100 µg/ml/well) was added to one well of each experimental point for 30 min and then ([125]I)rTIMP was added to all wells in a final 1-ml volume of EMEM containing 0.1% BSA.

To determine the effect of time and temperature on ([125]I)rTIMP binding, cells were incubated for various times up to 20 h with 150 ng/ml/well of ([125]I)rTIMP at 22°C or 4°C.

To determine equilibrium binding of ([125]I)rTIMP to cells at 22°C, the binding assays were performed using three wells per point and at various concentrations of ([125]I)rTIMP (5–2500 ng/ml/well) after 6-h incubation.

The assays were terminated by removing the binding solution and the cells were then washed twice with 2 ml of phosphate-buffered saline (PBS). The cells were then solubilized in 1 ml of 0.1% SDS in 0.1 M NaOH and radioactivity counted in a gamma scintillation counter at 47% efficiency.
Figure 1. Influence of rTIMP on keratinocyte and fibroblast growth. Primary cultures of normal human keratinocytes were seeded at 1.6 × 10⁶ cells/cm² on plastic dishes. Cells were cultured in EMEM containing 10% FCS, EGF, cholera toxin, and hydrocortisone, in the presence or absence of 10 µg/ml of rTIMP (rTIMP) was added 24 h after plating. Medium was changed three times a week and replaced by fresh culture medium with or without rTIMP, as appropriate. The cultures were studied for growth parameters before the stationary phase was reached, i.e., at preconfluence that occurred between days 5 and 12, according to the keratinocyte donor. Human skin fibroblasts from confluent or subconfluent cultures were used between the seventh and the ninth passages and seeded at 5 × 10⁶ cells/cm² in plastic flasks. Cells were cultured in EMEM containing 10% FCS in the presence or absence of 10 µg/ml of rTIMP (TIMP) was added 24 h after plating. The cultures were studied for cell growth evaluation before the stationary phase was reached, i.e., at preconfluence that occurred between days 3 and 5 according to the fibroblast strain. For each culture condition, the mean cell number from three samples was calculated. For each experiment, the rTIMP-treated and control number of cells were compared by Student t test for unpaired values. * Represents significant p values of (0.001 < p < 0.05). Control culture, □, TIMP-treated culture, ■.

Analysis of Binding Data: Each point represents the specific binding; best fit-curves were determined using an iterative non-linear regression program with a Gauss-Newton algorithm, according to Urien [29].

RESULTS

Effect of rTIMP on Cultured Keratinocytes and Fibroblasts

When keratinocytes were cultured on plastic dishes in medium supplemented with 10 µg/ml (0.46 µM) of rTIMP, a significant stimulation of cell growth was observed. The stimulatory effect of rTIMP ranged from 129–143% (Fig 1), depending on the keratinocyte cell strain, and was significant only during the non-confluent state of the culture. The increase in keratinocyte growth was induced at a concentration of rTIMP as low as 1 µg/ml (two of three strains studied); although all strains exhibited the effect at a 10 µg/ml concentration of rTIMP, no direct correlation between the increased amount of rTIMP added to the culture medium and the extent of keratinocyte growth could be observed. Importantly, when rTIMP was inactivated by reduction and carboxymethylation, the denatured protein was no longer capable of stimulating the growth of normal human keratinocytes in primary culture (data not shown).

Except for one fibroblast strain that showed a slight stimulation effect (127%) when cultured in the presence of 10 µg/ml of rTIMP, rTIMP did not significantly influence fibroblast growth (Fig 1).

Effect of rTIMP on Epidermal Growth in Skin Equivalents

Supplementation of the skin equivalent growth medium with rTIMP had an even more pronounced effect on epidermal growth. Enhanced proliferation of keratinocytes cultured on dermal equivalents was clearly evident at a concentration of rTIMP of 5 µg/ml (0.23 µM) and was associated with an increase in epidermal thickness after 17 d of culture (Fig 2 a, b).

By day 14 of culture, epidermal outgrowth was easily quantified by planimetry and by determination of the DNA content. Although both parameters were enhanced at an rTIMP concentration of 1 µg/ml (0.046 µM), significant increases compared to controls were observed only when rTIMP was used at a concentration of 5 µg/ml (Fig 3 a, b). The epidermal area and the epidermal DNA content increased 1.7 and 3.4 times, respectively, when 5 µg/ml rTIMP was added to the culture medium twice a week for 17 d.

The ratio of epidermal DNA content to the area of epidermal outgrowth, which is representative of the thickness of newly formed epidermis, was increased twofold in skin equivalents supplemented by 5 µg/ml of rTIMP (Fig 3c), confirming the morphologic observations. The increase in both cell numbers and cell layers was

Figure 2. Effect of rTIMP on epidermal growth in a living dermal equivalent; semi-thin sections of (a) control and (b) TIMP-treated skin equivalents (5 µg/ml) after 17 d of culture. Epidermalization was obtained from a 2-mm punch biopsy inserted into a human collagen matrix containing living fibroblast (DE), cultured in EMEM, 10% FCS, EGF, cholera toxin, and hydrocortisone in the presence or absence of 1 or 5 µg/ml of rTIMP. Note the area of dermal-epidermal separation in control sections (open arrow). There is a marked increase in the number of epidermal cell layers in TIMP-treated epidermis as compared to the control. Keratinocyte in mitosis (closed arrow). Bar, 20 µm, magnification ×430.
Figure 3. Effect of rTIMP on epidermal growth in a living dermal equivalent; quantitation of epidermal outgrowth after 17 d of culture. (a) Epidermal outgrowth area measured by planimetry, (b) Epidermal DNA content determined fluorimetrically, and (c) ratio of the epidermal DNA content/epidermal area giving an index of epidermal thickness. Supplementing the culture medium with 5 μg/ml of rTIMP (TIMPS) significantly increased the epidermal area and DNA content of the dermal equivalent confirming the histologic observations. TIMP1 and TIMP5 refer to cultures containing 1.0 and 5.0 μg/ml of rTIMP, respectively. Bar, ± standard error of the mean of the three samples. Comparison by Student t test for unpaired values: (a) for TIMPS, p = 0.02, (b) for TIMPS, p = 0.02, (c) for TIMPS, p = 0.11.

confirmed by automated image analysis (data not shown). These changes were also accompanied by modifications of cell parameters in the second and third layers relative to the controls cultured without rTIMP. In particular, the keratinocyte surface area was diminished twofold in rTIMP-treated skin equivalents together with a marked increase in the form factor (represents the morphologic evolution of the cell): i.e., 0.18 ± 0.08 for control and 0.42 ± 0.15 for rTIMP. In contrast, no modifications of cell parameters were observed between control and rTIMP-treated skin equivalents in the first cell layer. In addition, keratinocytes in the second and third cell layers appeared to be oriented perpendicular to the dermal-epidermal junction in skin equivalents cultured in the presence of rTIMP.

Influence of rTIMP on kECM Protein Production

Because the proliferative effect of rTIMP on keratinocytes might be related to its role in regulating the metabolism of ECM proteins elaborated by keratinocytes, we investigated whether proteins deposited by keratinocytes could be modified by rTIMP to further influence cell growth.

The growth rates of keratinocytes cultured on plastic or seeded on proteins deposited by keratinocytes were compared. Although human keratinocytes proliferate at a higher rate when cultured on ECM proteins produced by keratinocytes compared to cells cultured on plastic, no differences were observed in proliferation of keratinocytes grown on keratinocyte ECM proteins deposited in the presence or absence of rTIMP (Fig 4). Furthermore, only in late culture could low levels of latent proteolytic activity capable of limited degradation of this substrate be detected in the medium (data not shown).

Binding of (125I)rTIMP to Cultured Keratinocytes

The association of (125I)rTIMP and cultured keratinocytes was found to be time and temperature dependent. Although the association of the inhibitor with these cells proceeds at a higher rate at physiologic temperature compared to experiments performed at 22°C or 4°C, it could be complicated by secondary metabolic events such as internalization and degradation of the protein [27,28]. Therefore, in order to study the binding reaction itself, the association was studied at 22°C and at 4°C to eliminate these possible variables. Binding equilibrium between rTIMP and keratinocytes was reached at 22°C after 6–8 h; the kinetics of association was significantly reduced at 4°C, and 12–16 h of incubation was necessary to reach equilibrium (Fig 5).

Under steady-state conditions, the specific binding of (125I)rTIMP to cultured keratinocytes (i.e., total binding minus binding obtained in the presence of an excess of unlabeled inhibitor [100 μg/ml/well]) is shown in Fig 6a. When data were analyzed according to Urien [29], one class of high-affinity binding sites with Kd of 8.7 × 10^-8 M and 135,000 sites per cell was found (Fig 6b).

DISCUSSION

We have shown that rTIMP (0.02–4.65 μM) is capable of effectively stimulating the growth of normal human keratinocytes in primary cultures. In contrast, the growth of normal human skin fibroblasts was not significantly affected by rTIMP. In order to determine whether the effect of rTIMP on keratinocyte proliferation occurs only when cells are seeded on an inert support or also occurs in a tridimensional system, we studied the effect of rTIMP on the growth of keratinocytes cultured on living dermal equivalents (consisting of fibroblasts and matrix components). The skin equivalent, a tridimensional system described by Bell and associates [15] that we have modified slightly, has had wide pharmacologic and therapeutic application [16,17]. In this culture system, rTIMP at a concentration equal to or greater than 0.2 μM was able to strongly modify epidermal growth; a 3.4-times enhancement of keratinocyte proliferation, as determined by DNA content, and a twofold overall increase in epidermal thickness as quantitatively evaluated by planimetry and DNA measurements, was noted by day 17 of culture. Automated image analysis confirmed these results, in terms of an enhancement of both cell numbers and cell layers. Moreover, the comparison of cellular parameters, (cell perimeter, cell surface area, form factor, equivalent diameter), in the second and third layers of rTIMP-treated and untreated skin equivalents indicate that rTIMP may influence keratinocyte morphogenesis. The concentration of rTIMP used was equivalent to the mean value found in skin blisters [30] and exceeded the amount normally secreted by cultured keratinocytes but not by human fibroblasts [4].

In order to insure a basal growth of keratinocytes, it is necessary to supplement the culture medium of cells with factors known to influence cell proliferation, but because pure recombinant TIMP was used throughout these experiments, contamination of the prepa-
ration with an excess of growth factors does not occur and, therefore, is not responsible for the observed effect. We initially hypothesized that supplementation of keratinocyte culture medium with rTIMP might inhibit the degradation of ECM proteins produced by keratinocytes. This degradation could be catalyzed, in part, by ECM metalloproteinases secreted by growing cells [8,10,11,31,32] and thus influence keratinocyte growth. The concentration of rTIMP used can inhibit the low level of collagenase activity (and other metalloproteinases) constitutively secreted by the cells [33,34]. However, because supplementing the culture medium of keratinocytes with rTIMP does not modify the amount of keratinocyte ECM macromolecules deposited and as only low levels of latent ECM metalloproteinase activity were present in keratinocyte culture medium, we concluded that the growth-enhancing effect of TIMP on keratinocytes appears to be unrelated to its major function in the regulation of ECM degradation. We cannot exclude the possibility that rTIMP acts by binding to a plasma membrane matrix metalloproteinase that would interact with cells via its hemopexin-like domain [35].

It should also be noted that a second 21-kDa tissue inhibitor of metalloproteinases, TIMP-2, has recently been isolated from a variety of tumor cells and normal fibroblasts [36,37]. TIMP-2 and TIMP share significant homology in their amino acid sequences, although TIMP-2 does not cross-react with TIMP-specific antibodies. This inhibitor appears to show a selective affinity for pure, latent 72-kDa type IV procollagenase (gelatinase), which exists in a stable but noncovalent stoichiometric complex with TIMP-2. In contrast, TIMP binds only the active form of interstitial collagenase [6], but, interestingly, will also complex noncovalently with the 92-kDa type IV procollagenase (gelatinase) [8]. When sufficient quantities of the protein become available, it will be of interest to determine whether TIMP-2 can also stimulate keratinocyte growth.

Proteinase inhibitors are known to affect several cell functions that include: mitosis, motility, and proliferation [1]. Cystatin C (a thiol proteinase inhibitor), and human pancreatic secretory trypsin inhibitor (a serine proteinase inhibitor that also shares sequence homology with EGF), were both shown to enhance the mitotic response in 3T3 fibroblast [38-40]. In contrast, α1 proteinase inhibitor and soybean trypsin inhibitor, both serine proteinase inhibitors, have been shown to inhibit mitosis in lymphocytes [41]. In some instances, as with cystatin C, the target cell was shown to exhibit cell-specific receptors, which when occupied could trigger a series of secondary signals and gene expression [42]. Of importance is the observation that the structure of TIMP is identical to that of a protein having erythroid potentiating activity (EPA) [12], indicating that this single protein has at least two distinct activities [13,14]. EPA specifically stimulates the growth of human and murine peripheric blood- and bone-marrow-derived erythroid precursors, and it has been suggested [43] that cell growth may be modulated through EPA-specific cell-surface receptors.

Recombinant TIMP was therefore labeled with 125Iiodine and its interaction with cultured keratinocytes studied. Such labeling conditions did not modify the inhibitory capacity of TIMP [44]. The inhibitor binds in a saturable fashion to these cells, 106 keratinocytes being saturated with 14 ng (0.68 pmoles) of rTIMP at 22°C. It should be emphasized, however, that TIMP is constitutively produced by these cells in relatively large amounts (26–64 ng/106 cells [4,11,31]) and further occupation of cell-binding sites by the endogenous inhibitor could give a falsely low value for a saturation effect. Occupation of cell-binding sites by endogenous inhibitor could be partly responsible for the variation in the proliferative responses observed with different cell strains. It should also be noted that the experiments were performed in serum-free medium at 4°C or 22°C, conditions that virtually exclude the contribution of any cell-secreted proteins in this interaction, particularly ECM metalloproteinases, such as collagenase, and the further binding of rTIMP-collagenase complexes.

We have shown one class of specific rTIMP binding sites with a Kd of 8.7 nM. The specific rTIMP binding sites on keratinocytes are within the range of affinities reported for the EPA-specific cellsurface receptors on human and murine erythroid precursors, although the number of sites per cell is much higher [43]. The difference in the degree of differentiation between keratinocytes and blood- or bone-marrow-derived erythroid precursors could explain this discrepancy. At 22°C, rTIMP also bound to human skin fibroblasts but with an affinity threefold lower, possibly explaining the much lower growth-inducing effect of rTIMP on this cell type. Whether occupation of such TIMP binding sites may trigger sec-

Figure 5. Time course for binding of 125I-rTIMP to human adult keratinocytes at 22°C and 4°C. Cells were grown to confluence in 24-well plates and then washed with PBS. The cells were solubilized with 1 ml of 1% SDS in 0.1 M NaOH and counted in a gamma counter. Each point represents an average of triplicate determinations. Non-specific binding was 30–40% at both temperatures.

Figure 6. Steady-state binding of 125I-rTIMP to cultured human epidermal cells. At 22°C, standard binding assays were performed (four wells assayed for each concentration point) using various concentrations of 125I-rTIMP in the presence or absence of unlabeled rTIMP (100 μg/ml, well) after 6-h incubation. Specific binding was determined by subtracting non-specific binding from total binding. a: Binding curve showing saturation of binding (specific activity of 125I-rTIMP 2300 cpm/ng, wells contained 3.15 × 10^6 ± 0.15 × 10^6 cells/well). b: Inset shows a scatchard plot of the binding data.
ondary signals involved in the up or down regulation by growth factors is currently under investigation.

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REFERENCES


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