Requirement for Transglutaminase in the Activation of Latent Transforming Growth Factor-β in Bovine Endothelial Cells

Soichi Kojima,* Kiyomitsu Nara,§ and Daniel B. Rifkin,*‡

*Department of Cell Biology and Kaplan Cancer Center, New York University Medical School; ‡Raymond and Beverly Sackler Foundation Laboratory, New York, NY 10016; and §Department of Biological Sciences, Tokyo Institute of Technology, Ookayama, Meguroku, Tokyo 152

Abstract. A hitherto unknown function for transglutaminase (TGase; R-glutaminyl-peptide: amine γ-glutamyltransferase, EC 2.3.2.13) was found in the conversion of latent transforming growth factor-β (LTGF-β) to active TGF-β by bovine aortic endothelial cells (BAECs). The cell-associated, plasmin-mediated activation of LTGF-β to TGF-β induced either by treatment of BAECs with retinoids or by cocultures of BAECs and bovine smooth muscle cells (BSMCs) was blocked by seven different inhibitors of TGase as well as a neutralizing antibody to bovine endothelial cell type II TGase. Control experiments indicated that TGase inhibitors and/or a neutralizing antibody to TGase did not interfere with the direct action of TGF-β, the release of LTGF-β from cells, or the activation of LTGF-β by plasmin or by transient acidification. After treatment with retinoids, BAECs expressed increased levels of TGase coordinate with the generation of TGF-β, whereas BSMCs and bovine embryonic skin fibroblasts, which did not activate LTGF-β after treatment with retinoids, did not. Furthermore, both TGase inhibitors and a neutralizing antibody to TGase potentiated the effect of retinol in enhancing plasminogen activator (PA) levels in cultures of BAECs by suppressing the TGF-β-mediated enhancement of PA inhibitor-1 (PAI-1) expression. These results indicate that type II TGase is a component required for cell surface, plasmin-mediated LTGF-β activation process and that increased expression of TGase accompanies retinoid-induced activation of LTGF-β.

TISSUE type II transglutaminase (TGase; R-glutaminyl-peptide: amine γ-glutamyltransferase, EC 2.3.2.13) is a member of the TGase family that catalyze Ca^{2+}-dependent acyl transfer reactions between γ-carboxamide groups of the glutamine residues in peptides and either primary amines or ε-amino groups of the lysine residues in peptides, resulting in the formation of new γ-amides of glutamic acid or ε-(γ-glutamyl)lysine bonds and ammonia (Folk, 1980; Lorand and Conrad, 1984; Greenberg et al., 1991). The molecular structure of type II TGase has been reported (Ikura et al., 1988; Gentile et al., 1991; Nakanishi et al., 1991). Type II TGase has a wide distribution; it is found in liver (Folk, 1980; Ikura et al., 1988), epidermal cells (Lichti et al., 1985), erythrocytes (Signorini et al., 1988), macrophages (Chiocca et al., 1989; Gentile et al., 1991), and many cancer cells including HL-60 myeloid cells (Chiocca et al., 1989). Furthermore, various organs express type II TGase (Folk, 1980) due to its occurrence in ubiquitous cell types such as endothelial cells (ECs), smooth muscle cells (SMCs), and certain perivascular fibroblasts (Kojima et al., 1987; Greenberg et al., 1987; Korner et al., 1989; Thomázy and Fésüs, 1989; Gentile et al., 1991). Whereas physiological roles of two types of TGases have definitively been described, e.g., the formation of cross-linkages between fibrin molecules by plasma Factor XIIIa (Lorand and Conrad, 1984; Greenberg et al., 1991) and the formation of cross-linked envelopes during epidermal cell differentiation by tissue type I TGase (Thacher and Rice, 1985), the physiological role of type II TGase has not yet been established except for the formation of cross-links in erythrocyte membrane proteins (Folk, 1980; Lorand and Conrad, 1984). One putative role for type II TGase is an involvement in the regulation of cell growth and differentiation (Birckbichler and Patterson, 1978; Chiocca et al., 1989; Suedhoff et al., 1990). This hypothesis was derived from the observation that when cellular TGase levels are high, cell growth is suppressed and/or cell differentiation is induced. In some of the experiments designed to test this hypothesis, cells were treated with retinoids, compounds that have profound effects on the regulation of cell growth and differentiation (Roberts and Sporn, 1984), to increase TGase levels.

Recently, retinoids were also shown to increase the production of active TGF-β in osteoclasts (Oreffo et al., 1989), keratinocytes (Glick et al., 1989), and ECs (Kojima and Rifkin, 1993). The TGF-β family consists of a number of related, but functionally distinct, 25-kD homodimers

Please address all correspondence to Dr. D. B. Rifkin, Department of Cell Biology, New York University School of Medicine, 550 First Avenue, New York, NY 10016.

(Barnard et al., 1990; Roberts and Sporn, 1990). In humans, three subtypes (TGF-β1, -β2, and -β3) have been isolated (Roberts and Sporn, 1990). TGF-β has potent regulatory effects on both cell growth and differentiation. It can either promote or inhibit cell growth depending upon the cell type and culture conditions (Barnard et al., 1990; Roberts and Sporn, 1990). TGF-β also has been reported to increase type II TGase levels in normal human keratinocytes (George et al., 1990). One cell type whose growth is inhibited by TGF-β is ECs. TGF-β inhibits EC proliferation, migration, and, in bovine aortic ECs (BAECs), the production of plasminogen activator (PA), whereas it stimulates the production of PA inhibitor-1 (PAI-1) and cell matrix components such as collagen and/or fibronectin (Rifkin et al., 1991). A critical step in the regulation of TGF-β action is the activation of the latent molecule. TGF-β is synthesized and secreted by most normal and neoplastic cell types, including ECs, in a biologically latent, high molecular weight form (LTGF-β) that can not bind to TGF-β receptors (Barnard et al., 1990; Roberts and Sporn, 1990). LTGF-β consists of a noncovalent complex in which the 25-kD homodimeric active TGF-β is noncovalently associated with a dimer of its precursor propeptide called the latency associated peptide (LAP: 75-kD) based on its ability to sustain latency (Roberts and Sporn, 1990). LAP, in turn, is disulfide-bonded to a second, structurally unrelated protein of 135~180-kD called LTGF-β binding protein (Kanzaki et al., 1990). The nature of the activation mechanism of LTGF-β in vivo is unclear. In vitro, LTGF-β is activated by transient acidification (pH 2) or alkalization (pH 12), which probably disrupts the noncovalent interactions between active TGF-β and LAP, releasing the TGF-β molecule (Barnard et al., 1990; Roberts and Sporn, 1990), or by proteases, specifically plasmin, which may cleave LAP within its aminoterminal region and release active TGF-β (Lyons et al., 1988, 1990). Recently, our laboratory and the laboratory of P. D'Amore have described the activation of LTGF-β under physiological conditions in cocultures of ECs with either pericytes or SMCs (Sato and Rifkin, 1989; Antonelli-Orlidge et al., 1989). Both cell types were shown to produce LTGF-β and activation appeared to require cell–cell contact. Activation requires plasmin formed from serum plasminogen by PA (Sato and Rifkin, 1989; Sato et al., 1990; Kojima et al., 1991). The activation reaction occurs on the cell surface or matrix. The LTGF-β is localized at the cell surface by binding to the mannose 6-phosphate (M6P)/insulin-like growth factor-II receptor through M6P-containing carbohydrates in the LAP (Dennis and Rifkin, 1991), and through interaction with the LTGF-β–binding protein (Flaumenhaft et al., 1993).

Retinoid-induced LTGF-β activation in ECs is dependent upon plasmin as in the case of cocultures (Sato and Rifkin, 1989), and, indeed, retinoids increase surface PA/plasmin levels. However, the mechanism for LTGF-β activation by retinoids can not be ascribed solely to enhanced levels of PA/plasmin since a direct relationship between PA production and LTGF-β activation has not been observed. This implies the potential existence of an additional component(s) required for LTGF-β activation and whose level in ECs is upregulated by retinoids. Since another property of retinoids is their ability to increase expression of cellular type II TGase (Lichti et al., 1985; Kojima et al., 1987; Chiocca et al., 1989; Nara et al., 1989; Suedhoff et al., 1990), we have tested whether TGase is involved in the activation of LTGF-β in ECs. Here, we demonstrate that type II TGase is required for LTGF-β activation induced by retinoids as well as by cocultures of ECs with SMCs. The requirement for TGase in TGF-β formation is a hitherto unknown role of this enzyme and may explain the relationship between retinoids, TGase, and cell growth.

Glossary

αMEM	α modified MEM
αMEM-BSA	α MEM containing 0.1% BSA
αTGaseIgG	antitransglutaminase antibody
αTGF-βIgG	antitransforming growth factor-β antibody
BAECs	bovine aortic endothelial cells
BESFs	bovine embryonic skin fibroblasts
BSMCs	bovine smooth muscle cells
CM	conditioned medium
CS	calf serum
DAPBT	2-[3-(diallylamino)propionyl]benzothiophene
DMDC	dimethylated dansylcadaverine
ECs	endothelial cells
L682777	1,3,4,5-tetramethyl-2[(2-oxopropyl)thio]imidazolium chloride
L683685	1,3-dimethyl-4,5-diphenyl-2[(2-oxopropyl)thio]-imidazolium trifluoromethylsulfonate
L683696	1,3-dimethyl-4-phenyl-2[(2-oxopropyl)thio]imidazolium chloride
LAP	latency associated peptide
LTGF-β	latent transforming growth factor-β
PA	plasminogen activator
SMCs	smooth muscle cells
TGase	translutaminase

Materials and Methods

Materials

All-*trans*-retinol, retinoic acid, bacitracin, cystamine, monodansylcadaverine, and BSA were purchased from Sigma Chem. Co. (St. Louis, MO). Stock solutions of retinoids were prepared every 2 wk in ethanol and stored at −20°C under nitrogen gas. Stock solutions were serially diluted into culture medium to yield a final ethanol concentration of 0.5%. This concentration of ethanol did not affect the production or activation of LTGF-β (data not shown). 2-[3-(diallylamino)propionyl]benzothiophene (DAPBT) and dimethylated dansylcadaverine (DMDC) were generous gifts from Dr. Laszlo Lorand (Northwestern University, Evanston, IL). The inhibitors, 2[(2-oxopropyl)thio]imidazolium derivatives: 1,3,4,5-tetramethyl-2[(2-oxopropyl)thio]imidazolium chloride (L68277), 1,3-dimethyl-4,5-diphenyl-2[(2-oxopropyl)thio]imidazolium trifluoromethylsulfonate (L683685), and 1,3-dimethyl-4-phenyl-2[(2-oxopropyl)thio]imidazolium chloride (L683-696) were kindly supplied from Dr. Andrew M. Stern (Merck Sharp & Dohme Research Laboratories, West Point, PA). Recombinant human TGF-β1 (rTGF-β1) was a generous gift from Genentech, Inc. (South San Francisco, CA). Antibody against porcine TGF-β (αTGF-β IgG), which has previously been shown to neutralize porcine, human, and bovine TGF-β1 and -β2 (Sato and Rifkin, 1989; Roberts et al., 1990), was obtained from R&D Sys., Inc. (Minneapolis, MN). The preparation of antibody against bovine EC type II TGase (αTGase IgG) was previously described (Nara et al., 1989). Briefly, cytosol was prepared from retinol-treated bovine ECs (10 μM retinol, 2 d) and cytosolic proteins were separated by preparative SDS-PAGE. The 80-kD band of TGase, located by Coomassie brilliant blue staining of the gel, was excised from the gel and the gel slice containing TGase was homogenized with Freund's complete adjuvant and injected subcutaneously into a rabbit. Immunizations and bleedings were continued biweekly. IgG fraction was isolated from the serum using protein A–Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The antibodies were shown to be immunospecific by Western blot of BAEC cytosol and microsequencing of the protein that was isolated by monitoring the immunoreactivity with this antibody.

Cell Culture

BAECs were isolated and grown in α modified MEM (αMEM) (GIBCO BRL, Gaithersburg, MD) containing 10% calf serum (CS) (ICN Biomedicals Inc., Costa Mesa, CA). Bovine aortic smooth muscle cells (BSMCs) and embryonic skin fibroblasts (BESFs) were isolated and grown in DME (Flow Labs. Inc., McLean, VA) containing 10% CS.

Preparation of Conditioned Medium

Conditioned medium (CM) was prepared as described (Kojima et al., 1991; Kojima and Rifkin, 1993). For retinoid experiments, BAECs were grown to confluence in 35-mm dishes, the cultures were rinsed with PBS, pH 7.4, and incubated in 1 ml of serum-free αMEM containing 0.1% BSA (αMEM-BSA), and retinoid or vehicle (0.5% ethanol) for 24 h. Thereafter, the medium was aspirated, the cultures were washed with PBS, and the cells incubated in 1 ml of αMEM-BSA without retinoid for an additional 12 h to produce CM. For coculture experiments, BAECs and BSMCs were seeded separately in 35-mm dishes (8 cm^2) at a density of 5×10^4 cells/cm^2 in αMEM containing 10% CS, or 3.2×10^5 BAECs and 0.8×10^5 BSMCs were seeded in the same 35-mm dishes in αMEM containing 10% CS. After a 2-h incubation to permit the cells to attach, the cultures were rinsed with PBS and incubated in 1 ml of αMEM-BSA for an additional 6 h to produce CM. The CM was centrifuged to remove cell debris and used in assays to detect either TGF-β or LTGF-β. For [^{3}H]thymidine incorporation assays, CM was prepared in Eagle's modified MEM containing 0.1% BSA.

Wound Migration Assay for TGF-β

Wound assays for BAEC migration were carried out as described previously (Kojima et al., 1991). After a denuded area was made in a confluent monolayer using a razor blade, the cultures were incubated in the presence of the additions to be tested for 24 h. The cells were fixed, and the number of cells that migrated from the original edge of the wound counted. This number is inversely proportional to the amount of TGF-β present in CM, since TGF-β suppresses BAEC migration (Heimark et al., 1986). This assay can be used to detect concentrations of TGF-β as low as 0.4–0.8 pM (Sato et al., 1990). The data are presented as the number of cells that have migrated >125 μm from the original edge in order to exclude those cells which moved across the origin before TGF-β had an effect. The number represents the average obtained by counting the cells in six random fields from each of two replicate dishes.

Cellular PA Assay for TGF-β

This assay was based upon the observation that TGF-β suppresses PA levels in ECs (Saksela et al., 1987) and can be used to measure the concentration of TGF-β present in CM (Flaumenhaft and Rifkin, 1992). Confluent monolayers of BAECs grown in 96-well culture plates were washed with PBS and incubated in 35 μl of CM for 4 h. The cells were washed with PBS, cellular PA extracted into 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1, and PA levels of the extracts measured using the ^{125}I-labeled fibrin plate assay (Gross et al., 1982). The amount of TGF-β in the CM was determined by comparison to a standard curve prepared with various concentrations of rTGF-β1 in αMEM-BSA. The amount of LTGF-β in the CM was determined in a similar manner after the CM was acidified (pH 2, 1 h at room temperature) followed by neutralization. This assay can be used to measure TGF-β in the 0.08–2.4 pM range. The specificity of the inhibition of PA was validated by controls employing αTGF-β IgG added to the CM. Data are expressed as the amount of TGF-β per 10^6 cells to compensate for the reduction of cell number in retinol-treated cultures (~15–25% reduction from control cultures; Kojima et al., 1986). For example, when the concentration of TGF-β in 1 ml of CM, collected from confluent cultures of 10^6 cells in a 35-mm dish, is 1 pM (25 pg/ml), this is expressed as 1 fmol/10^6 cells.

[^{3}H]Thymidine Incorporation Assay for TGF-β

The inhibition of [^{3}H]thymidine incorporation by CCL-64 mink lung epithelial cells as an assay for TGF-β was carried out according to the method of Danielpour et al. (1989). A suspension of CCL-64 cells (2×10^5 cells/ml) was made in DME containing 0.2% FCS and 10 mM Hepes. 40 ml of this suspension was transferred to each well of a 96-well culture plate. After preincubation for 2 h, 35 μl of CM was added directly to each well and the cultures were incubated for 22 h. Thereafter, 15 μl of [^{3}H]thymidine (10 μCi/ml) was added directly to each well, and the cultures were incubated for 4 h at 37°C. Cells were fixed by incubating dishes for 1 h at room temperature with 160 μl of methanol: acetic acid (3:1) directly added to the radioactive medium, washed twice with 250 μl of 80% methanol, trypsinized, and solubilized into 1% SDS-trypsin solution. Radioactivity in the cell lysate was measured with a Beckman LS3801 β-scintillation counter. The amount of TGF-β in the CM was determined by comparison to a standard curve prepared with various concentrations of rTGF-β1 in Eagle's modified MEM containing 0.1% BSA. This assay can be used to measure TGF-β in the range of 0.08–2.4 pM. Data are expressed as the amount of TGF-β per 10^6 cells as described before.

Preparation of Cytosol and Membrane Extract Fractions

Cells were scraped from the culture dish with a rubber policeman and washed three times with cold Ca^{2+} and Mg^{2+}-free Hepes buffered saline (CMFH buffer: 129 mM NaCl, 5 mM KCl, 0.3 mM Na_2HPO_4, 1 mM $NaHCO_3$, 5 mM glucose, and 25 mM Hepes, pH 7.4). The washed cells were ruptured in cold CMFH buffer containing 40 μM leupeptin and 2 mM PMSF using ultrasonication on ice. The supernatant (cytosol) was obtained by centrifugation of the homogenate at 15,000 *g* for 15 min at 4°C. The pellet was washed with the same buffer, solubilized in CMFH buffer containing 10 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 40 μM leupeptin and 2 mM PMSF, centrifuged at 15,000 *g* for 15 min, and the supernatant collected as the soluble membrane extract.

TGase Assay

TGase activity was measured as the Ca^{2+}-dependent incorporation of ^{14}C-putrescine (Amersham Corp., Arlington Heights, IL) into N,*N*-dimethylcasein by a modification of the method described by Nara et al. (1989). The reaction mixture consisted of CMFH buffer containing 5 mM $CaCl_2$, 1 mg/ml N,*N*-dimethylcasein, 20 mM dithiothreitol, 0.8% (vol/vol) glycerol, 250 μM ^{14}C-putrescine (0.45 μCi), and the sample, in a final volume of 300 μl. The reaction mixtures were incubated for 1 h at 37°C and reactions stopped by the addition of 600 μl of 16.7% TCA with 100 μl of 2% BSA. The precipitate was collected on Whatman GF/C glass filters, washed three times with 2 ml of 10% TCA, and the radioactivity measured by liquid scintillation counting. TGase activity was expressed as nanomoles of putrescine incorporated into casein in 1 h at 37°C per mg of protein of sample. Protein concentration was measured by microtiter plate BCA (Pierce, Rockford, IL) assays using BSA as the standard.

Reverse Fibrin Autography

Reverse fibrin autography was carried out by the method of Erickson et al. (1984). Briefly, CM was prepared from BAECs as described in the legend for Fig. 6, concentrated 10-fold on Microcon concentrator (Amicon, Beverly, MA), and proteins in CM fractionated through SDS-PAGE with 10% resolving gels. Gels were washed twice with 2.5% Triton X-100 and then with saline, and applied onto fibrin-agar gel containing plasminogen and urokinase. The PAI-1 was visualized as a lysis-resistant opaque band after incubation of the gel at 37°C for 3 h.

Results

Effect of Inhibition of TGase Activity on Formation of TGF-β by Retinoid-treated ECs

To test the hypothesis that TGase is involved in the activation of LTGF-β by retinoid-stimulated BAECs, a TGase inhibitor, cystamine (Lorand and Conrad, 1984), was included with retinol in the medium of wounded cultures of BAECs and cell migration was monitored (Fig. 1). In this system, the generation of TGF-β induced by retinoids can be measured as the inhibition of cell migration. The addition of retinol to wounded cells in the absence of cystamine inhibited cell migration by ~40%. The inclusion of αTGF-β IgG abol-

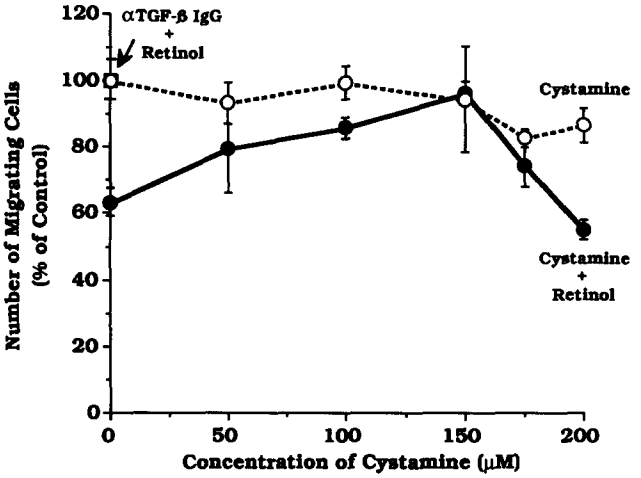

Figure 1. Dose-dependent inhibitory effect of cystamine on retinol-induced suppression of BAEC migration. Confluent BAECs were wounded as described in Materials and Methods. The cells were allowed to migrate overnight in the presence of increasing concentrations of cystamine with and without 2 μM retinol, and the monolayers were fixed, stained, and the number of cells that had moved >125 μm from the original wound margin counted. Data are expressed as a percent of control using fresh medium containing 0.5% ethanol. The number of cells that had moved >125 μm in the control was 52. (○) Increasing amounts of cystamine alone. (●) Retinol (2 μM) with increasing amounts of cystamine. (■) Retinol (2 μM) with αTGF-β IgG (10 μg/ml).

ished the inhibitory effect of retinol, indicating that the inhibition of migration observed in the presence of retinol was due to TGF-β. When migration in the presence of retinol plus cystamine was monitored, the number of migrating cells increased with increasing concentrations of cystamine until 150 μM and decreased at higher concentrations. The inclusion of cystamine alone at concentrations <150 μM had little effect on cell migration. The inhibitory effect of concentrations of cystamine >150 μM plus retinol was probably due to toxicity. Similar results were obtained in this assay using retinol plus two other TGase inhibitors, bacitracin and monodansylcadaverine (Maxfield et al., 1979; Lorand and Conrad, 1984) (data not shown). These results suggest that the inclusion of TGase inhibitors abolished retinol's capacity to stimulate the formation of TGF-β.

To corroborate this hypothesis, we next included TGase inhibitors during the preparation of CM from retinol-treated or untreated BAECs, and measured the effect of the CM on BAEC migration in wound assays (Fig. 2). CM from retinol-treated cells inhibited test cell migration by 35% (sample *2*). This inhibition was due to TGF-β since it was abrogated by inclusion of αTGF-β IgG (sample *11*). Whereas CM prepared from untreated cells in the presence of each TGase inhibitor had little effect on migration (samples *3*, *5*, and *7*), inclusion of these inhibitors in the medium during the preparation of CM from retinol-treated cells suppressed the generation of inhibitory activity (samples *4*, *6*, and *8*). In addition, the inclusion of αTGase IgG also alleviated the inhibitory effect of retinol on cell migration (sample *10*). The effect of cystamine was also observed in the inhibition of [^{3}H]thymidine incorporation by mink lung cells as a measurement of TGF-β (Fig. 3). As seen in the other assays, retinol exposure induced TGF-β formation (sample *2*) and this was blocked by the inclusion of cystamine in the initial culture medium (sample *4*). The effect of retinol was also blocked by αTGF-β IgG (sample *5*). Furthermore, the effect of the TGase inhibitors, DAPBT, L682777, L683685, and L683696 was tested (Table I). These inhibitors are thought to react specifically with the active site of TGase and to suppress TGase activity (Lorand et al., 1987; Barsigian et al., 1991). CM was prepared from retinol-related BAECs in the presence of the four TGase inhibitors as well as monodansylcadaverine and dimethylated dansylcadaverine (DMDC), and the amount of active TGF-β present in CM was determined by the PA assay. DMDC was used as a noninhibitory control for monodansylcadaverine. Whereas DMDC did not affect retinol-induced formation of TGF-β, all of the TGase inhibitors tested suppressed TGF-β formation to the levels obtained in untreated cell CM. The effect of the TGase inhibitors and αTGase IgG was also observed with cells treated with retinoic acid (data

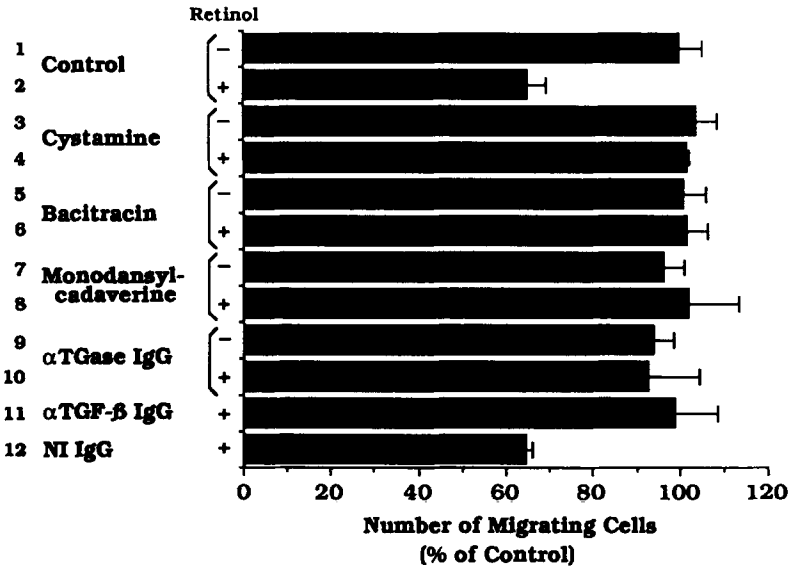

Figure 2. Effects of TGase inhibitors and anti-TGase antibody on retinol-induced formation of TGF-β. Confluent BAECs were incubated with 0.5% ethanol (vehicle; samples *1*, *3*, *5*, *7*, and *9*) or with 2 μM retinol (samples *2*, *4*, *6*, *8*, and *10*) for 24 h and CM was prepared in the presence of 100 μM TGase inhibitors, cystamine (samples *3* and *4*), bacitracin (samples *5* and *6*), and monodansylcadaverine (samples *7* and *8*) or 100 μg/ml of neutralizing antibodies to TGase (samples *9* and *10*) as described in Materials and Methods. The CM was tested for TGF-β activity in wound migration assays using BAECs. Cell migration was expressed as a percent of control using CM from untreated cells. The number of cells that had moved >125 μm in the control was 80. The formation of TGF-β in CM of retinol-treated cells was verified by addition of αTGF-β IgG (10 μg/ml; sample *11*) or nonimmune (*NI*) IgG (10 μg/ml; sample *12*) to the CM from retinol-treated cells.

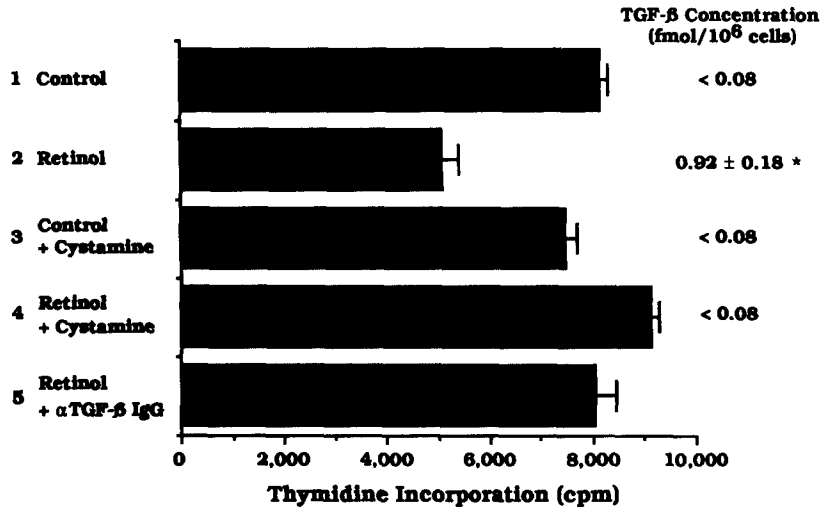

Figure 3. Cystamine inhibition of TGF-β formation in retinol-treated BAECs measured by mink lung cell [^{3}H]thymidine incorporation assays. Confluent BAECs were treated with 2 μM retinol as before and CM was prepared in the presence and the absence of 100 μM cystamine. The CM was tested for TGF-β activity in mink lung cell [^{3}H]thymidine incorporation assays as described in Materials and Methods. Sample *1*, CM from control cells; sample *2*, CM from retinol-treated cells; sample *3*, CM from control cells prepared in the presence of cystamine; sample *4*, CM from retinol-treated cells prepared in the presence of cystamine; and sample *5*, CM from retinol-treated cells with αTGF-β IgG (10 μg/ml). The concentrations of TGF-β in each CM determined by this assay are also listed. *0.92 fmol (23.1 pg)/10^{6}cells is equivalent to 14.3 pg/ml (0.57 pM) of CM/0.62 × 10^6 cells/35-mm dish.

Table I. Effect of TGase Inhibitors on Formation of TGF-β in Retinol-treated BAECs

Cell culture	TGF-β
	fmol/10^{6}cells
Experiment 1	
Control	0.08 ± 0.01
Retinol + DMSO	1.10 ± 0.12*
Retinol + DAPBT	<0.08
Retinol + MDC	<0.08
Retinol + DMDC	0.77 ± 0.11
Experiment 2	
Control	<0.08
Retinol + DMSO	0.90 ± 0.09
Retinol + L682777	0.21 ± 0.03
Retinol + L683685	<0.08
Retinol + L683696	<0.08

Confluent BAECs were treated with 2 μM retinol for 24 h as before. CM was prepared in the presence of either 0.5% dimethylsulfoxide (DMSO), DAPBT (100 μM), monodansylcadaverine (MDC; 100 μM), or DMDC (100 μM) in Experiment 1, or 0.5% DMSO, L682777 (50 μM), L683685 (50 μM), or L683696 (50 μM) in Experiment 2 and the amount of active TGF-β was determined by PA activity assay as described in Materials and Methods. Data are expressed as the amount per 10^6 cells.
* 1.10 fmol (27.5 pg)/10^6 cells is equivalent to 22.3 pg/ml (0.89 pM) of CM/0.81 × 10^6 cells/35-mm dish.

not shown). Inhibitors and antibody did not affect the inhibition of cell migration or the suppression of PA levels by either rTGF-β or CM (data not shown). These results suggested that TGase is involved in the formation of TGF-β in retinoid-treated BAECs.

Specificity of Retinoid Effects

Because retinoids are known to increase TGase levels in certain cells (Kojima et al., 1987; Chiocca et al., 1989; Nara et al., 1989; Suedhoff et al., 1990), we measured the levels of TGase in both membrane and cytosolic fractions of BAECs (Table II). Treatment with retinol caused a 2.5-fold increase in cytosolic TGase and a 4.3-fold increase in membrane activity. Exposure of BAECs to retinol also caused a twofold increase in PA levels but only a 20% increase in total (active and latent) TGF-β. The effect of retinol showed some cell specificity since the stimulation of TGase in BSMCs and BESFs was much lower than that observed in BAECs as were the levels of PA. The effects of retinol on LTGF-β activation by these three cell types were similar to its effects on TGase and PA expression. Retinol induced the formation of active TGF-β only in BAECs. Only retinol-treated BAECs expressed significantly increased levels of PA, TGase, and ac-

Table II. Comparison between the Effects of Retinol on TGase and PA Levels and Secretion and Activation of LTGF-β in BAECs, BSMCs, and BESFs

		TGase activity		PA activity		Amount of TGF-β	
Cell	Retinol	Cytosol	Membrane extracts	Medium	Cell lysate	Active	Total
		nmole putrescine/h/mg protein		*U/10^6 cells*	*U/mg protein*	*fmol/10^6 cells*	
BAEC	−	335 ± 48	53 ± 9	3.3 ± 0.7	1.7 ± 0.3	<0.08	15.2 ± 2.7
	+	825 ± 40	229 ± 19	6.8 ± 0.8	4.0 ± 0.6	0.75 ± 0.09	19.0 ± 1.2
BSMC	−	13.2 ± 1.3	4.8 ± 0.0	0.02 ± 0.00	0.73 ± 0.07	0.10 ± 0.03	25.0 ± 1.2
	+	26.5 ± 0.2	6.8 ± 2.0	0.06 ± 0.01	0.49 ± 0.04	<0.08	19.3 ± 2.6
BESF	−	0.07 ± 0.01	0.24 ± 0.02	0	0.05 ± 0.01	0.12 ± 0.02	12.7 ± 3.4
	+	0.07 ± 0.01	0.48 ± 0.15	0	0.13 ± 0.03	0.11 ± 0.01	14.8 ± 1.8

Confluent BAECs, BSMCs, and BESFs were treated with 2 μM retinol for 24 h as before. CM, cell lysate, cytosol, and membrane extracts were prepared from each cell, and amounts of active and total (active and latent) TGF-β present in CM. PA activity levels in both CM and cell lysate, and TGase activity levels in both cytosol and membrane extracts were measured as described in Materials and Methods. Data are expressed as either the amount and activity per 10^6 cells or activity per mg protein of sample.

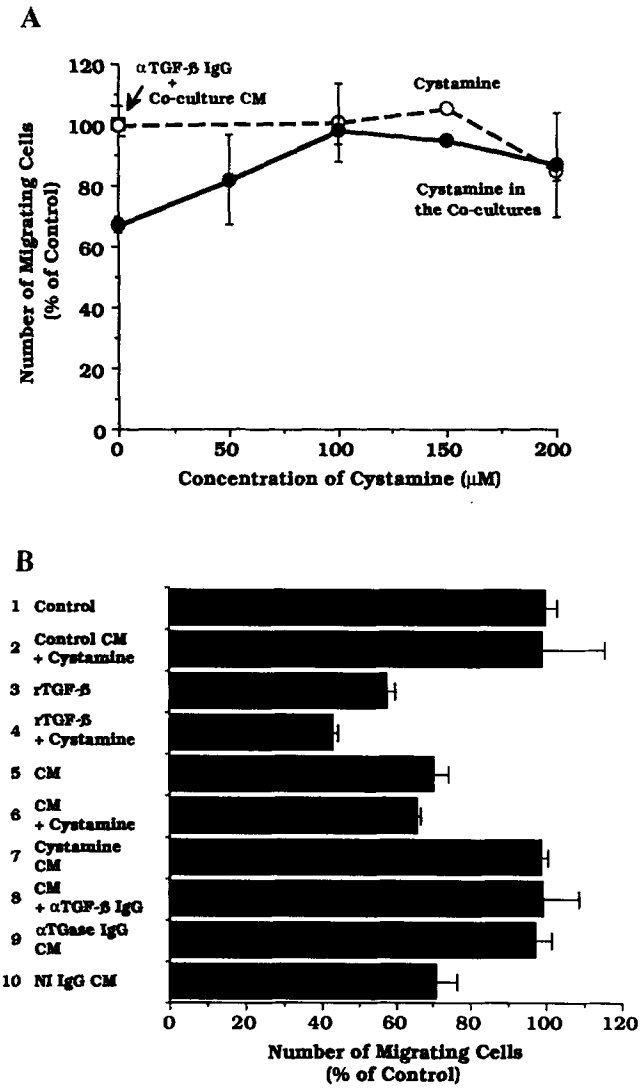

Figure 4. Inhibition of TGF-β formation in cocultures of BAECs and BSMCs by cystamine and anti-TGase antibody. (*A*) Dose dependency of cystamine inhibition: CM was prepared from homotypic and heterotypic cultures of BAECs and BSMCs in the presence of increasing concentrations of cystamine as described in Materials and Methods. The CM was added to wounded BAEC monolayers, and cell migration was quantitated as before. The number of cells that had moved >125 μm was expressed as a percent of control using 4:1 mixture of BAEC CM and BSMC CM. The number of cells that had moved >125 μm in the control was 50. (○) Mixture of BAEC and BSMC CM (4:1) prepared in the presence of increasing amounts of cystamine. (●) Coculture CM prepared in the presence of increasing amounts of cystamine. (■) Coculture CM prepared in the absence of cystamine to which αTGF-β IgG (10 μg/ml) has been added. (*B*) Control experiment: Wound assays of BAEC migration were performed as before testing the following additions: sample *1*, control CM that was a 4:1 mixture of BAEC CM and BSMC CM; sample *2*, cystamine (100 μM) added to control CM; sample *3*, rTGF-β (2 pM) added to fresh medium; sample *4*, cystamine (100 μM) plus rTGF-β (2 pM) added to fresh medium; sample *5*, coculture CM; sample *6*, cystamine (100 μM) added to coculture CM; sample *7*, coculture CM prepared in the presence of cystamine (100 μM); sample *8*, coculture CM supplemented with αTGF-β IgG (10 μg/ml); sample *9*, coculture CM prepared in the presence of αTGase IgG (100 μg/ml); and sample *10*, coculture CM prepared in the presence of NI IgG (100 μg/ml). The number of cells that had moved in the control was 62.

tive TGF-β concomitantly, although the total amount of TGF-β produced was similar among the three cell types regardless of the treatment.

Involvement of TGase in Formation of TGF-β by Cocultures

We previously documented that cocultures of BAECs and BSMCs activate LTGF-β (Sato and Rifkin, 1989). Therefore, we next tested whether cystamine blocked TGF-β formation in cocultures. A result similar to that illustrated in Fig. 1 was obtained (Fig. 4 *A*). Increasing concentrations of cystamine added to the medium during the preparation of coculture CM abrogated the ability of the CM to suppress test cell migration. Control experiments (Fig. 4 *B*) indicated that cystamine did not block the effect of rTGF-β (sample *4*) or CM (sample *6*) when it was added directly to wounded cells. Cystamine was effective only when included during the preparation of CM (sample *7*). Similar results were obtained using αTGase IgG instead of cystamine during coculture activation of LTGF-β (Fig. 4 *B*, sample *9*). These results indicate that cystamine and αTGase IgG block the formation of TGF-β in the coculture system as well as in the retinoid system.

Effects of TGase Inhibitor and Antibody on LTGF-β Production

To quantify the effect of TGase inhibitors (cystamine, DAPBT, and L683685) and αTGase IgG on the production

Table III. Effect of TGase Inhibitor, and αTGase IgG on Production of Active and Latent TGF-β in CM of BAECs Either Treated with Retinol (Experiment 1) or Cocultured with BSMCs (Experiment 2)

Cell culture	Active TGF-β	Total TGF-β
	fmol/10⁶ cells	
Experiment 1		
Control	<0.08	24.7 ± 3.2
Retinol	0.56 ± 0.15	31.0 ± 1.3
Retinol + Cystamine	0.09 ± 0.02	26.0 ± 2.5
Retinol + DAPBT	<0.08	19.5 ± 3.4
Retinol + DMDC	0.48 ± 0.07	19.4 ± 2.2
Retinol + L683685	0.12 ± 0.03	32.6 ± 8.1
Retinol + αTGase IgG	0.11 ± 0.01	24.3 ± 4.3
Retinol + NI IgG	0.78 ± 0.14	26.4 ± 1.1
Experiment 2		
Control	<0.08	48.0 ± 5.0
Coculture	0.90 ± 0.04	41.2 ± 7.8
Coculture + Cystamine	< 0.08	42.5 ± 6.5
Coculture + DAPBT	0.08 ± 0.04	36.1 ± 3.0
Coculture + DMDC	0.79 ± 0.12	33.7 ± 9.1
Coculture + L683685	<0.08	52.9 ± 1.9
Coculture + αTGase IgG	<0.08	43.5 ± 5.5
Coculture + NI IgG	0.87 ± 0.02	44.0 ± 5.0

(*Experiment 1*) Confluent BAECs were treated with 2 μM retinol for 24 h as before. CM was prepared in the presence and the absence of either cystamine (100 μM), DAPBT (100 μM), DMDC (100 μM), L683685 (50 μM), αTGase IgG (100 μg/ml), or NI IgG (100 μg/ml), and amounts of active and total (active plus latent) TGF-β present in CM were determined by PA activity assays as described in Materials and Methods. (*Experiment 2*) CM was prepared from cocultures of BAECs and BSMCs in the presence and the absence of the same inhibitors or antibody as used in Experiment 1 and amounts of active and total TGF-β were determined.

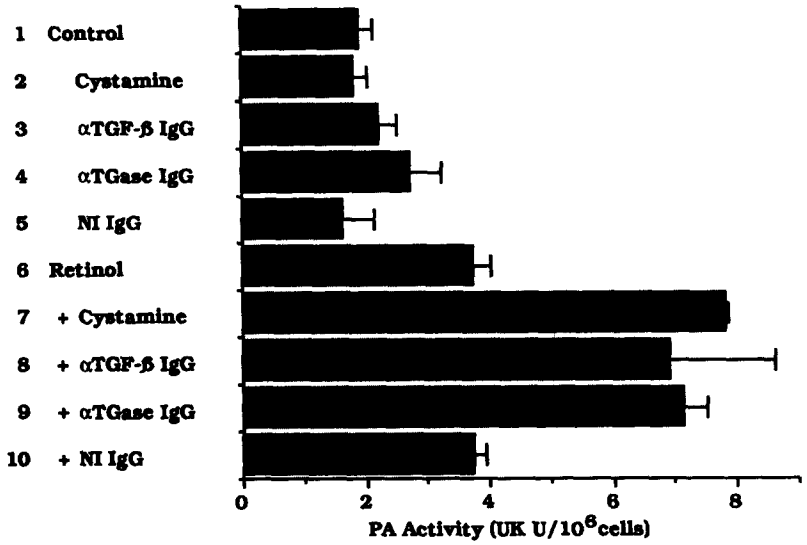

Figure 5. Effect of cystamine and anti-TGase antibody on PA levels as an inhibitor of endogenous formation of TGF-β. Confluent BAECs were treated with retinol or vehicle in the presence and the absence of either cystamine, αTGF-β IgG, αTGase IgG, or NI IgG for 2 d in αMEM-BSA. PA activity levels in each medium were assayed as described in Materials and Methods and expressed as urokinase (UK) units per 10^6 cells. Samples *1–5*, vehicle (0.5% ethanol); samples *6–10*, retinol (2 μM). Samples *2* and *7*, cystamine (100 μM); samples *3* and *8*, αTGF-β IgG (10 μg/ml); samples *4* and *9*, αTGase IgG (100 μg/ml); and samples *5* and *10*, NI IgG (100 μg/ml).

of LTGF-β, CM was prepared in the presence of TGase inhibitors or αTGase IgG from either retinol-treated BAECs or cocultures of BAECs and BSMCs, and the amounts of both active and total TGF-β present in CM were measured. The results are listed in Table III. In both systems, retinol-treated cells or cocultures (Experiments 1 and 2, respectively), cystamine, L683685, and αTGase IgG reduced the amount of active TGF-β without significantly changing total TGF-β content, indicating that these compounds blocked the formation of TGF-β by suppressing LTGF-β activation and not by suppressing the secretion of LTGF-β. DAPBT suppressed the formation of TGF-β but with a reduction in total TGF-β production of 25–35%. However, it is unlikely that this decrease of LTGF-β secretion accounts for 100% inhibition of active TGF-β formation, since a similar degree of reduction in total TGF-β secretion was observed with DMDC which did not completely suppress the activation of LTGF-β. Furthermore, TGase inhibitors and αTGase IgG did not interfere with the activation of LTGF-β in CM either by exogenous plasmin (0.8 U/ml, 2 h at 37°C) or by transient acidification (pH 2, 1 h at room temperature) (data not shown).

Enhancement of PA Levels in ECs by Simultaneous Addition of Retinol and Inhibitor of LTGF-β Activation

Retinoids have been shown to enhance PA synthesis in ECs. This effect is often accompanied by increased expression of PAI-1 (Kojima et al., 1986; Kooistra et al., 1991). We have proposed that the coinduction of PAI-1 with PA in retinoid-treated ECs is the result of TGF-β formation induced by retinoids followed by increased PAI-1 expression due to TGF-β activity (Kojima and Rifkin, 1993). If this hypothesis is correct and if the formation of TGF-β requires TGase, the effect of retinoids on PA levels should be augmented in the presence of TGase inhibitors since PAI-1 expression should not be increased. This was tested by an experiment in which BAECs were treated with a combination of retinol and either cystamine, αTGF-β IgG, αTGase IgG, or NI IgG and the PA activity levels of CM were measured (Fig. 5). Whereas cystamine alone did not increase PA levels (sample *2*) and retinol alone increased them by 100% (sample *6*), these two agents in combination increased PA levels by 260% (sample *7*). A similar result was obtained with the combination of retinol and αTGF-β IgG (sample *8*) or retinol and αTGase IgG (sample *9*). NI IgG did not enhance the effect of retinol (sample *10*). Controls of αTGF-β IgG, αTGase IgG, and NI IgG alone had no effect on basal PA production (samples

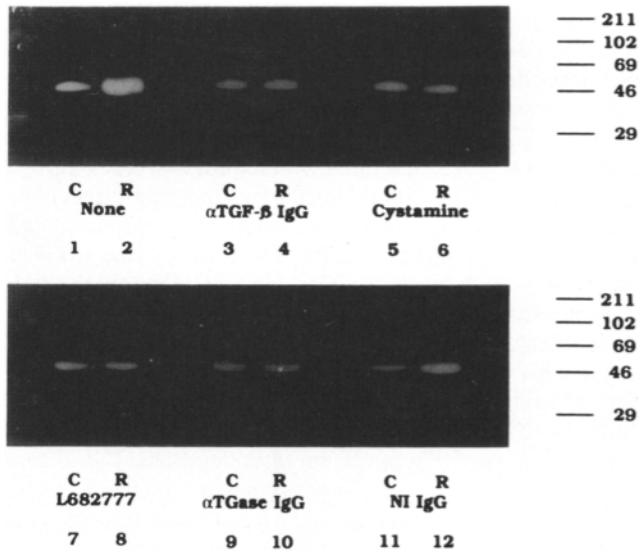

Figure 6. Suppression of retinol-induced augmentation of PAI-1 levels by TGase inhibitors or anti-TGase antibody. Confluent BAECs were treated with retinol or vehicle in the presence and the absence of either αTGF-β IgG, cystamine, L682777, αTGase IgG, or NI IgG for 24 h in αMEM containing 0.1% gelatin. Medium was changed and further incubated for another 24 h as before except that gelatin was omitted from medium. This later medium was collected, concentrated, and subjected to SDS-PAGE. PAI-1 was then detected by reverse fibrin autography. Odd number lanes, control (*C*; 0.5% ethanol); Even number lanes, retinol (*R*; 2 μM). Lanes *3* and *4*, αTGF-β IgG (50 μg/ml); lanes *5* and *6*, cystamine (100 μM); lanes *7* and *8*, L682777 (100 μM); lanes *9* and *10*, αTGase IgG (100 μg/ml); and lanes *11* and *12*, NI IgG (100 μg/ml).

3–5). To confirm that the potentiating effect of αTGase IgG on retinol-induced PA expression is via suppression of increased PAI-1 levels after retinol-induced TGF-β formation, the amounts of PAI-1 present in CM of cells treated in different ways were measured by reverse fibrin zymography (Fig. 6). The enhanced production of PAI-1 in retinol-treated cultures can be seen by comparing lanes *1* and *2*. When cultures were treated with retinol and either αTGF-β IgG (lane *4*), cystamine (lane *6*), L682777 (lane *8*), or αTGase IgG (lane *10*), the normally observed increase in PAI-1 did not occur. None of these treatments alone affected basal levels of PAI-1 production (lanes *3*, *5*, *7*, and *9*). Finally, the retinol-induced increase in PAI-1 was not blocked by NI IgG (lane *12*). These results support the hypothesis that TGase is a component required for LTGF-β activation in retinol-treated BAECs.

Discussion

The present study describes a novel function for type II TGase as a component required for LTGF-β activation. We have demonstrated that TGase inhibitors of two different classes, substrate competitor (monodansylcadaverine) and active site-directed inhibitors (cystamine, DAPBT, L682777, L683685, and L683696) as well as a neutralizing antibody to type II TGase abrogate the activation of LTGF-β observed with both retinoid-treated BAECs and BAEC/BSMC cocultures. These inhibitors and the neutralizing antibody to TGase do not affect the activity of TGF-β, the release of LTGF-β, or the activation of LTGF-β by plasmin or transient acidification. The concentrations of TGase inhibitors, cystamine, bacitracin, and monodansylcadaverine and antibody to TGase required for complete inhibition of LTGF-β activation, are consistent with those required for direct inhibition of TGases derived from BAECs (data not shown), guinea pig liver (Lorand and Conrad, 1984), and CHO cells (Maxfield et al., 1979); whereas the concentrations of DAPBT, L682777, L683685, and L6383696 required to block TGF-β production were 10–20-fold higher than those for direct inhibition of BAEC TGase (data not shown). Only retinol-treated BAECs, but not BSMCs or BESFs, both activate LTGF-β and express high levels of TGase and PA. The observation indicates that retinoids induce LTGF-β activation in BAECs through enhancement of both PA and TGase levels. Further support for the involvement of TGase in LTGF-β activation derives from the observation that inhibitors of TGase and an antibody to TGase potentiate retinol-induced PA production presumably via the suppression of endogenous TGF-β formation that normally results in an increase of PAI-1 levels. This effect was obvious after a 24-h treatment of cells with retinol.

Although we have demonstrated that the increase of both PA and TGase levels in ECs by retinoids is associated with LTGF-β activation in homotypic BAEC cultures, we cannot adopt this as an explanation for LTGF-β activation by cocultures of ECs with SMCs as neither PA nor TGase levels increase dramatically during the time of coculture of BAECs and BSMCs (data not shown). However, it is possible that these enzymatic activities are increased at specific sites where ECs and SMCs are in contact. Preliminary data indicate that coculture induces LTGF-β activation more rapidly (2–6 h) than do retinoids (18–36 h). Thus, a rapid change occurs upon coculture of ECs and SMCs, resulting in TGase- and plasmin-mediated activation of LTGF-β, whereas retinoid-induced activation follows an enhancement of TGase and PA synthesis that takes place over a period of hours. It is also noteworthy that we were not able to induce LTGF-β activation by adding guinea pig liver TGase to homotypic cultures of BAECs (data not shown). This suggests two possibilities: one, there is a species specificity for TGase action and two, there are additional requirements for activation. These possibilities are presently being examined.

The regulation of cell growth and differentiation has emerged as a putative function for type II TGase as two groups have reported that TGase activity increases with decreasing cell growth (Birckbichler and Patterson, 1978; Milhaud et al., 1980). However, little is known about the molecular mechanisms whereby type II TGase might control cell growth (Thacher and Rice, 1985; Lichti et al., 1985; Chiocca et al., 1989; Suedhoff et al., 1990). Although it is possible that type II TGase controls cell growth through its GTPase activity (Lee et al., 1989), the regulation of phospholipase A_2 and its inhibitor (Cordella-Miele et al., 1990; Ando et al., 1991), or the modulation of extracellular matrix function by cross-linking matrix proteins such as fibronectin, collagen, fibrinogen/fibrin, proteoglycan, and vitronectin (Martinez et al., 1989; Kinsella and Wight, 1990; Sane et al., 1991), our findings may directly explain the negative relationship between type II TGase and cell growth by linking TGase activity with the formation of TGF-β, a potent growth inhibitor. This hypothesis predicts that older cells produce higher levels of TGF-β and that TGF-β is a growth inhibitor for these cells. This is currently being tested.

It will be of interest to determine if cell-associated TGase is required for the formation of active TGF-β in other systems such as osteoclasts and keratinocytes treated with retinoids (Oreffo et al., 1989; Glick et al., 1989), ECs treated with bleomycin (Phan et al., 1991), and human breast cancer cells treated with anti-estrogens (Colletta et al., 1991). We have shown that TGase is required for LTGF-β activation observed when BAECs are treated with basic fibroblast growth factor (data not shown). Thus, in three systems in which ECs have been shown to produce active TGF-β, a requirement for TGase exists.

According to our model for LTGF-β activation, the activation takes place on the cell surface or matrix where the components required for activation form an assemblage of molecules that promotes the reaction (Dennis and Rifkin, 1991; Flaumenhaft et al., 1993). Therefore, TGase, normally found intracellularly, must exist on the cell surface in order to participate in the activation process. The ability of the αTGase IgG to inhibit LTGF-β activation is consistent with this proposal. Although we have no direct evidence to verify the extracellular deposition of TGase, Martinez et al. (1989) and Sane et al. (1991) recently demonstrated the cross-linking of fibrinogen and vitronectin, respectively, using human umbilical vein EC suspensions and suggested the existence of cell-associated TGase in ECs. Also, the existence of extracellular matrix-bound TGase was reported in wounded embryonic human lung fibroblasts (Upchurch et al., 1991). The mechanism by which TGase is involved in LTGF-β activation remains to be elucidated. Bendixen et al. (1991) recently reported that TGase cross-links plasminogen to fibronectin. It is possible that TGase concentrates plasminogen on the extracellular matrix. Furthermore, it is also possible that the

LTGF-β is cross-linked to membrane protein(s) via LAP or LTGF-β binding protein by the type II TGase. Preliminary experiments indicate that semipurified LTGF-β without binding protein as well as binding protein portion of LTGF-β from human platelets is a substrate for guinea pig liver TGase (Kojima, S., unpublished observation).

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