Plasminogen Activator and Collagenase Production by Cultured Capillary Endothelial Cells

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ABSTRACT   Cultured bovine capillary endothelial (BCE) cells produce low levels of collagen-olytic activity and significant amounts of the serine protease plasminogen activator (PA). When grown in the presence of nanomolar quantities of the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA), BCE cells produced 5–15 times more collagenolytic activity and 2–10 times more PA than untreated cells. The enhanced production of these enzymes was dependent on the dose of TPA used, with maximal response at 10^{-7} to 10^{-8} M. Phorbol didecanoate (PDD), an analog of TPA which is an active tumor promoter, also increased protease production. 4-O-methyl-TPA and 4α-PDD, two analogs of TPA which are inactive as tumor promoters, had no effect on protease production. Increased PA and collagenase activities were detected within 7.5 and 19 h, respectively, after the addition of TPA. The TPA-stimulated BCE cells synthesized a urokinase-type PA and a typical vertebrate collagenase.

BCE cells were compared with bovine aortic endothelial (BAE) cells and bovine embryonic skin (BES) fibroblasts with respect to their production of protease in response to TPA. Under normal growth conditions, low levels of collagenolytic activity were detected in the culture fluids from BCE, BAE, and BES cells. BCE cells produced 5–13 times the basal levels of collagenolytic activity in response to TPA, whereas BAE cells and BES fibroblasts showed a minimal response to TPA. Both BCE and BAE cells exhibited relatively high basal levels of PA, the production of which was stimulated approximately threefold by the addition of TPA. The observation that BCE cells and not BAE cells produced high levels of both PA and collagenase activities in response to TPA demonstrates a significant difference between these two types of endothelial cells and suggests that the enhanced detectable activities are a property unique to bovine capillary and microvessel endothelial cells.

Capillary proliferation in vivo is marked by fragmentation of the capillary basal lamina and subsequent migration and proliferation of distinct endothelial cell populations in response to stimuli (2). New blood vessel formation, or angiogenesis, can be studied in a number of systems (9, 10), and is promoted by angiogenic factors derived from both normal tissues (4, 11), and tumors (2, 9). Since the formation of capillaries is marked by both the destruction of the basal lamina and the invasion of cells through interstitial tissue, capillary formation may require the elaboration of proteases to degrade the proteins of the basal lamina and interstitial stroma. Therefore, we have proposed (20) that angiogenesis requires the secretion of proteases and have initiated experiments to characterize the pro-

teases produced by endothelial cells in response to various stimuli.

Previous work has shown that endothelial cells derived from large vessels of several tissues and species synthesize at least two extracellular proteases: the serine protease plasminogen activator (PA) and vertebrate interstitial collagenase (19). Endothelial cells isolated from bovine aorta (13, 14), rabbit vena cava (17), and human umbilical vein (14) secrete variable amounts of PA, an enzyme which converts plasminogen to plasmin, a neutral protease with broad substrate specificity. The amount of PA activity is dependent upon the species and source of endothelium (14), the level of cellular inhibitors (17), and on the exact culture conditions (15). However, significantly

higher levels of PA production can be induced in many endothelial cells by the inclusion of the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) in the growth media (14). In contrast, the basal level of collagenase production by endothelial cells is quite low (19). However, in the presence of TPA, human umbilical vein endothelial cells produce significant amounts of a latent collagenase, which, upon activation, is capable of degrading types I, II, and III collagens (19). Bovine vein endothelial cells also have been reported to secrete low levels of a type IV collagenase (16). However, no single endothelial cell type has been shown to synthesize significantly increased amounts of both collagenase and PA in response to TPA stimulation.

The endothelial cells involved in neovascularization and therefore of greatest interest in terms of their protease production are the capillary endothelial cells. Thus, we examined the synthesis of PA and collagenase by cultured bovine capillary endothelial (BCE) cells. To determine if collagenase and PA synthesis could be stimulated in BCE cells, we also investigated the modulation of these two enzymes in response to TPA, a known inducer of PA in endothelial cells (17). Since the responses of endothelial cells derived from large vessels and capillaries may be quite different, the levels of PA and collagenase activities produced by both BCE and bovine aortic endothelial (BAE) cells were compared.

MATERIALS AND METHODS

Cell Culture

BCE were isolated from bovine adrenal glands from freshly slaughtered calves and cloned as described by Folkman et al. (8). Growth medium consisted of an equal mixture of modified Eagle's medium (α-MEM with ribonucleosides; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) and tumor-conditioned medium (see below) supplemented with 10% (vol/vol) donor calf serum (Flow Laboratories, Inc., Rockville, MD) and 1% (vol/vol) antibiotic-antimycotic (Gibco Laboratories). Only those clones which uniformly produced Factor VIII–related antigen were maintained in liquid nitrogen for later use.

Large vessel endothelial cells were obtained from the aortae of the same animals used above. BAE cells were prepared as described previously (22). The cells were grown in Dulbecco's modified Eagle's medium (low glucose DME, Gibco Laboratories) containing 10% (vol/vol) donor calf serum unless otherwise stated.

Fibroblasts were obtained from bovine embryonic skin (BES fibroblasts) using the same procedure as described for the preparation of chick fibroblasts (5). The cells were grown in DME medium with 10% (vol/vol) donor calf serum, unless otherwise stated.

Dr. J. Folkman (Childrens Hospital Medical Center, Boston, MA) kindly supplied mouse sarcoma 180 cells. These cells were grown in DME with 10% donor calf serum. The spent medium from confluent cultures was used as the source of tumor-conditioned medium as described by Folkman et al. (8). Cells used in all experiments were between fifth and tenth passage.

Immunofluorescence for Factor VIII – related Antigen

Cells were plated on 12-mm gelatin-coated sterile cover slips in 35-mm culture dishes. At confluence, the cover slips were removed and fixed in 100% methanol (5 min, −20°C), washed in PBS, and incubated for 40 min at 37°C with a 1:100 dilution of rabbit antiserum to bovine Factor VIII (provided by Dr. E. Davie, University of Washington). The cover slips were washed with PBS (three times for 15 min each) and incubated for an additional 40 min at 37°C with rhodamine conjugated anti-rabbit IgG (1:120 dilution in PBS; N. L. Cappel Laboratories, Cochranville, NJ). After further washing, the cover slips were mounted on microscope slides and examined by fluorescence microscopy at × 312 with a Leitz Orthomat microscope.

Preparation of Cells for Experiments

Cells were seeded on 35-mm gelatin-coated dishes (1.5% gelatin [Sigma Chemical Co., St. Louis, MO] in calcium and magnesium-free PBS) and grown to confluence in their respective growth media unless otherwise indicated. At confluence, the cells were washed twice with PBS and incubated for 18 h in DME with 10% (vol/vol) acid-treated (17) calf serum depleted of plasminogen (7). The cells were then washed twice with PBS and cultured for an additional 24 h in DME containing 5% (vol/vol) calf serum (depleted of plasminogen) and the agent to be tested. This protocol was found to effectively reduce the basal PA levels of BCE cells. The postculture medium was collected, centrifuged at 400 g for 10 min to remove cells and cellular debris, and stored at −20°C. The cell monolayers from duplicate cultures were washed three times with cold PBS, scraped into PBS, and pelleted by centrifugation at 400 g for 10 min. The cell pellets were solubilized in 250 μl of 0.1 M Tris-HCl, pH 8.1, 0.5% Triton X-100 and frozen at −20°C until total cell protein and PA assays were performed. Protein determinations were performed by the method of Lowry et al. (18) using BSA as a standard.

Plasminogen Activator Assay

Aliquots of Triton X-100-extracted cells (usually 0.5–1.0 μg cell protein) were added to 500 μl of 0.1 M Tris-HCl, pH 8.1, containing 125 μg BSA and 4 μg of calf plasminogen (5). This mixture was added to dishes coated with ^{125}I-fibrin and incubated at 37°C for 1–2 h (5). Aliquots (100 μl) were removed and counted in a Packard 5210 gamma scintillation counter. The amount of radioactivity released by BCE plasminogen activator was related to the amount released by 0.001 Plough units of human urokinase standard run with all assays (Leo Pharmaceutical Products, Ballerup, Denmark). The reaction was stopped when <20% of the total radioactivity was released from the plates. Plasminogen activator assays of serum-free conditioned medium were performed as described above except that aliquots of medium were used rather than cell lysates.

Molecular Weight Determinations of PA

SDS PAGE was performed as described by Crowe et al. (5). Samples were mixed with an equal volume of nonreducing sample buffer and loaded on an SDS 9% polyacrylamide slab gel with a 3% polyacrylamide stacking gel. Electrophoresis was performed at 10 mA. After electrophoresis was completed, the gel lanes were sliced into 1-mm sections. The slices were incubated for 18 h in 250 μl of 0.25% Triton X-100 in water. An equal volume of 0.2 M Tris-HCl, pH 8.1, with 250 μg BSA and 8 μg of calf plasminogen was added to each tube. The contents of each tube were assayed for PA activity as above.

Molecular weight standards consisted of iodinated BSA, aldolase, and chymotrypsinogen and were applied to tandem lanes in the same gel under reducing conditions. The gel was cut into 1-mm slices and each slice was counted for radioactivity in the gamma counter.

Concanavalin A –Sepharose Chromatography

A column (1 ml) of concanavalin A (Con A)–Sepharose was equilibrated with 1 M NaCl containing 3.4 mM phosphate, pH 7.5, and 0.01% (vol/vol) Tween-80. Sample elution was carried out with 0.6 M α-methylmannoside in the same buffer. Fractions of 600 μl were collected and 25-μl aliquots were assayed for PA activity. All column procedures were performed in the cold. Serum-free conditioned media from confluent BCE control and TPA-treated (2.3 × 10^{-7} M) cultures were the sources of bovine PA. Cells were grown to confluence in complete tumor-conditioned medium, incubated for 18 h in medium containing 10% acid-treated calf serum (without plasminogen), and maintained for 23 h in DME containing 10% tryptose phosphate broth with or without TPA. The latter conditioned medium was collected, centrifuged at 400 g, and frozen at −70°C until use. Tissue-type PA partially purified from serum-free conditioned medium from Bowes human melanoma cells was kindly provided by D. Weil (New York University Medical Center). Human urokinase was obtained from Leo Pharmaceutical Products. All samples were dissolved in or dialyzed into the equilibrating buffer and were applied to the column in a total volume of 5 ml. The PA activity of the applied samples ranged from 5 to 25 Plough units.

Collagenase Assay

Postculture media were thawed and activated as described by Moscatelli et al. (19). Latent collagenase was activated by treating aliquots of medium (100 μl) with trypsin (0.5 mg/ml) for 30 min. The trypsin was inactivated by adding soybean trypsin inhibitor to a final concentration of 5 mg/ml. For each sample containing >5% serum, the concentration of trypsin necessary to achieve complete activation of collagenase was determined. The collagenase in the activated conditioned media was assayed for its ability to cleave ^{125}I-collagen-Sepharose, according to the method of Moscatelli et al. (19). One unit of collagenolytic activity is defined as the amount of collagenase required to degrade 1 μg of collagen in 1 h at 25°C.

Chemicals

RESULTS

Cloned adrenal BCE cells, isolated by the procedure of Folkman et al. (8), were judged to be microvascular endothelial cells by the following criteria. First, the cells had a typical nonoverlapping cuboidal morphology and contained large nuclei with prominent nucleoli (Fig. 1 A). Bovine capillary and aortic endothelial cells approaching confluence had similar cuboidal morphologies, as shown in Fig. 1 A and C. Second, all of the BCE cells produced Factor VIII–related antigen, as determined by indirect immunofluorescence. Third, BCE cells required tumor-conditioned medium for optimal growth (8). All experiments were performed only with cells derived from clones which displayed all three of these properties and thus were considered to be authentic capillary endothelial cells.

Protease Production by BCE Cells

Confluent cultures of BCE cells maintained under normal culture conditions exhibited levels of cell-associated PA activity comparable to and usually greater than the PA activity of aortic endothelial cells (13, 14) (Table I). The basal amounts of PA found in BCE and BAE cells were relatively high compared to other nontransformed cell types, such as BES fibroblasts. Like BAE cells (15), the cell-associated PA activity of BCE cells in vitro was found to vary with growth conditions, such as cell density, the species and concentration of serum in the culture medium, and the specific clone of BCE cells. Under these same growth conditions, low levels of collagenolytic activity were detected in the medium conditioned by BCE, BAE, and BES cells.

TPA-induced Changes in BCE Cells

Since TPA has been shown to increase PA synthesis (14) in some large vessel endothelial cells, and collagenase in others (19), the ability of TPA to modulate production of both of these enzymes in BCE cells was investigated. Within 3 h after the addition of 10^{-7} M TPA to cultures of BCE, there was a dramatic change in cell shape (Fig. 1 A and B). The flattened cobblestonelike cells became more refractile and displayed numerous cell processes. The cells maintained this appearance as long as TPA was present in the culture fluid. Similar morphological changes were observed when BAE cells were treated with TPA (Fig. 1 C and D).

To determine the amount of TPA required to maximally induce protease secretion by BCE cells, confluent cultures were
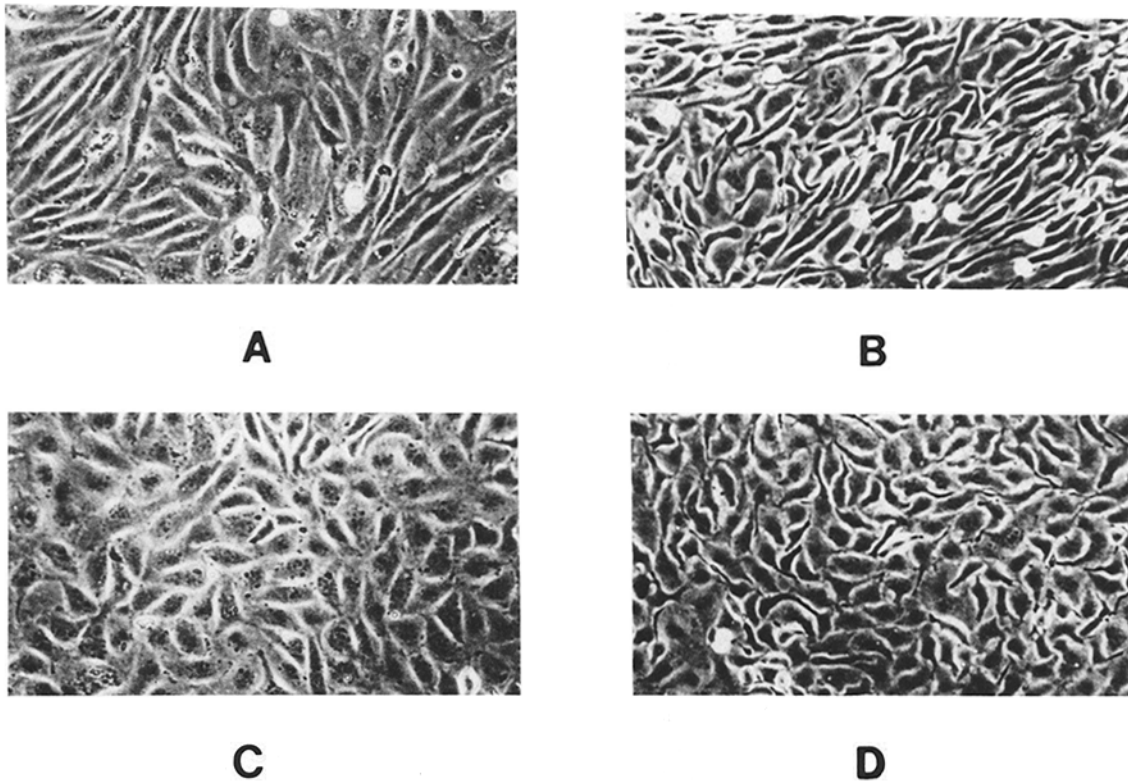

A

B

C

D

FIGURE 1 The morphology of unstimulated and TPA-stimulated bovine endothelial cells. Bovine capillary and aortic endothelial cells were grown to confluence in their respective media and incubated with or without 2×10^{-7M} TPA. Photographs of untreated and TPA-treated cultures were taken using a phase-contrast microscope, at $\times$ 125, 2.5 h after the addition of TPA. (A) BCE cell control. (B) BCE cell with TPA. (C) BAE cell control. (D) BAE cell with TPA.

TABLE I

Effect of TPA on PA and Collagenase Production by BCE, BAE, and BES Cells

	PA activity		Collagenase activity	
	−TPA	+TPA	−TPA	+TPA
	mU/μg protein		mU/culture	
Bovine capillary endothelium	7.2	31.0	21.4	72.4
Bovine aortic endothelium	4.7	8.8	10.9	13.5
Bovine embryonic skin	0.0	0.0	11.5	13.6

Confluent cultures of BCE, BAE, and BES cells were prepared for experimental use as described in Materials and Methods. Duplicate cultures were incubated for 23 h with or without 2×10^{-7} M TPA. The culture fluids were collected, activated with trypsin, and assayed for collagenase activity. The cell layers from the same cultures were extracted with Triton X-100 and the extracts were assayed for PA. The data represent the average of duplicate measurements.

incubated for 23 h with various concentrations of TPA. The PA levels in the cell layer and in the medium of cultures incubated with different TPA concentrations are shown in Fig. 2. After the 23-h incubation, most of the PA produced by BCE cells was found in the medium. However, there was an excellent correspondence between the stimulating effects of TPA on the amount of cell-associated PA and on secreted PA with the maximum increase in both cases occurring at 2×10^{-8} M TPA. With 10^{-9} M TPA there was a slight stimulation of cell-associated PA which was not reflected in secreted PA. This apparent difference may be the result of PA inactivation in the medium at low concentrations of PA. In general, the changes in cell-associated PA reflected changes in total PA and could be used conveniently to monitor changes in PA production. Moreover, as has been found with other cells, all of the collagenase produced by BCE cells was secreted into the culture medium; no activity was found associated with the cell layer. Thus, PA and collagenase could be assayed in the same culture since PA could be measured in the cell layer and collagenase in the culture fluid.

When the PA activity of the cell layer and the total collagenolytic activity secreted into the culture medium were measured, a slight enhancement of both PA and collagenase activities occurred in the presence of 2×10^{-9} M TPA; maximal stimulation of both enzyme activities was obtained with 2×10^{-8} M TPA (Fig. 3). The degree of stimulation of these enzymes was variable from clone to clone but was always at least two- to tenfold above the basal levels of untreated cultures. The reason for this variation is unknown and is presently being investigated. The dose response, with respect to both PA and collagenase production, of BCE cells to TPA was similar to that observed previously for other cells (17, 19, 24).

To investigate whether the increased protease production was the result of an overall increase in RNA and protein synthesis, the rates of RNA and protein synthesis in unstimulated and TPA-stimulated BCE cells were compared. RNA and protein synthesis rates were estimated from the rates of incorporation of ^{3}H-uridine and ^{35}S-methionine into acid-insoluble material. Untreated BCE cells and BCE cells that had been exposed to TPA for 20 h were given a 2-h pulse of ^{3}H-uridine or a 5-h pulse of ^{35}S-methionine. The amount of ^{3}H-uridine or ^{35}S-methionine incorporated into acid-insoluble material was determined by standard procedures (5). In TPA-treated cultures, 25% less ^{3}H-uridine was incorporated into acid-insoluble material than in untreated cultures. However,

there was no difference between TPA-treated and untreated BCE cells in the incorporation of ^{35}S-methionine. Thus, the increased production of proteases cannot be attributed to an overall increase in the rate of protein synthesis.

To investigate the effects of TPA analogs on PA and collagenase production by BCE cells, BCE cells were incubated for 24 h with three analogs. The active tumor promoter phorbol-12,13-didecanoate (PDD) stimulated cell-associated PA and collagenase activities to the same extent as TPA (Table II). Two analogs of TPA which are inactive as tumor promoters, 4-O-methyl-12-O-tetradecanoyl phorbol-13-acetate (4-O-methyl TPA) and 4-α-phorbol-12,13-didecanoate (4-α-PDD), had little or no effect on these activities at 2×10^{-7} M (Table II). Thus, the specific induction of PA and collagenase by analogs of TPA correlated with their tumor promoting potential.
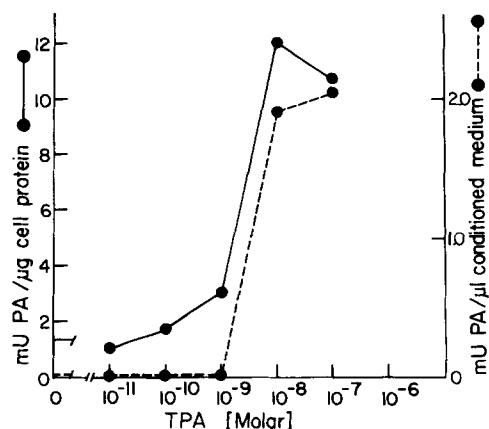

FIGURE 2 Induction of cell-associated and secreted PA by TPA. BCE cells were grown to confluence and prepared for experimental use as described in Materials and Methods. The cultures were incubated for 24 h in serum-free DME containing the indicated concentrations of TPA. The culture fluids from duplicate cultures were collected and assayed for secreted PA. The cells from the same cultures were extracted with 0.1 M Tris, pH 8.1, 0.5% Triton X-100 and the extracts assayed for cell-associated PA.
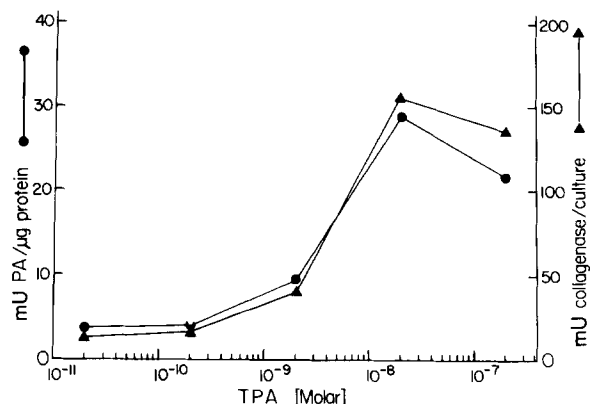

FIGURE 3 TPA-induced PA and collagenase production by BCE cells. BCE cells (passage 8) were grown to confluence in complete tumor-conditioned medium and prepared for experimental use, as described in Materials and Methods. The cultures were incubated for 23 h in DME with 5% calf serum (depleted of plasminogen) and various concentrations of TPA. The culture fluids from duplicate cultures were collected, activated with trypsin, and assayed for collagenase. The cells from the same cultures were extracted with Triton X-100 and the extracts were assayed for PA.

Kinetics of Protease Production

The time course of the TPA-induced stimulation of PA and collagenase production by BCE cells was determined. Confluent BCE cultures were incubated with fresh media with or without 2×10^{-7} M TPA, and at regular intervals the cell-associated PA and secreted collagenase levels were measured. The PA activity of unstimulated BCE cells increased slightly over the 25-h period (Fig. 4): the magnitude of this increase was variable from experiment to experiment. However, when BCE cells were exposed to TPA, a significant increase in PA activity above the values obtained with unstimulated BCE cells was detected by 7.5 h.

Unstimulated BCE cells secreted low levels of collagenase (Fig. 4). However, within 19 h after the addition of the tumor promoter, increased amounts of collagenase activity were detected in the culture fluids of TPA-treated cells (Fig. 4). The collagenase continued to accumulate in the medium of TPA-treated cultures for up to 48 h. A second dose of TPA did not further enhance the rate of collagenase secretion, suggesting that the cells were synthesizing collagenase at a maximum rate.

Table II

Effect of TPA Analogs on BCE Cell PA and Collagenase Production

	PA activity	Collagenase activity
	mU/µg protein	mU/culture
Control (untreated)	6.6	10.5
TPA	21.7	136.0
PDD	24.1	131.0
4-O-methyl TPA	6.7	16.5
4-α-PDD	5.8	15.8

Confluent BCE cell cultures on 35-mm dishes were exposed for 23 h to TPA or its analogs at a concentration of ~2 × 10⁻⁷ M. The culture fluids from duplicate cultures were collected, activated with trypsin, and assayed for collagenase activity. The cell layers from the same cultures were extracted with detergent and the extracts were assayed for PA. The data represent the average of duplicate measurements.
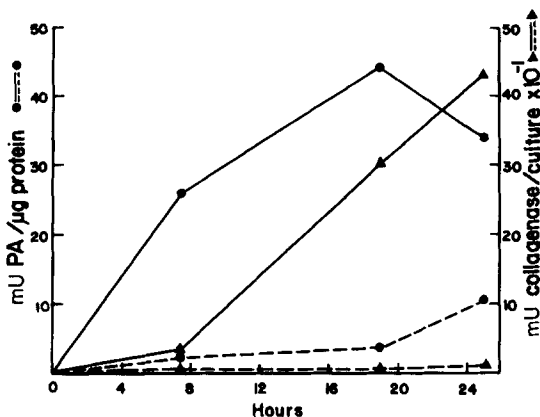

FIGURE 4 Time course of TPA stimulation of PA and collagenase production by BCE cells. BCE cells (passage 8) were grown to confluence in complete tumor-conditioned medium and prepared for experimental use, as described in Materials and Methods. Duplicate cultures in 35-mm culture dishes were incubated with or without 2 × 10⁻⁷ M TPA in DME containing 5% calf serum depleted of plasminogen. At 7.5, 19, and 25 h after the addition of TPA culture fluids were collected, activated with trypsin, and assayed for collagenase. The cells from the same cultures were extracted in Triton X-100 and the extracts were assayed for PA. Broken lines represent untreated cultures; continuous lines represent TPA-treated cultures.
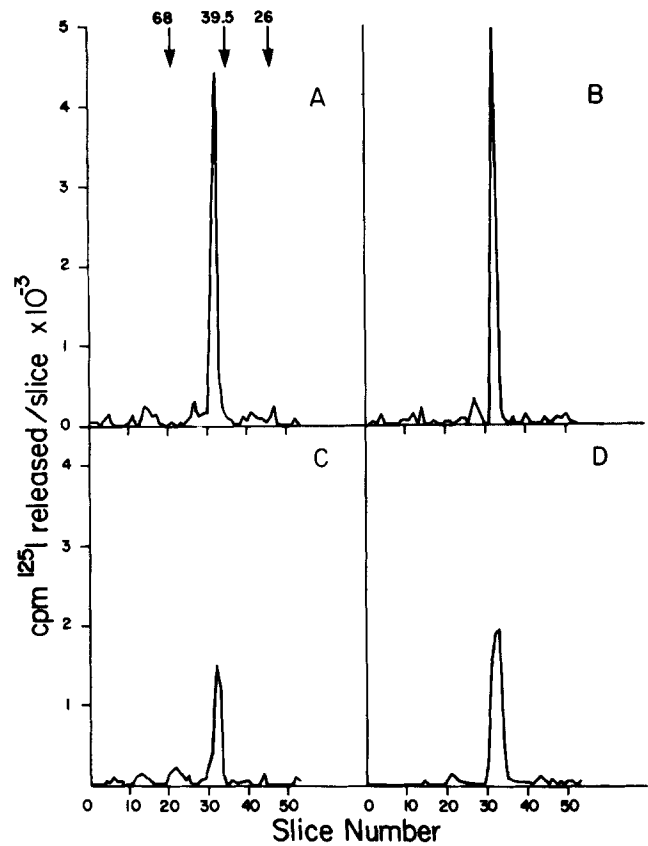
FIGURE 5   PAGE of PA from BCE and MDBK cells. BCE and MDBK cells were grown in their respective media for 24 h with or without 2 × 10⁻⁷ M TPA. The cells were washed, collected, and solubilized in Triton X-100. Aliquots of the cell extracts (20–60 mU PA) were applied to SDS 9% polyacrylamide slab gels and run under nonreducing conditions. The gel lanes were sliced (1 mm) and each slice was assayed for PA activity, as described. Iodinated molecular weight standards were applied to the same gel, under reducing conditions. (A) BCE cell control. (B) BCE cell with TPA; (C) MDBK cell control. (D) MDBK cell with TPA.

The ability to detect increased PA production before detecting an increase in collagenase production (Fig. 4) may reflect the greater sensitivity of the PA assay compared to the collagenase assay (19, 20, 23).

Characterization of BCE Cell PA and Collagenase

Two classes of immunologically noncross-reactive PA have been described, the tissue-type and the urokinase-type (21). The tissue-type PA has a greater molecular weight than the urokinase-type PA, is a glycoprotein, and binds to fibrin (3, 21). Urokinase differs from tissue-type PA in its sugar content and does not bind to fibrin. Since tissue-type PA has been isolated from the plasma of cadavers and has been presumed to be a product of endothelial cells, the type of PA made by untreated and TPA-stimulated BCE cells was investigated.

The PA synthesized by both unstimulated and TPA-stimulated BCE cells appears to be predominantly the urokinase-type based on the following data. The apparent molecular weight of the cell-associated PA from untreated (Fig. 5A) and TPA-stimulated (Fig. 5B) BCE cells, as judged by SDS PAGE, was 42,500 ± 1,500 daltons. This is in close agreement with the estimated molecular weight of the urokinase-type PA produced by BAE cells in vitro (15, 26). Moreover, the molecular weight
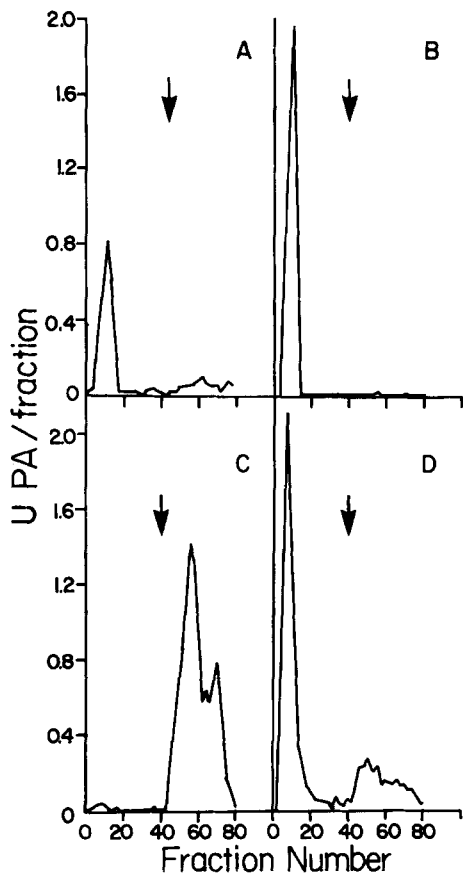
FIGURE 6 Con A–Sepharose chromatography of PAs. Samples with PA activity (5–25 U) were applied to a column of Con A–Sepharose. The arrow indicates the start of α-methylmannoside elutions. Fractions were assayed for PA as described in the text. (A) control BCE conditioned medium, (B) TPA-treated BCE conditioned medium, (C) partially purified conditioned medium from human melanoma cells, and (D) human urokinase.

of the BCE PA was identical to the molecular weight of the PA produced by untreated (Fig. 5C) and TPA-stimulated (Fig. 5D) Madin-Darby bovine kidney (MDBK) cells. This is suggestive evidence that the BCE cell PA is the urokinase-type, since other species of kidney cells have been shown to synthesize the urokinase-type PA in vitro (1). Furthermore, like human urokinase, the BCE cell PA is not retained by Con A–Sepharose, while human tissue activator binds to this resin (Fig. 6). While not conclusive, the present data suggest that under these in vitro culture conditions BCE cells elaborate predominately a urokinase-type PA. Experiments are now in progress to further substantiate that the major BCE cell PA is urokinase-type.

The collagenase synthesized by TPA-stimulated BCE cells was also characterized. All of the collagenase was secreted in a latent form and could be activated with trypsin or with the sulfhydryl reagent mersalyl (19). At 25°C, the activated BCE collagenase cleaved iodinated type I guinea pig skin collagen at a single site, resulting in the characteristic TC_A and TC_B fragments which are three-quarters and one-quarters, respectively, of the length of the intact molecule (Fig. 7). Table III lists the effects of protease inhibitors on the activity of the BCE cell collagenase. The collagenase appeared to be a metalloprotease, as it was inhibited by EDTA but was unaffected by inhibitors of serine proteases (antipain, DFP, Trasylol^R), aspartate proteases (pepstatin), or thiol proteases (N-ethylmal-
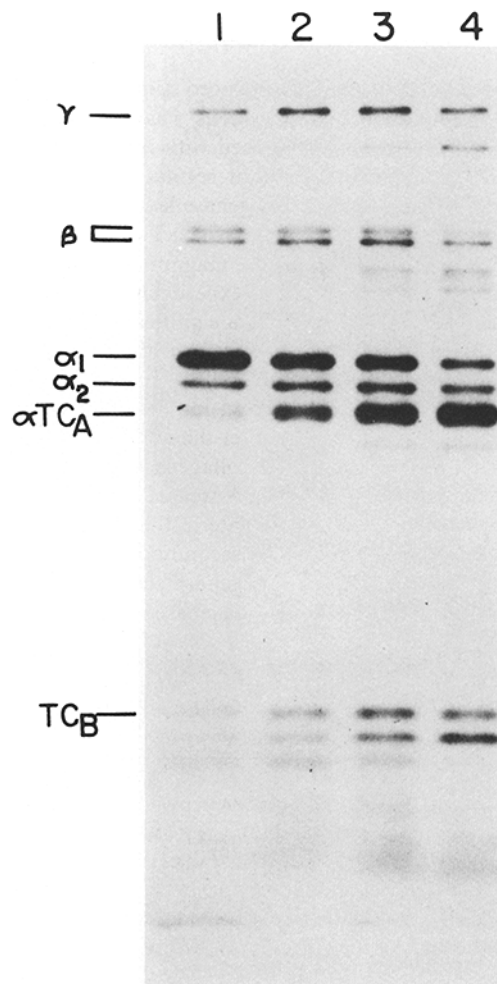

FIGURE 7 Digestion of collagen by BCE cell collagenase. BCE cells were incubated with 10^{-7} M TPA for 24 h. The medium was removed from the cultures and activated with trypsin and the trypsin was inhibited with a tenfold excess of Trasylol^R. Aliquots of the medium containing 12 mUnits of collagenase activity were mixed with 3 × 10^{-2} μCi ^{125}I-guinea pig skin collagen (0.35 μCi/μg) and incubated at 25°C for various periods of time. The samples were then reduced and boiled, loaded on an SDS 5–16% polyacrylamide gradient gel, and electrophoresed. The dried gel was exposed to Kodak X-O-mat film for 72 hr at −70°C. Lanes: 1, control; 2–4, samples incubated at 25°C for 2.5, 5, and 12 h, respectively.

TABLE III

Effect of Protease Inhibitors on BCE Cell Collagenase Activity

Additions	Activity remaining
	%
None	100
EDTA (10 mM)	0
Bovine calf serum	
(1% vol/vol)	5.9
(2% vol/vol)	0
Antipain (100 μg/ml)	105
DFP (3.75 mM, added twice)	87.0
Trasylol^R (100 μg/ml)	99.4
Pepstatin (100 μg/ml)	90.7
N-Ethylmaleimide (10 mM)	93.6

Activated post-culture medium (0.1 ml) containing 5.5 mU of BCE collagenase activity was mixed with 0.4 ml of 0.1 M HEPES, pH 7.6, 5 mM CaCl$_2$, 0.5 mg/ml BSA, and the indicated concentrations of protease inhibitors. Collagenase activity was measured after incubating the samples at room temperature for 30 min.

eimide) (Table III). The enzyme was inactivated by collagenase inhibitors present in calf serum (Table III). Thus, the collagenase secreted by TPA-treated BCE cells had properties similar to those of collagenases obtained from other tissues (12) and from human umbilical vein endothelial cells (19).

Protease Activities in Other Cells

The ability of TPA to induce PA and collagenase activities in BCE cells was compared to its ability to induce these enzymes in BAE cells and BES fibroblasts. BCE and BAE cells and BES fibroblasts were grown under identical conditons and incubated in the presence or absence of 2×10^{-7} M TPA for 23 h. The cell-associated PA levels of both untreated and TPA-treated cultures are shown in Table I. As mentioned previously, BCE cells and BAE cells from untreated cultures exhibited relatively high basal levels of PA compared to PA in BES fibroblasts. The addition of TPA to the culture medium resulted in a two- to fourfold stimulation over basal levels of PA in BCE and BAE cells but had no effect on PA in BES fibroblasts. Although BAE cells have been reported to produce high levels of PA, BCE cell levels were even higher, both in the presence and absence of TPA (Table I). BES fibroblasts produced little or no PA under either condition.

The amount of collagenolytic activity released into the culture fluids by each of these cell types are shown in Table I. BCE cells were the only cells to produce significantly increased amounts of activity in the presence of TPA compared to untreated cultures. It is possible that the low levels of collagenolytic activity associated with BAE cells may be related to the production of a collagenase inhibitor. BAE cells were found to secrete an inhibitor of collagenase, but we were unable to detect an increase in the secretion of this collagenase inhibitor when cells were treated with TPA (Fig. 8). BCE cells did not produce an inhibitor of collagenase when assayed under these same conditions. The inhibitor produced by the BAE cells is different than that associated with the latent collagenase, as trypsin treatment of the culture fluid did not inactivate the inhibitor. The lack of a significant increase in inhibitor levels after exposure of the cells to TPA would argue that the lack of effect of TPA on BAE cell collagenolytic activity is not the result of an excess production of collagenase inhibitor. How-
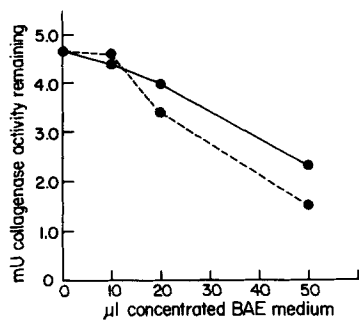
FIGURE 8 Inhibition of BCE collagenase by BAE postculture medium. BAE cells were grown to confluence and prepared for experimental use as described in Materials and Methods. Four plates of BAE cells were incubated for 24 h in DME without serum. The medium on two of the plates also contained 10^{-7} M TPA. The culture fluids were collected, dialyzed, concentrated tenfold, and assayed for collagenase inhibitory activity. The concentrated postculture medium was mixed with 4.6 mU of trypsin-activated BCE collagenase in 0.4 ml 0.1 M HEPES, pH 7.6, 5 mM $CaCl_2$, 0.5 mg/ml BSA and incubated at room temperature for 30 min. The collagenase activity remaining in the sample was determined. The continuous line represents the residual collagenase activity in samples containing concentrated postculture medium from untreated BAE cells. The broken line represents the residual collagenase activity in samples containing concentrated postculture medium from TPA-treated BAE cells.

ever, the coordinate increase in both collagenase and inhibitor cannot be excluded. However, the ability of BCE cells to increase the production of collagenolytic activity in response to tumor promoters is a major difference between endothelial cells derived from bovine adrenal capillaries and bovine aortae.

DISCUSSION

In this paper we have characterized the modulation by TPA of collagenase and PA production by BCE cells. TPA causes a two to tenfold increase in the amount of enzymatic activity produced by BCE cells. The PA produced appears to be of the urokinase-type as determined by its molecular weight and its inability to bind to Con A–Sepharose. The rather high basal levels of PA production by BCE cells was surprising but are consistent with the previous reports of high basal levels of PA synthesized by aortic endothelial cells from several animal species (13, 14). The collagenase produced is found exclusively in a latent form. This is in agreement with what has been observed with other types of endothelial cells (20) and appears to be a property of most collagenases produced in vitro (12). We have not yet been able to detect either a type IV or type V collagenase activity in TPA-stimulated BCE cell culture fluids (Moscatelli, D., unpublished observations). This may be due to the fact that these two enzymes are normally produced in very low amounts and/or are membrane bound. More sensitive methods of detection may indicate that TPA stimulates the synthesis of types IV and V collagenase as well. Experiments to test this are currently in progress.

The TPA-induced stimulation of collagenase and PA activities was rapid and was also seen with PDD, an active analog of TPA. In contrast, nontumor promoting derivatives such as 4-O-methyl TPA and 4 α-PDD did not stimulate enzyme production.

The inability of TPA to stimulate collagenolytic activities in cultures of BAE cells is extremely interesting, for this may indicate an important physiological difference between BCE and BAE cells in vitro. The precise nature of the difference between BCE and BAE cells with respect to collagenase production is unknown. We found that BAE cells secrete an inhibitor of BCE collagenase. TPA might simulate the coordinate production of both collagenase and collagenase inhibitor resulting in no significant increase in collagenolytic activity even though there was an increase in enzyme production. At the present time our techniques do not permit us to differentiate between increased production of enzyme and increased production of activity. However, regardless of the mechanism for the lack of response of BAE cells in respect to collagenolytic activity, the differences between the responses of BAE and BCE cells to TPA differentiate the two cell types.

The responses of BCE cells is also significantly different from the human umbilical cord endothelial cells studied previously (19). These human cells responded to TPA by increasing the production of collagenolytic activity with no increase in PA production. Therefore, only BCE cells demonstrate an increase of both collagenolytic and PA activities after exposure to TPA.

Several differences between the growth requirements of capillary and large vessel endothelial cells have been described. Folkman et al. (8) demonstrated that BCE cells required tumor-conditioned medium for survival and proliferation, whereas BAE cells grew at equal rates in regular or tumor-conditioned medium. Davison and Karasek (6) found a growth-promoting

effect of dibutyryl cAMP on human dermal foreskin microvascular endothelial cells in vitro, in marked contrast to the lack of response of human umbilical vein endothelial cells to this drug. Based on the known functional differences of capillary and large vessel endothelial cells, biochemical differences between these two cell types are not surprising. There are probably many other biochemical differences which remain to be described.

We have proposed that the stimulation of angiogenesis by specific molecules requires the secretion of latent collagenase and PA (20). With respect to this model, two aspects of the results described in the present paper should be emphasized. First, BCE cells are capable of synthesizing increased amounts of both latent collagenase and PA when exposed to TPA. This is in contrast to two other types of endothelial cells, BAE cells and human umbilical vein endothelia cells, which do not exhibit the coordinated increase in production of both enzymes in response to TPA. Human umbilical vein endothelial cells increase only the production of latent collagenase when exposed to TPA (19), while BAE cells increase only PA synthesis when exposed to TPA (14).

Second, the ability of BCE cells to respond to angiogenic stimuli can potentially be used to identify and purify angiogenic stimulators. In this regard, it should be stated that TPA has not been described to be an angiogenic agent. However, given the strong inflammatory nature of this compound, it would not be surprising if it were angiogenic. Since the sarcoma 180 cells used to condition the growth medium used for BCE cells do produce an angiogenic factor (25), we were somewhat surprised at the rather low levels of proteases produced by control BCE cells. This may be due in part to a reversal of the stimulation during the 1-d conditioning period before assays were initiated as well as the fact that the angiogenic factor may be produced in less than optimal amounts under the conditions used to normally culture the sarcoma 180 cells. Experiments to test the effects of bona fide angiogenic stimulators on protease production by BCE cells as well as other aspects of this model are currently underway.

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