Plasminogen Activator Production by Human Retinal Endothelial Cells of Nondiabetic and Diabetic Origin

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The authors examined the effect of insulin-like growth factor I (IGF I), epidermal growth factor (EGF), and acidic fibroblast growth factor (AFGF) on the synthesis by human retinal endothelial cells (HREC) of plasminogen activators (PA; tissue-type [t-PA] and urokinase-type [u-PA]) and plasminogen activator inhibitor (PAI). Immunologic and functional assays for t-PA, u-PA, and PAI were conducted with cell lines derived from three diabetics and three nondiabetic controls. Confluent HREC of nondiabetic origin did not respond to IGF I (100 ng/ml) with any change of t-PA antigen in the medium (10.7 ± 1.1 ng/ml unstimulated versus 10.1 ± 0.8 ng/ml stimulated, \( P \) not significant). Likewise AFGF and EGF caused no significant change of t-PA levels. Both IGF I and EGF caused a significant increase of t-PA from HREC of diabetic origin (9.6 ± 0.8 ng/ml unstimulated versus 16.6 ± 1.9 ng/ml IGF I-stimulated, \( P < 0.001 \), and 14.6 ± 2.7 ng/ml EGF-stimulated, \( P < 0.005 \)). Supplementation of AFGF had no effect on HREC of diabetic origin. In confluent cultures, only small quantities of u-PA were detected. After wounding confluent cultures, u-PA activity was associated with cells migrating from the wound edges. Functional PA activity was also measured by chromogenic assay. Results further supported a predominance of t-PA activity being produced by confluent HREC in culture. These results suggest that modulation of PA production by HREC is influenced by exposure to growth factors, by the state of confluency, and the origin of the cells (diabetic versus nondiabetic).

Neovascularization, whether it occurs in the eye or other tissues, develops as a series of sequential steps. The earliest step identified morphologically is the local degradation of basement membranes of the parent vessel, usually a small capillary. This degradation causes gaps in the basement membrane and permits egress of vascular endothelial cells, which then migrate from the wall of the existing vessel to form new vascular sprouts. Dissolution of the basement membrane requires production by vascular endothelial cells of several enzymes, including plasminogen activators (PA). These convert plasminogen to the serine proteinase, plasmin, which causes proteolysis of many extracellular matrix components, including laminin and fibronectin. Plasmin can also activate procollagenase.

Most investigators agree that the endothelial cell alone is capable of expressing all the information required for forming a new vascular network. Circulating hormones and locally produced growth factors modulate both protease production and endothelial cell migration.

Previous studies from our laboratory suggest the involvement of insulin-like growth factor I (IGF I) in diabetic retinopathy. We found that (1) IGF I concentrations in the vitreous of diabetics are greater than in controls, (2) both serum and vitreous IGF I concentrations are greater when proliferation is proceeding at a rapid rate, and (3) vitreous concentrations of IGF I in diabetics are within a concentration range known to affect multiplication and differentiation of several cell types. Additional supporting evidence for this postulate includes King et al.'s observation that retinal endothelial cells, but not endothelial cells from the aorta, have specific receptors for IGF I and respond to it with a threefold increase in the incorporation of tritiated thymidine into DNA. More recent studies from our laboratory indicate that IGF I also has a chemotactic effect on endothelial cells.

The current study considers the influence of IGF I and other selected growth factors on the synthesis of...
PAs and their natural inhibitor (PAI) by human retinal endothelial cells (HREC). These effects were compared in HREC derived from nondiabetic and diabetic subjects.

Materials and Methods

Source and Preparation of Cultures

Human eyes were obtained from the National Disease Research Interchange within 36 hr of death. Only tissue from persons less than 65 years of age was used, and for these studies, we used three pairs of eyes from diabetics and three pairs from control subjects. The diabetics were known to be insulin dependent with proliferative retinopathy. On arriving in the laboratory, the eyes were soaked in minimal essential medium (MEM) containing 2% amphotericin B (Gibco, Grand Island, NY), 2% streptomycin-penicillin, and 1% gentamicin at 4°C for 30 min. The eyes were placed on sterile gauze, and 3 ml of povidone-iodine solution (Sherwood, Mahwah, NJ) was pipetted over each eye. After 3 min, the globe was punctured approximately 2 mm from the limbus, a circumferential incision was made, and the anterior chamber was removed. The liquified vitreous was then removed using a pipette, and a vitreous spatula was used to loosen the vitreous adherent to the anterior retina.

When all the vitreous was removed, the retina was gently removed from the layer of retinal pigment epithelium and cut at the optic nerve. The retina was then removed with surgical tweezers and placed on a 53-μm nylon mesh filter (Fisher, Lexington, MA). Both retinas were placed on one filter and washed with 400–500 ml of MEM containing antibiotics and an antifungal agent as described. The retinas were then chopped into small pieces and pipetted into a 25-ml flask containing 1 U/ml of collagenase (Sigma, St. Louis, MO) in MEM containing 5% bovine serum albumin. Retinas were then mechanically agitated using a magnetic stirring bar and allowed to digest for approximately 60–70 min or until no tissue fragments could be seen. Typically, the “clear” MEM became slightly opaque due to the digested retina. Collagenase was neutralized by the addition of 20 ml of MEM containing 20% horse plasma. The cells were then washed free of tissue PA (t-PA) Studies

Immuno logic staining: For these studies, HREC were sparsely plated in 25-cm² flasks and allowed to attach. To prevent distortion of cell morphology, Bouin’s solution was immediately added to the flasks to create a 1% solution of fixative in the growth medium of cells. After fixation for 1 hr the flask was washed three times with phosphate-buffered saline (PBS), and the rabbit anti-human t-PA antibody
(American Diagnostica, New York, NY) was added for 30 min. The antibody was diluted 1:100 with PBS. To the control flask unimmunized rabbit serum was added at the identical dilution as the antibody. Each flask was then rinsed in three changes of PBS, followed by a 3% solution of hydrogen peroxide for 5 min. This once again was followed by a rinse (three times) in PBS. Protein-A-Peroxidase (Boehringer Mannheim Biochemicals, Indianapolis, IN) prepared at a 1:200 dilution in PBS was then added to the flask for 30 min, followed by a rinse in three changes of PBS. A 0.004% diaminobenzidine solution was added for 10 min followed by a water wash for 5 min.17

Quantitative immunologic assay: After the cells reached confluency, the conditioned medium was collected for assays in the following manner. First, the cells were washed and then incubated for 24 hr in 2 ml of serum-free medium in the presence and absence of the test substance. At the end of the incubation, the culture medium was collected. The cells were washed three times with warm PBS. From selected dishes, cells were harvested by scraping them into 0.5 ml of 0.2% Triton X-100 (Sigma) in 0.1 M Tris HCl (Schwarz Mann Biotech, Cleveland, OH). The cells in the remaining dishes were used for determination of cell number. The culture medium and cell lysates were centrifuged. Aliquots (15 ml) of both the culture medium and the cell lysates were used in the immunologic assay.

The Immubind-5 t-PA enzyme-linked immunosorbent assay (ELISA; American Diagnostica) was used to quantify t-PA antigen. This kit measures both free activator antigen and circulating activator-inhibitor complexes. It uses the double-antibody principle, which is unique for its design in using quenching and normal antibodies as a control for t-PA specificity. This method allows the elimination of false positive results when measuring t-PA antigen in test samples. The assay measures t-PA antigen in a range of 0.015–0.3 ng/well. Maximal sensitivity is 0.07 ng t-PA/ml, which allows for considerable dilution if necessary.

Undiluted conditioned medium (10 μl) was added to a well prefilled with goat anti-human t-PA immunoglobulin and incubated for 3 hr. During the incubation period the t-PA antigen in the sample binds to the anti-t-PA immunoglobulin. Horseradish peroxidase (HRP)-labeled anti-t-PA is added in the next step and incubated for 2 hr. The conjugate binds to free antigenic determinants on the t-PA molecule and formed a coat–antibody t-PA-conjugate sandwich. The wells were washed thoroughly to remove excess conjugate and other bound material. The HRP substrate was then added. The rate at which the substrate was converted to a colored product was proportional to the amount of HRP conjugate bound to u-PA. To determine the amount of u-PA in the cell lysate or conditioned medium, the samples were plotted against a standard curve generated with commercial u-PA.19 The antigen is expressed as ng/ml of medium conditioned by 10^6 cells, and its levels for the cell lysates are expressed as ng of antigen/mg of protein.

PAI

The F1-5 ELISA (Monozyme, Charlottenlund, Denmark) was used to quantify PAI-1 antigen. This method is based on the double-antibody principle and determines only free PAI-1, not complexes of PAI-1 and t-PA. The detection limit of this assay is 1 ng/ml of PAI-1. All samples were diluted 1:4 to fall within the detection limit of the assay.18 The PAI antigen is expressed as ng/ml secreted by 10^6 cells. All immunologic data were evaluated by student t-test and expressed as mean ± standard deviation.

Wounding and Substrate Overlay of Confluent Monolayers

This technique allows for direct visualization of proteolysis around migrating cells.20,21 Confluent monolayers of HREC in 35-mm culture dishes were wounded with a 5-mm wide rubber policeman.
Wounded cultures were washed three times with PBS, and fresh complete medium was replaced. Twenty-four hours after wounding, monolayers were fixed with 95% ethanol for 2 min at room temperature and then overlaid with a mixture containing 2% casein, 0.8% agar, plasminogen, amiloride (1 mM), or affinity-purified anti-human t-PA antibodies (10 μg/ml). Amiloride or anti-human t-PA antibodies were added to the overlay mixture to characterize whether t-PA or u-PA was being produced by wound-edge cells. In selected experiments, imipenem C (10 μg/ml) was added to inhibit cell division.

PA Activity Assay

The PA activity was quantified by incubating acidified samples of conditioned medium with a plasmin-specific chromogenic substrate and measuring enzymatic activity. The conditioned medium collected for the immunologic analysis was subsequently analyzed in this functional assay. Cells were at confluence when the serum-free medium was removed for analysis. A modification of the assay described by Chmielewska et al. was used. To a 100-μl aliquot of conditioned medium, 5 μl of 50% glacial acetic acid was added, and samples were incubated for 10 min at room temperature. A 10-μl aliquot of the sample was diluted with 490 μl of 0.05 M Tris buffer (pH 8.1) containing 0.01% Tween 80 (BioRad, Richman, CA), purified human plasminogen, and a chromogenic substrate, H-d-norleu-l-hexahydrotyr-l-lys-pNA (American Diagnostica). Ten microliters of cyanogen bromide fragments of fibrinogen was added. A standard curve was obtained by adding 490 μl of 0.05 M Tris buffer (pH 3.9) to 0.1 M sodium acetate buffer (pH 3.9) at 20,000 g under conditions developed to optimize the separation of the various cell types. The endothelial cells were collected as clumps of cells at a density of 1.035 g/ml. Exhaustive washing to remove the Percoll completely resulted in improved cell growth. We routinely recovered 5 x 10⁵ cells from a pair of cadaveric eyes with more than 90% of these cells being viable by trypan blue dye exclusion. Cell attachment was significantly improved with the use of fibronectin-coated flasks. Within hours of plating, small islands of endothelial cells attached to the growth surface, but no evidence of actual growth appeared for 3–4 days. However, these cells then demonstrated excellent proliferative capability using the serum- and growth factor-supplemented medium. The use of plasma-derived serum for the culture of microvascular cells was essential in selecting for the growth of endothelial cells because plasma-derived serum does not support the growth of either smooth muscle cells or pericytes.

As shown in Figure 1, cultures maintained for up to ten passages did not show observable morphologic changes or loss of mitotic vigor. No morphologic differences could be detected between cultures of nondiabetic and diabetic origin at the light-microscopic level. Although no attempt was made to determine the maximum number of passages possible or the maximum growth rate, we did not observe increased pleomorphism and declining growth potential until passage 10. With passage numbers greater than 10, an increase in cytoplasm-to-nuclear ratio, increased vacuolization, and decreased mitotic rate occurred. These changes were equally apparent in HREC of diabetic and nondiabetic origin. We used only early passages (3–5) for all experiments.

Results

The initial isolation step yielded short segments of microvessels. Because both endothelial cells and pericytes are present in these microvessels, the isolated microvessels were treated with collagenase to remove the basement membrane and pericytes embedded in the basement membrane. The gradient centrifugation separated the endothelial cells from other cell types and debris. A 50% Percoll gradient was established in small (10-ml) polycarbonate tubes with a Ti50 rotor at 20,000 g under conditions developed to optimize the separation of the various cell types. The endothelial cells were collected as clumps of cells at a density of 1.035 g/ml. Exhaustive washing to remove the Percoll completely resulted in improved cell growth.
Final cell density on reaching confluence averaged $2 \times 10^4$ cells/cm$^2$. The cell identity was verified by the presence of Factor VIII antigen and incorporation of acetylated low-density lipoprotein (Fig. 2). These cells were routinely free of pericyte and pigment epithelial cell contamination as judged by two independent investigators.

Immunohistochemical staining using protein-A-peroxidase with rabbit anti-human antibodies located t-PA mainly in the perinuclear area, representing the Golgi (Fig. 3).

**PA Studies Using Confluent Cultures of HREC**

All studies were conducted with cell lines derived from six subjects (three diabetic and three controls) using immunologic and functional assays for t-PA, u-PA, and PAI.

**Immunologic Studies of t-PA Antigen**

Figure 4 shows the levels of t-PA antigen in conditioned medium of confluent HREC from diabetic and nondiabetic subjects after supplementation of serum-free medium with either IGF I, epidermal growth factor (EGF), or acidic fibroblast growth factor (AGF) for 24 hr. All results are expressed as ng/ml of t-PA antigen secreted by $10^6$ cells. Basal formation of t-PA antigen was similar for HREC of control and diabetic origin. The HREC of nondiabetic origin did not respond to IGF I (100 ng/ml) with any change of t-PA production ($10.7 \pm 1.1$ ng/ml unstimulated versus $10.1 \pm 0.8$ ng/ml stimulated).

**Fig. 2.** Acetylated low-density lipoprotein labeled with 1,1'dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate was added to low-density cultures of human retinal endothelial cells to assess purity of these isolates (original magnification $\times 500$).
Fig. 3. Immunohistochemical staining using protein-A-peroxidase with rabbit anti-human antibodies demonstrating t-PA localization in the perinuclear area representing the Golgi. The nuclei of these cells are unstained as is much of their cytoplasm (original magnification X1000).

Although small decreases of t-PA antigen occurred with EGF and AFGF supplementation, these were not statistically significant. For cells of diabetic origin, IGF I caused a significant increase of t-PA secretion (9.6 ± 0.8 ng/ml unstimulated versus 16.6 ± 1.9 ng/ml stimulated, P < 0.001). Likewise EGF caused an increase in t-PA antigen levels over basal levels (14.6 ± 2.7 ng/ml versus 9.5 ± 0.8 ng/ml, P < 0.01). The AFGF caused no significant increase in t-PA secretion (9.5 ± 0.8 ng/ml unstimulated versus 8.9 ± 0.4 stimulated).

In HREC cultures of diabetic origin, time-course studies of t-PA release showed similar kinetics of accumulation with IGF I and EGF. The accumulation of t-PA antigen in the serum-free medium began several hours after growth factor addition (lag time, 4–6 hr). The t-PA rose rapidly over the first 12–16 hr and more slowly over the subsequent 8–12 hr. Cultures continued to release small amounts of t-PA between 24–48 hr, essentially stabilizing by 24 hr. In the presence of serum (1.25–5%), however, the synthesis and secretion of t-PA antigen could be demonstrated up to 72 hr, at which point the medium was removed and replenished. In this fresh culture medium, the accumulation of t-PA antigen could again be detected. The long-term effect of IGF I supplementation on t-PA antigen production was studied by maintaining cultures in the same flasks for 15 days (representing five media changes). The IGF I-supplemented cultures continued to produce significant amounts of t-PA antigen; however, a minimum of 5% serum was required to ensure cell viability.

The 4–6-hr delay that occurred between growth factor addition and enhanced t-PA antigen release suggested that this increase depended on other metabolic events. To determine whether protein synthesis or RNA synthesis was involved in the stimulation of t-PA release, cultures of diabetic origin were treated with either cycloheximide (2 μg/ml) or dactinomycin (1 μg/ml) at selected times after IGF I (100 ng/ml) addition. At 16 hr the medium was removed, and the amount of t-PA antigen in the medium was determined.

Extremely low levels of t-PA antigen (0.56 ± 0.02 ng/ml) were detected when cycloheximide was added 3 hr after IGF I treatment. The final t-PA concentration (at 16 hr) was 2.2 ± 0.19 ng/ml and 4.1 ± 0.39 ng/ml when the drug was added 6 and 9 hr after IGF I, respectively. The final t-PA antigen concentration in cultures treated with IGF I alone was 13.9 ± 0.51 ng/ml (at 16 hr). Dactinomycin treatment resulted in

Fig. 4. Tissue plasminogen activator antigen levels in conditioned media of human retinal endothelial cells from diabetic and nondiabetic individuals. Following 24 hr of incubation with the test substance of interest, t-PA antigen present in the serum-free conditioned media was measured by ELISA technique. t-PA antigen is expressed as ng/ml of conditioned media secreted by 10^6 cells. Each value represents the mean of nine samples. Cell cultures from diabetic individuals supplemented with either IGF I or EGF produced significantly more t-PA antigen than control cultures. The cultures derived from nondiabetic subjects did not respond with a significant change of t-PA. Error bars represent standard deviations. See Results section for statistical analysis.
lowered t-PA levels (2.5 ± 0.12 ng/ml) when added 3 h after IGF I. A minimal effect was noticed when RNA synthesis was stopped 6 or 9 hr after IGF I addition (12.4 ± 0.63 ng/ml and 13.6 ± 0.58 ng/ml, respectively).

Immunologic Studies of u-PA Antigen

The u-PA antigen levels were measured in the conditioned medium from confluent cultures of HREC, and u-PA levels were uniformly low. Growth-factor supplementation of cultures of nondiabetic origin did not result in an increase of u-PA levels (control 0.49 ± 0.03 ng/ml, IGF I 0.48 ± 0.02 ng/ml, EGF 0.49 ± 0.04 ng/ml, and AFGF 0.51 ± 0.01 ng/ml; \( P = \) not significant for all studies). Growth-factor supplementation of cultures of diabetic origin likewise did not result in a change in u-PA levels compared with basal (data not shown).

Because only low levels of u-PA antigen were detected in conditioned medium and u-PA activity is known to be associated with the cell membrane, u-PA antigen levels were measured in cell lysates from selected experiments and were expressed as ng of antigen/µg of cell protein. Levels of u-PA expression were not different (nondiabetic unstimulated 0.054 ± 0.002 versus IGF I-stimulated 0.085 ± 0.002, \( P = \) not significant; diabetic unstimulated 0.094 ± 0.002 versus IGF I-stimulated 0.088 ± 0.004, \( P = \) not significant).

To determine whether u-PA was present in these cells before culturing, microvessels were isolated from four pairs of eyes (two of diabetic origin and two of nondiabetic origin) and cell lysates prepared and assessed for the presence of u-PA antigen. In these cell lysates, extremely low levels were detected (in the 0.1-ng range).

Immunologic Studies of PAI

The fibrinolytic "state" of endothelial cells is a reflection of the balance between the activities of PAs and those of their natural inhibitors. Agents that alter the fibrinolytic state may do so by changing PA, PAI, or both.\(^{22}\) The inhibitor is an antiactivator and can neutralize the activity of both t-PA and u-PA but not that of plasmin.

As shown in Figure 5, basal production of PAI antigen was greater in diabetic cultures than in nondiabetic cultures (8.5 ± 1.5 ng/ml versus 6.1 ± 0.1 ng/ml, \( P < 0.05 \)), and IGF I augmented PAI antigen secretion in cultures of diabetic origin (11.6 ± 3.4 ng/ml, \( P < 0.001 \)). Supplementation with EGF also increased PAI secretion in cultures of diabetic origin, 12.9 ± 2.6 compared with 8.5 ± 1.5 ng/ml in control cultures, \( P < 0.01 \). However AFGF had no significant effect on PAI antigen levels (7.1 ± 1.9 ng/ml). In HREC of nondiabetic origin, both AFGF and EGF supplementation significantly depressed PAI secretion (nondiabetic unstimulated 6.1 ± 0.01 ng/ml versus AFGF supplemented 3.9 ± 0.4 ng/ml, \( P < 0.005 \), and versus EGF supplemented 3.4 ± 0.3 ng/ml, \( P < 0.001 \)). However IGF supplementation did not significantly affect PAI levels in nondiabetic HREC (6.8 ± 1.0 ng/ml). These studies were done with passage 3–5 cells.

Fig. 5. Plasminogen activator inhibitor antigen levels in conditioned media of human retinal endothelial cells from diabetic and nondiabetic individuals. Following 24 hr of incubation with the test substance of interest, the PAI antigen present in the conditioned media was measured by ELISA technique. IGF I and EGF supplementation resulted in an increase of PAI secretion in diabetic cell lines; AFGF had no effect. AFGF and EGF significantly depressed PAI production from nondiabetic human retinal endothelial cells but not diabetic human retinal endothelial cells. Each value represents the mean of nine samples; error bars represent standard deviation. See Results section for statistical analysis.

Functional PA Activity in Confluent Cultures of HREC

Detection of antigen by ELISA methods allows precise quantitative differences to be detected. It does not, however, give an indication of functional activity. Functional PA activity in conditioned medium and cell lysates was therefore measured by a chromogenic assay.

In HREC of diabetic origin, IGF I and EGF supplementation increased the fibrinolytic activity detected in the HREC conditioned medium as measured by the chromogenic assay. All values were corrected for cell counts and represent the quantity of PA activity produced by 10⁶ cells. The total enzymatic activity found in the conditioned medium was 8.0 ± 0.6 IU/ml in diabetic unstimulated, 10.7 ± 0.8 IU/ml in IGF I-stimulated, 9.8 ± 0.5 IU/ml in EGF-stimulated, and 7.4 ± 0.7 IU/ml in AFGF-stimulated, (\( P < 0.05 \) for IGF I only). In HREC of nondiabetic origin, growth-factor supplementation had a minimal effect on the amount of PA activity.
activity generated (nondiabetic unsupplemented 8.0 ± 0.7 IU/ml, IGF I-supplemented 8.2 ± 0.8 IU/ml, EGF-supplemented 6.8 ± 0.5 IU/ml, and AFGF-supplemented 7.3 ± 0.7 IU/ml, \( P = \text{not significant} \) for all comparisons).

The chromogenic assay measures plasmin generated but does not determine whether the activity is generated by t-PA or u-PA. The ELISA studies support predominantly t-PA generation. However, it was possible that the small quantity of u-PA was actually generating the plasmin detected by the chromogenic assay.

To determine whether u-PA or t-PA was generating the plasmin measured in the colorimetric assay, amiloride was added to inhibit the u-PA-catalyzed hydrolysis of the chromogenic substrate. The PA activity produced by HREC under confluent conditions represents t-PA; there was no change in PA activity in assays with or without amiloride (data not shown).

These studies demonstrated the release of both t-PA and u-PA from these endothelial cells, albeit t-PA in much greater quantities than u-PA when cells were at confluence. The biologic significance of the release of both u-PA and t-PA from these cells is unknown. However, the conditions modifying the expression of one PA versus the other may help elucidate their function. We therefore asked whether these cells could be stimulated to produce u-PA under specific conditions. Confluent layers of endothelial cells were wounded and the monolayers overlaid with a casein-agar mixture containing plasminogen. Twenty-four hours after this mechanical wounding, zones of caseinolysis along the edges of the wound were macroscopically evident as dark bands of lysis against a gray-white background under dark-field illumination.

At higher magnification, the lysis was seen to be confined to the edge of the wounded monolayer and corresponded to an area ten to 15 cells deep (Fig. 6). No lysis was observed when monolayers were overlaid immediately after wounding, demonstrating that lysis was not due to preformed enzyme released by dead or damaged cells lining the wound edge. This wound-associated lysis was evident when the cells were overlaid 24 hr after wounding and persisted until the wound had completely closed.

To determine whether u-PA or t-PA was causing the caseinolysis, the drug amiloride, which competitively inhibits the catalytic activity of u-PA without affecting the activity of t-PA or plasmin, was incorporated into the overlay mixture. These experiments were done on fixed cells to avoid the possible effects of amiloride related to inhibition of the Na+/H+ antiport system. Amiloride incorporated into the overlay completely inhibited caseinolysis along the wound edge (Fig. 7, left). In contrast, affinity-purified anti-human t-PA antibodies did not inhibit wound-edge caseinolysis (Fig. 7, right). Thus, the plasminogen-dependent caseinolysis observed in association with migrating HREC at the wound edge is due to cell-associated u-PA activity in the migrating cells. Wounding has been shown to induce both cell migration and cell division. Wound-edge caseinolysis was observed in cultures treated with mitomycin C
Fig. 7. Cultures were overlaid 24 hr after wounding with a mixture containing casein, agar, plasminogen, and amiloride (left), or casein, agar, plasminogen, and anti-t-PA antibodies (right). Under dark-field illumination, the zones of caseinolysis appear as a dark band (arrows) on a grey/white background, revealing the production of u-PA by the cells migrating from the wound edge. (data not shown). These results suggest that the lysis is not dependent on cell proliferation but is a property of cell migration.

Discussion

Vascular endothelial cells have the ability to release the serine proteinase, PA, which catalyzes the conversion of plasminogen to plasmin. Although other peptidases are recognized, PA is present in the growing vasculature of the embryo and, in addition, can generate other enzymes capable of disrupting matrix such as collagenase III. Because plasmin produced by endothelial cells is postulated to be important in the net turnover of the subendothelial matrix, alterations in control of this enzyme may be important in pathologic processes such as diabetes.

The factors controlling the release of PAs from endothelial cells are poorly understood. Their synthesis and release are inducible cellular functions subject to modulation by hormones and drugs.

The current studies were done to explore the release of PA activity and subsequent generation of plasmin from human retinal microvessel endothelial cells and to examine PA activity in cell cultures derived from diabetic eyes. The production of PA by these cells was assessed in three ways: (1) by measurement of t-PA antigen, u-PA antigen, and PAI antigen in confluent cultures of HREC using ELISA techniques; (2) by measurement of functional PA activity generated in confluent cultures of HREC; and (3) by the wounded monolayer model.

In confluent cultures, the expression pattern of PA was stable; that is, t-PA was always expressed in confluent cultures of endothelial cells derived from different individuals, be they diabetic or non-diabetic. The cells derived from diabetic subjects were maintained under identical culture conditions as those of nondiabetic origin. All experiments were performed in serum-free medium; however, growth factor-supplemented medium was used in the primary isolation of these cells and immediately after plating of the cells. The effect this may have had on the number of receptors for specific growth factors is not known. However, all t-PA and PAI levels were measured under steady-state conditions.

Our comparative data (Fig. 4) were obtained under identical conditions at confluence and in serum-free medium. The HREC of diabetic origin expressed more t-PA, and this level of expression was stable over the passages studied (3-5). These observations suggest that during the finite period of metabolic derangement (lifetime of the individual) cellular modifications occur that propagate a “memory” of the diabetic state and that these cellular modifications can be maintained in culture by isolated cells. These observations are supported by the clinical observations that despite reversal of hyperglycemia, using intensive insulin treatment or pancreatic transplantation, characteristic features of microvascular disease progress or persist.

In conditioned medium from cell lines of diabetic origin, the ELISA method indicates that the t-PA antigen responses after IGF I and EGF supplementation were significantly greater compared with basal levels. However, supplementation with these growth factors also resulted in an increase of PAI levels in the conditioned media. Use of the chromogenic assay also showed the presence of functional activity; however,
PAI was inactivated by acidification before measurement of the plasmin generated. Recent studies by Speiser et al.¹¹ show the activation of plasminogen by t-PA can occur on the surface of microvascular endothelial cells from human omental tissue in the presence of PAI activity. Thus a shift in the secretion rate of t-PA and PAI in favor of free t-PA is not required for the generation of fibrinolytic activity.

The t-PA response using confluent HREC cultures derived from diabetic patients demonstrates that diabetic microvascular cells intrinsically respond to IGF I and EGF stimulation with significantly greater t-PA secretion than nondiabetic microvascular cells. However IGF I supplementation resulted in a significantly greater release of t-PA antigen (compared with unstimulated levels in the cells of diabetic origin) than did EGF.

The synthesis and secretion of t-PA antigen by HREC proceeded linearly for up to 24 hr in serum-free medium; then a plateau was reached. This probably reflects some metabolic constraint placed on the cells by being in the serum-free environment. In serum-containing medium, synthesis and secretion of t-PA was maintained for up to 15 days.

Production of PA activity by endothelial cells can be regulated at various levels. An increase in PA activity may reflect new synthesis of t-PA by the endothelial cells. However, many physiologic experiments indicate a fast release of t-PA from a cellular storage pool. With stimulation of the endothelial cells, an immediate and localized increase in fibrinolytic potential occurs. This did not occur in our experiments; there was a delay (4–6 hr) before enhanced t-PA release. This slow response is consistent with an effect on RNA synthesis, protein synthesis, or a response to specific cell-cycle events that depend on the mitogenic properties of IGF and EGF rather than a release of an available intracellular t-PA pool.

Experiments using cycloheximide indicate that protein synthesis is involved. Simultaneous addition of cycloheximide and IGF I to cultures resulted in a reduction of t-PA levels in the culture medium as measured at 16 hr. In cycloheximide-treated cultures, however, the t-PA concentrations at 3, 6, or 9 hr after the addition of growth factor approximated the levels at these same times in cultures treated with growth factor alone, indicating that the t-PA detected in cycloheximide-treated cultures was released before the drug was added. The suppression of RNA synthesis using dactinomycin resulted in lower t-PA levels after growth factor treatment. However, dactinomycin was only effective when added up to 6 hr after addition of growth factors. Therefore, we concluded that if RNA synthesis is necessary for the increase of t-PA detected in the medium, then the RNA involved was synthesized during the first 6 hr after growth-factor addition. Thus, the slow response of enhanced t-PA release may be a general phenomenon involving RNA and/or protein synthesis, clearly indicating the absence of available intracellular pools of PA.

The u-PA studies indicated that little u-PA antigen was detected in the conditioned medium of confluent HREC and that the growth factors tested did not increase the levels detected in conditioned media from these cultures. However, one possible explanation for this is that u-PA is cell associated and t-PA is released into the medium, which would agree with the work of other investigators.⁴²,⁴³ However, our data do not support this; uniformly low u-PA antigen concentrations were detected not only in conditioned medium but also in the cell lysates formed from these confluent cultures.

The u-PA has important functional differences from t-PA. The latter binds to fibrin and to other components of extracellular matrices, and this interaction can increase its catalytic activity. The former binds to specific plasma membrane receptors, a configuration that focuses its activity on the close environment of the cell surface. There is little doubt that the two enzymes play distinct roles in the generation of extracellular proteolysis.

Our data suggest that HREC are capable of synthesizing both types of PAs. Under conditions of confluence, the predominant, if not exclusive, PA detected is t-PA. Under conditions of migration—as demonstrated in the wounded monolayer model—the HREC of both diabetic and nondiabetic origin are capable of expressing u-PA activity. The experimental system used involves cell fixation for the identification of the PAs expressed by migrating cells. This allowed only for the characterization of cell-associated enzymes. Cell-associated activity was not inhibited by anticatalytic anti-t-PA antibodies in unfixed cells, thereby providing evidence that secreted t-PA does not account for increased catalytic activity at the wound edge. The use of amiloride and anti-t-PA antibodies therefore demonstrates that wound-associated proteolysis is due to increased levels of u-PA in migrating endothelial cells.

The studies using mitomycin C demonstrate that cell division is not required for u-PA activity. The mechanism responsible for the change in expression of PA activity in HREC may be occurring via reorganization of the endothelial cell cytoskeleton mediating a signal to the cell nucleus. The affect of growth factors on the expression of u-PA activity remains to be studied, but previous reports demonstrate that growth factors stimulate endothelial cell migration and thus could indirectly result in u-PA expression by these cells.

Therefore, cautious interpretation of experiments
done at subconfluency is warranted because a mixed population of cells exists in this state. Some are stationary and probably secreting t-PA only, and others are migrating to fill in the empty surface of the culture dish. These latter cells, which are actively migrating, predominately secrete u-PA as demonstrated by the experiments using the wounded monolayer.

In conclusion, our studies indicate selective differences in the response of cells isolated from diabetic and nondiabetic subjects. The reason for the difference in production of t-PA and PAI by HREC diabetic individuals, however, cannot be determined from the current studies. It is highly unlikely this represents a difference of the HREC of diabetics in adjusting to the in vitro milieu. The cells studied were between passages 3–5 and were not the primary isolate. A more likely explanation is that there is either an inherent or an acquired difference in the basic control of the plasminogen activator-inhibitor system in HREC of diabetic origin. We know of no evidence indicating an inherent difference for any cellular parameter thus far examined in diabetics, not only for HREC but for a wide variety of other cells.

Acquired differences due to nonenzymatic glycosylation can alter enzyme activity, binding of regulatory molecules, cross-linking of proteins, macromolecular recognition of endocytosis, and most importantly, the function of nucleic acid. Selective alterations of DNA by nonenzymatic glycosylation cause highly characteristic genetic anomalies both in the experimental animal and humans. For example, the incidence of spina bifida is 50- to 100-fold greater than normal in humans exposed to high glucose levels in the first trimester, and the cardiac abnormalities both in humans and experimental animals induced by high glucose levels tend to be specific types. Such verified observations may imply the susceptibility of some genetic control mechanisms and the relatively poor susceptibility of others. Viewed in this manner, one might predict a similar susceptibility of the plasminogen activator-inhibitor system.

In summary, our data show a variation in t-PA and PA responses of diabetic HREC compared with controls. The significance of these findings must be clarified by in vivo studies.

**Key words:** plasminogen activators, microvascular endothelial cells, retina, insulin-like growth factor I, diabetic retinopathy

**References**

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