CONTROL OF PROLIFERATION OF HUMAN VASCULAR ENDOTHELIAL CELLS

Characterization of the Response of Human Umbilical Vein Endothelial Cells to Fibroblast Growth Factor, Epidermal Growth Factor, and Thrombin

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ABSTRACT

Because the response of human endothelial cells to growth factors and conditioning agents has broad implications for our understanding of wound healing, angiogenesis, and human atherogenesis, we have investigated the responses of these cells to the fibroblast (FGF) and epidermal growth factors (EGF), as well as to the protease thrombin, which has been previously shown to potentiate the growth response of other cell types of FGF and EGF. Because the vascular endothelial cells that form the inner lining of blood vessels may be expected to be exposed to high thrombin concentrations after trauma or in pathological states associated with thrombosis, they are of particular interest with respect to the physiological role of this protease in potentiating cell proliferation.

Our results indicate that human vascular endothelial cells respond poorly to either FGF or thrombin alone. In contrast, when cells are maintained in the presence of thrombin, their proliferative response to FGF is greatly increased even in cultures seeded at a density as low as 3 cells/mm^2. Human vascular endothelial cells also respond to EGF and thrombin, although their rate of proliferation is much slower than when maintained with FGF and thrombin. In contrast, bovine vascular endothelial cells derived from vascular territories as diverse as the bovine heart, aortic arch, and umbilical vein respond maximally to FGF alone and neither respond to nor bind EGF. Furthermore, the response of bovine vascular endothelial cells to FGF was not potentiated by thrombin, indicating that the set of factors controlling the proliferation of vascular endothelial cells could be species-dependent.

The requirement of cultured human vascular endothelial cells for thrombin could explain why the human cells, in contrast to bovine endothelial cells, are so

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difficult to maintain in tissue culture. Our results demonstrate that by using FGF and thrombin one can develop cultures of human vascular endothelial cells capable of being passage repeatedly while maintaining a high mitotic index. The stock cultures used for these studies have been passed weekly with a split ratio of 1 to 10 and are currently in their 30th passage. These cultures are indistinguishable from earlier passages when examined for the presence of Weibel-Palade bodies or Factor VIII antigen. We conclude that the use of FGF and thrombin can prevent the precocious senescence observed in most human endothelial cell cultures previously described.

KEY WORDS vascular endothelium · EGF, FGF · thrombosis · aging

The importance of the vascular endothelium in thrombosis has long been recognized (9). However, the mechanisms and factors controlling the regeneration of the endothelium after injury have remained poorly understood. An obstacle to the study of the regenerative capacity of the endothelium has been the difficulty encountered in establishing long-term cultures of endothelial cells and in maintaining these cells at a low enough density to study the factors controlling their proliferation (10, 24).

Recently, fibroblast growth factor (FGF), a mitogen extracted from pituitary and brain, has been found to promote the survival and proliferation of bovine vascular endothelial cells in low density cultures (13, 15, 16, 20). The use of FGF has permitted the development of cloned lines of bovine endothelial cells. The development of such bovine cells lines, though important for our understanding of endothelial cell function and metabolism, may have less clinical relevance than would corresponding endothelial cell lines of human origin. The use of cloned human endothelial cell lines could lead to the elucidation of the mitogenic factors controlling the proliferative response of the human endothelium after denudation, but the development of such cloned cell lines has been hampered by the difficulties encountered in obtaining cultures that could be passaged repeatedly without losing the ability to proliferate, and by the inability to maintain human vascular endothelial cells at a low enough cell density to permit cloning (10).

The relative lack of success in maintaining human endothelial cell cultures so that they may grow vigorously for long periods of time (and with a high mitotic index) when passaged at low density could be explained by the use of cells derived from the umbilical vein. This vascular territory could be preprogrammed to senesce rapidly after birth because of its unique function as a prenatal link between the mother and the fetus. Alternatively, the difficulty in culturing these cells could result from inadequate conditions under which they are maintained in vitro. Because growth factors such as FGF and the epidermal growth factor (EGF) have been found to delay the onset of senescence and terminal differentiation of several types of cultured cells (14, 26, 30), and because the response of human endothelial cells to growth factors and conditioning agents has broad implications for our understanding of wound healing, angiogenesis, and human atherogenesis, we have investigated the responses of these cells to both FGF and EGF, as well as to the protease thrombin, which has been shown to potentiate the growth responses of other cell types to FGF and EGF (3, 37). Since vascular endothelial cells form the inner lining of blood vessels, they could be expected to encounter high thrombin concentrations after trauma or in pathological states associated with thrombosis, and are therefore of particular interest with respect to the physiological role of this protease in potentiating cell proliferation.

Our results indicate that human vascular endothelial cells respond poorly to FGF or to thrombin alone. However, when cells are maintained in the presence of thrombin, their proliferative response to FGF is greatly increased, even in cultures seeded at a density as low as 300 cells/cm^2. Human vascular endothelial cells also respond to EGF plus thrombin, although their rate of proliferation is much slower than when maintained with FGF plus thrombin. These results contrast with those obtained with cultures of bovine vascular endothelial cells derived from the umbilical vein, the fetal heart, or the aortic arch of the calf. In all cases, the cells responded maximally to FGF alone but not to EGF. Furthermore, the response of bovine cells to FGF is not potentiated by throm-

bin. These results indicate that the set of factors controlling the proliferation of vascular endothelial cells may not be the same for all mammalian species, and that the precocious senescence observed with human endothelial cell cultures derived from the umbilical vein is not an invariably programmed event, but rather reflects the nutrient limitations of the medium in which the cells are maintained.

MATERIALS AND METHODS

FGF was purified from bovine pituitary glands and bovine brains as described in references 12 and 15. Both pituitary and brain FGF yielded a single band on polyacrylamide gel electrophoresis at pH 4.5. No bands were observed at pH 8.5. For reasons of economy, FGF from the brain was used in the experiments reported here. The same results, however, could be obtained with FGF from pituitary.

EGF was purified as described by Savage and Cohen (28) from the submaxillary glands of adult, male, Swiss-Webster mice that had been given subcutaneous injections of testosterone propionate (1 mg/animal per day) for 8 days. The final preparation yielded a single band on polyacrylamide gel electrophoresis at pH 8.5, and three amino acids, lysine, alanine, and phenylalanine, were absent from the final preparation (28). The biological activity of the EGF (assayed by measuring its potency in stimulating DNA synthesis in human foreskin fibroblasts) equaled that of a reference preparation received from Dr. Stanley Cohen, Vanderbilt University.

Highly purified human α-thrombin (2,900 units/mg) was a generous gift from Dr. John Fenton (Division of Laboratories and Research, New York State Dept. of Health, Albany, New York).

Tissue Culture of Endothelial Cells

Endothelial cells were obtained from the heart and aortic arch of adult cattle as previously described (16, 20). The endothelial cells from the bovine umbilical vein and human umbilical vein were obtained by collagenase digestion as described by Jaffe et al. (24). Briefly, the human umbilical cord was washed with ethanol, the end was cut evenly, and the vein was cannulated. The vein was then perfused with phosphate-buffered saline (PBS) until it was clear of blood and clots. It was then filled with 0.05% collagenase (Worthington Biochemical Corp., Freehold, N. J.) in PBS, tied closed, and kept in a 37°C water bath for 15 min. It was then kneaded gently between fingers to help loosen the human endothelial cells, which were then harvested by passing *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES)-buffered medium 199 supplemented with 20% human serum through the vein, forcing the collagenase solution and the free endothelial cells out of the cord and into a 10-cm tissue culture dish. FGF (1 μg/ml) and thrombin (2 μg/ml) were then added. The plates were maintained at 37°C for 6 h in a humidified incubator to allow the cells to plate. The cells were then rinsed and incubated in fresh medium. Stock cultures were kept in HEPES-buffered medium 199, supplemented with 10% human serum (Irvine Serum Co.), 100 ng/ml FGF, and 1 μg/ml thrombin. The medium was changed every 3 days for optimal growth. Tissue culture dishes were purchased from BioQuest, BBL & Falcon Products, Becton Dickinson & Co., Cockeysville, Md.

Bovine umbilical endothelial cells were obtained by the same method, except that the medium was Dulbecco's modified Eagle's (DME) supplemented with 10% calf serum (Grand Island Biological Co., Grand Island, N. Y.) and 100 ng/ml of FGF, without thrombin.

The bovine aortic endothelial cells used were either early passages of uncloned cells or the cloned cell lines already described (16, 20). The human endothelial cells were used between passages 3 to 15, the cultures being passaged every week with a split ratio of 1 to 10.

Electron Microscopy

For electron microscopy, cells were grown in tissue culture dishes as previously described, and fixed *in situ* with 2.5% glutaraldehyde-0.1 M cacodylate buffer (pH 7.2) for 20 min at room temperature. After being washed with the same buffer, the cells were postfixed in 2% OsO_4 in 0.1 M cacodylate (pH 7.2) for 20 min at room temperature, stained with 2% uranyl acetate in 3% ethanol, dehydrated with ethanol, and finally embedded in Epon 812 on the plate to give a layer 1–2 mm thick. When the Epon had cured, the plastic dish was peeled away from the Epon and areas of interest were cut from the mold and glued to Epon blocks. Then sections were cut parallel to the monolayer, stained with lead citrate, and examined in a Hitachi HU-12 electron microscope.

Fluorescence Microscopy

The presence of Factor VIII antigen in the human endothelial cell cultures was determined by indirect immunofluorescence. Rabbit antibodies against human Factor VIII were obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. Fluorescein-labeled goat anti-rabbit IgG was obtained from Meloy Laboratories Inc., Springfield, Va.

The endothelial cells were plated in Lab-Tek tissue culture chambers (Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.) in HEPES-buffered medium 199, supplemented with 10% human serum, 100 ng/ml of FGF, and 1 μg/ml of thrombin. When the cells reached confluence, the chambers were removed and the slides were washed for 5 min in PBS. The cells were then fixed for 5 min in ethanol:methanol (1:1) and dried. The dry cells were rehydrated in PBS for 15 min, excess PBS was removed, and a drop of 1:100 or

1:1,000 dilution of either rabbit anti-Factor VIII serum or normal rabbit serum sufficient to cover the slide was added. The slide was incubated for 45 min at room temperature and then washed for 1 h with PBS, with four changes of buffer. The staining and washing procedure was then repeated with a 1:50 dilution of fluorescein-conjugated goat anti-rabbit IgG. The washed slides were covered with one drop of PBS and a coverslip and examined in a Zeiss photomicroscope equipped with an epi-illumination system and a Mercury vapor lamp with a 440-nm filter. Photographs were taken with Kodak Tri-X film.

Bovine endothelial cells were similarly identified on the basis of the presence of Factor VIII antigens as already described (16).

Cell Growth Measurements

BOVINE VASCULAR ENDOTHELIAL CELLS: Confluent cultures were suspended with 0.25% trypsin. Trypsinization was stopped with 30% horse serum, and the cell suspension was distributed in dishes containing 5 ml of DME with 10% calf serum so that 10,000 or 20,000 cells were plated per 6-cm dish. After 18 h the medium was removed and the cells were rinsed once with DME and incubated in 5 ml of medium containing 10% calf serum and the various mitogens to be tested. FGF or EGF was added to the cultures every other day, thrombin every 4 days. Triplicate cultures were trypsinized and counted with a Coulter counter every day or every other day. All experiments were repeated six to seven times over a period of a year, with identical results.

HUMAN VASCULAR ENDOTHELIAL CELLS: Confluent cultures were trypsinized as described for the bovine endothelial cells, and the cell suspension was distributed in 6-cm dishes containing 5 ml of HEPES-buffered medium 199 with 10% human serum so that 10,000 cells were plated per 6-cm dish. After 18 h the medium was removed, the cells were rinsed once with HEPES-buffered medium 199, and 5 ml of medium containing 10% human serum and the various mitogens to be tested were added to the cultures. FGF or EGF was added to the cultures every other day, and thrombin every 4 days. Media were changed every week. Cells were counted every other day, as already described. All experiments were repeated six to seven times over a period of a year, with identical results.

Iodination of EGF

The lactoperoxidase technique (32) was used to label the EGF with ^{125}I. The labeled protein was separated from unreacted $Na^{125}I$ by passage of the iodination mixture through a Sephadex G-25 column (1 × 50 cm) equilibrated and eluted with a buffer containing 0.1 M phosphate (pH 7.4) and 0.15 M sodium chloride. The labeled EGF was stored frozen in the presence of 0.1% albumin. The specific activity of the ^{125}I-EGF was 110,000–230,000 cpm/ng.

^{125}I-EGF Binding Assay

Confluent cultures in 6-cm dishes were washed twice with 4-ml aliquots of binding medium which consisted of DME containing 0.1% bovine serum albumin (BSA) 10^{-7} M KI, 50 mM *N,N-bis*-(2-hydroxyethyl)-2-aminethanesulphonic acid (BES) adjusted to pH 6.8. The cells were incubated at 37°C with 2 ml of the binding medium containing ^{125}I-EGF at the required concentration. After incubation, unbound radioactivity was removed by washing the cells four times with cold (5°C) Tris-saline buffer at pH 7.4. To detach the cells, 2 ml of a 0.025% solution of trypsin in Tris-saline buffer minus calcium and magnesium were added to each dish, and the dishes were incubated at 37°C for 10 min. A 0.2-ml aliquot of the cell suspension was removed for cell counting (Coulter counter). The remaining contents of the dishes were transferred to counting vials, and the radioactivity was measured with a Beckman 310 series gamma counter.

RESULTS

Characterization of Human Endothelial Cells

The cells cultured from human umbilical vein show the characteristic morphology of endothelial cells. When maintained at confluence for extended periods of time in medium containing 10% human serum and 1 μg/ml FGF, the cells become tightly packed but show no tendency to overlap or overgrow one another (Fig. 1). To confirm that these cells were derived from endothelium, they were examined by immunofluorescent staining for the presence of Factor VIII antigen, reported to be characteristic of endothelial cells (2, 23, 25). Positive staining for Factor VIII antigen was observed in the perinuclear region in most of the human umbilical vein cells (Fig. 2*a*). Higher magnification (Fig. 2*b*) reveals distinct foci indicating a vesicular localization of the antigen. With the exception of bovine vascular endothelial cells, which exhibit the same fluorescence pattern as human cells, positive staining for Factor VIII antigen has not been observed in any other cultures examined, including granulosa cells, luteal cells, adrenal cortex cells, foreskin fibroblasts, vascular smooth muscle cells, corneal endothelial cells, and corneal or lens epithelial cells. Electron microscope examination of the cytoplasm of the human endothelial cells revealed abundant rough endoplasmic reticulum which reflects the high secretory activity of these cells. Also observed were free ribosomes, microtubules 250 Å in diameter, as well as numerous micropinocytotic vesicles 500–

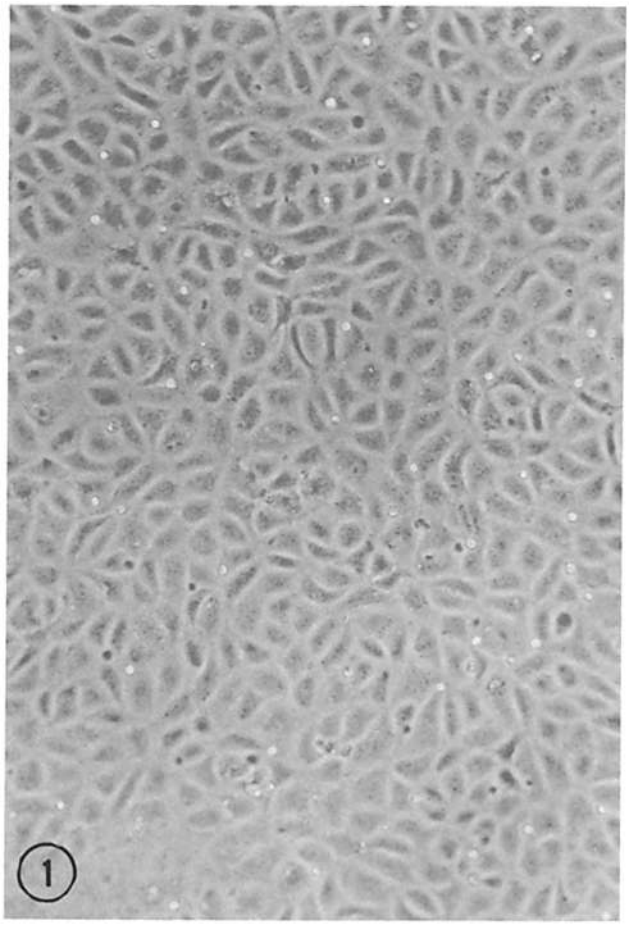

FIGURE 1 Morphology of human endothelial cells. Cells were grown as described in the text and maintained at confluence in the presence of 100 ng/ml FGF and 2 μg/ml thrombin. × 100.

1,000 Å in diameter. Small bundles of 60–70 Å diameter filaments and 100 Å filaments were observed at the cell peripheries. Weibel-Palade bodies were numerous (Fig. 3 C). Bovine endothelial cells derived from either the aortic arch (Fig. 3 A) or the umbilical vein display (Fig. 3 B) the same general structural features as the human umbilical vein endothelial cells but do not have Weibel-Palade bodies. This indicates that Weibel-Palade bodies are not a universal marker for endothelial cells.

The Effect of FGF, EGF, and Thrombin on the Proliferation of Human Vascular Endothelial Cells

Endothelial cells obtained by collagenase treatment of human umbilical veins will proliferate when cultured in HEPES-buffered medium 199 supplemented with either FGF (1 μg/ml) or EGF (100 ng/ml), provided the medium is supplemented for 10–20% human serum. The cells, however, when cultured in medium containing 10–35% bovine or fetal bovine serum grow poorly, even in the presence of FGF and EGF. The requirement for human serum for optimal growth of human endothelial cells is not surprising, for, although FGF and EGF show little species-specificity themselves, the growth response of any cell type is mediated by conditioning factors present in the serum which may be species-specific. Because, for primates, many hormones are known to be effective only on cells of the same species from which they were isolated, it is likely that human endothelial cells require agents found only in human serum that allow them to respond maximally to FGF and/or EGF.

Thrombin did not, by itself, have a pronounced

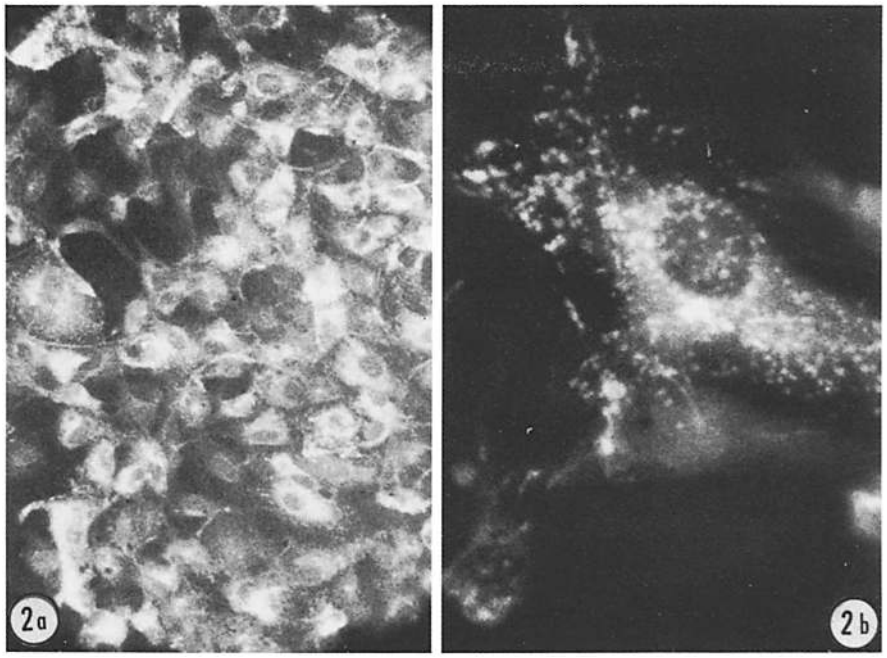

FIGURE 2 Immunofluorescent staining for Factor VIII antigen in cultured human endothelial cells. Cultures grown in the presence of 100 ng/ml FGF, 2 μg/ml thrombin, and 10% human serum were treated as described in reference 16. Factor VIII antiserum diluted 1:1,000. Cultures treated with normal rabbit antiserum at 1:100 dilution did not fluoresce. (*a*) × 100; (*b*) × 400.

effect on the proliferation of vascular endothelial cells. Because it has been previously observed that thrombin is able to potentiate the response of cells to other growth factors, including FGF and EGF (37), we decided to test the response of human endothelial cells to FGF and EGF in the presence and absence of thrombin. Fig. 4 A shows the response of these cells to various concentrations of FGF with and without thrombin. It can be seen that thrombin acts to potentiate the effect of FGF such that the dose-response is displaced by more than two orders of magnitude. For example, the increase in cell number observed when cultures are incubated with 1 ng/ml FGF plus 1 μg/ml thrombin exceeds that observed with 100 ng/ml FGF and no thrombin. Whereas thrombin alone induces only a threefold increase in cell number after 15 days, cells incubated in the presence of thrombin plus FGF increase more than 100-fold (Fig. 4 A). Although thrombin does potentiate the effect of saturating concentrations of FGF, the most ponounced effects of thrombin are observed when subsaturating doses of FGF are employed.

As noted before, EGF effectively stimulates the proliferation of human vascular endothelial cells, although to a lesser extent than does FGF (Fig. 4 B). Although lower doses of EGF than of FGF are required to induce a proliferative response, the maximal cell numbers obtained are never so great as with FGF. Thrombin can be seen (Fig. 4 B) to potentiate the response to EGF in much the same way as with FGF, that is, by allowing the cells to respond to reduced concentrations of the growth factor.

When cultures of human umbilical vein endothelial cells are treated with saturating doses of either FGF (1 μg/ml) or EGF (100 ng/ml), the addition of thrombin does not significantly shorten the population-doubling time (Fig. 5). This is in contrast to the effect of thrombin on cells maintained with subsaturating concentrations of EGF or FGF (Fig. 4), and indicates that the effect of thrombin is to sensitize the cells to low doses of mitogens rather than to affect directly the cycling time of the cells.

The Effect of FGF, EGF, and Thrombin on the Rate of Proliferation of Bovine Vascular Endothelial Cells

We have also investigated the effect of thrombin on the growth and survival of a cloned line of

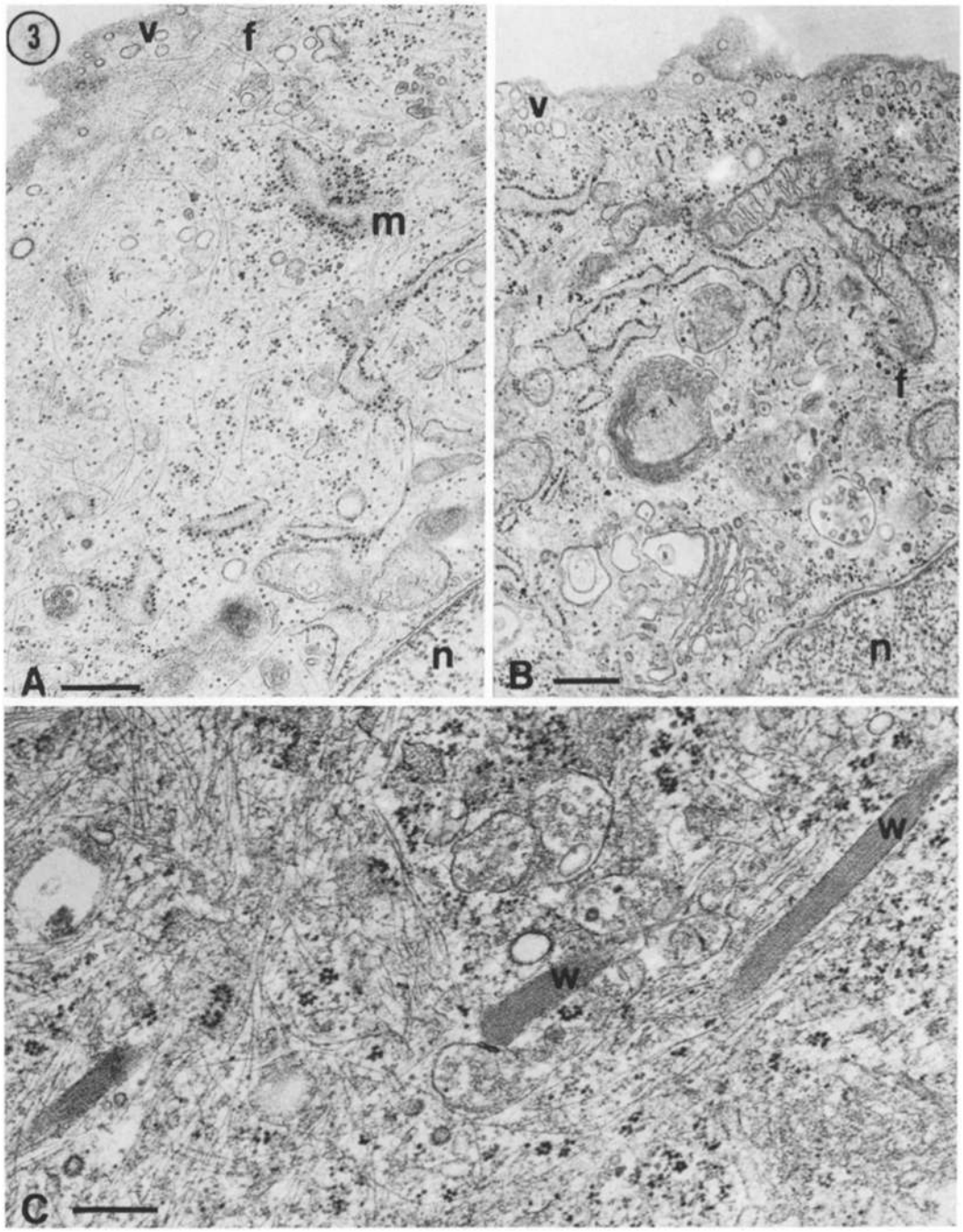

FIGURE 3 Electron micrographs of vascular endothelial cells derived from (A) the adult bovine aortic arch, (B) the bovine umbilical vein, (C) the human umbilical vein. (A) Numerous vesicles (*v*) are seen near the cell surface, 60–70 Å and 100 Å filaments (*f*) are evident, as are microtubules (*m*). Numerous ribosomes and polysomes are seen in the cytoplasm; (*n*), nucleus. × 25,000. Bar, 0.5 μm. (B) The same structural features can be seen as in (A). × 25,000. Bar, 0.5 μm. (C) Numerous Weibel-Palade bodies (*W*) can be seen in the cytoplasm; other structural features are similar to those seen for adult bovine aortic arch (A) and the bovine umbilical vein (B). × 37,000. Bar, 0.5 μm.

endothelial cells derived from bovine aortic arch. In contrast to the human endothelial cells, growth of the bovine cells is not affected by EGF (Fig. 6), and the response to FGF is not potentiated by thrombin (Fig. 7 B). It should be noted that the bovine cells are much more sensitive to FGF than are human cells. Bovine cells respond to FGF at 1 ng/ml and reach saturation at 100 ng/ml. The effect of thrombin on the human endothelial cells, therefore, is to shift the log dose response to that found naturally with the bovine cells. This may represent an optimal response that cannot be improved upon by further addition of conditioning factors. With EGF, the human cells are responsive to concentrations of 1 ng/ml or greater, and are potentiated by the presence of thrombin, while the bovine cells are unresponsive to doses between 0.1 ng/ml and 100 ng/ml in the presence or absence of thrombin (Fig. 7 B).

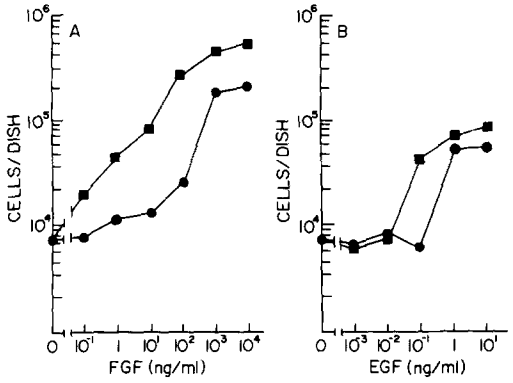

FIGURE 4 Effect of thrombin on the response of human endothelial cells to FGF and EGF. 10,000 cells were plated in 6-cm dishes in HEPES-199 with 10% human serum and maintained in the presence of (A) FGF or (B) EGF in the presence (■—■) or absence (●—●) of thrombin (2 μg/ml). On day 15, triplicate cultures were trypsinized and counted. Controls treated only with thrombin contained 2.2 × 10^4 cells. Standard deviation did not exceed 10% of the mean.

Because the differential response of bovine versus human vascular endothelial cells to EGF and to the potentiating effects of thrombin could be due to their differing sites of origin (aortic arch and umbilical vein), we have compared the mitogenic effects of EGF and FGF on bovine vascular endothelial cells derived from diverse vascular territories including fetal, as well as adult, bovine heart, aortic arch, and umbilical vein. In no case were we able to obtain a mitogenic effect of EGF, although the cells did respond quite well to FGF

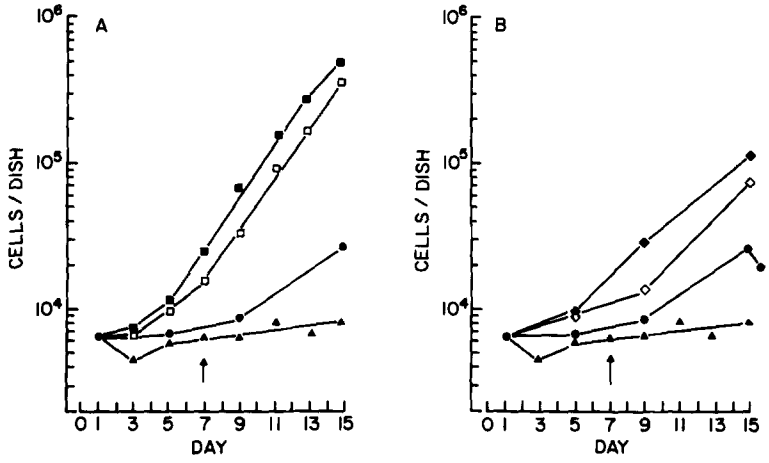

FIGURE 5 Growth rate of human endothelial cells maintained in the presence of saturating concentrations of FGF and EGF with or without thrombin. Cells were plated at 10,000 cells/6-cm dish in HEPES-buffered medium 199 supplemented with 10% human serum. (A) The cells were maintained in the presence of FGF alone (□—□), FGF + thrombin (■—■), thrombin alone (●+●), or without any addition (▲+▲). FGF was added every other day at a concentration of 1 μg/ml. Thrombin was added every 4 days at a final concentration of 2 μg/ml. The media were changed weekly (↑). Standard deviation did not exceed 10% of the mean. (B) The cells were maintained in the presence of EGF alone (◇—◇), EGF plus thrombin (◆—◆), thrombin alone (●+●), or without any addition (▲+▲). EGF was added every other day at a final concentration of 100 ng/ml. Thrombin was added every 4 days at a final concentration of 2 μg/ml. The media were changed weekly (↑). Standard deviation did not exceed 10% of the mean.

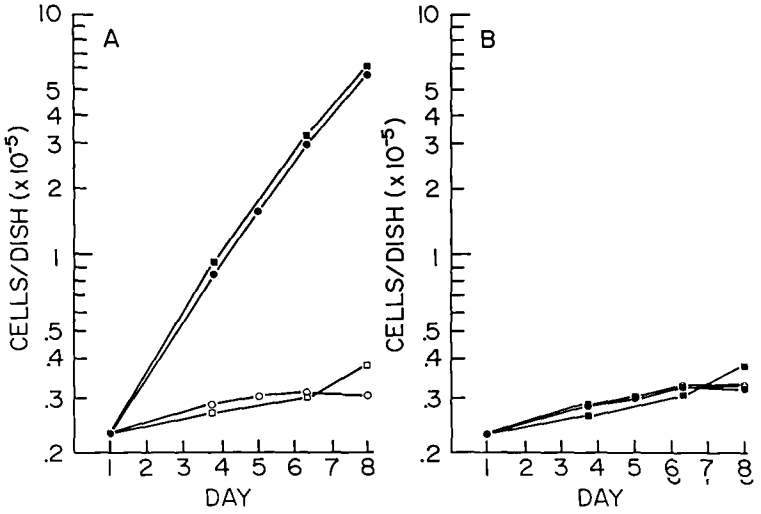

FIGURE 6 Growth rate of bovine endothelial cells maintained in the presence of FGF or EGF with or without thrombin. Vascular endothelial cells derived from the adult aortic arch (56 passages, 336 generations) were plated at 740 cells/cm² in 6-cm dishes in DME supplemented with 10% calf serum (16). (A) The cells were maintained in the presence of FGF alone (●–●), FGF + thrombin (■–■), thrombin alone (□+□), or without any addition (○+○). FGF was added every other day at a final concentration of 100 ng/ml, and thrombin was added every 4 days at a final concentration of 2 μg/ml. Standard deviation did not exceed 10% of the mean. (B) Same as (A), but the cells were maintained in the presence of EGF alone (●–●), EGF + thrombin (■–■), thrombin alone (□- -□), or without any addition (○–○). EGF was added at a final concentration of 100 ng/ml, and thrombin at 2 μg/ml. Standard deviation did not exceed 10% of the mean.

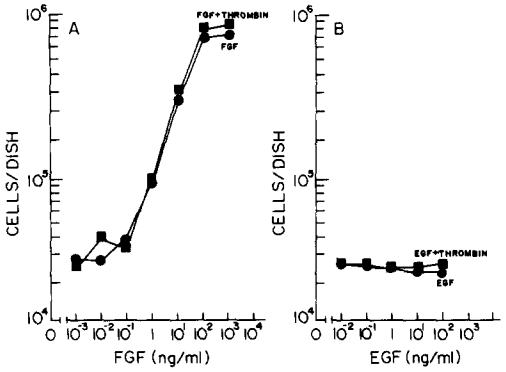

FIGURE 7 Effect of thrombin on the response of bovine endothelial cells to FGF and EGF. Cells were plated at 370 cells/cm² in 6-cm culture dishes in Dulbecco's modified Eagle's medium containing 10% calf serum. FGF (A) and EGF (B) were added at the indicated concentrations every other day, and thrombin (2 μg/ml) was added on day 4. After 7 days, triplicate cultures were trypsinized and counted. Controls treated with thrombin alone contained 3.6×10^4 cells. Standard deviation did not exceed 10% of the mean.

(Fig. 8). Likewise, thrombin did not have any potentiating effect on any of the bovine cultures maintained in the presence of either EGF or FGF.

In the experiments described above, human cells grown in human serum were found to be responsive to EGF and to thrombin, whereas bovine cells in bovine serum were not. To determine whether these different responses might be due to differences in the sera, we have repeated these experiments using human serum with the bovine cells to see if they might respond to EGF or thrombin under these conditions. As shown in Table I, bovine endothelial cells maintained in human serum responded to FGF and not at all to either thrombin or EGF.

EGF-Binding Sites in Human and Bovine Vascular Endothelial Cells

The lack of responsiveness of bovine vascular endothelial cells to EGF could result either from a constitutive defect (lack of receptors for the mitogen) or from culture conditions that could make the cells unresponsive to the mitogenic stimulus. An example of the latter situation is found with corneal epithelial cells. When maintained in tissue culture, these cells do not respond to EGF, although specific EGF-receptor sites can be readily detected (19). In contrast, when the

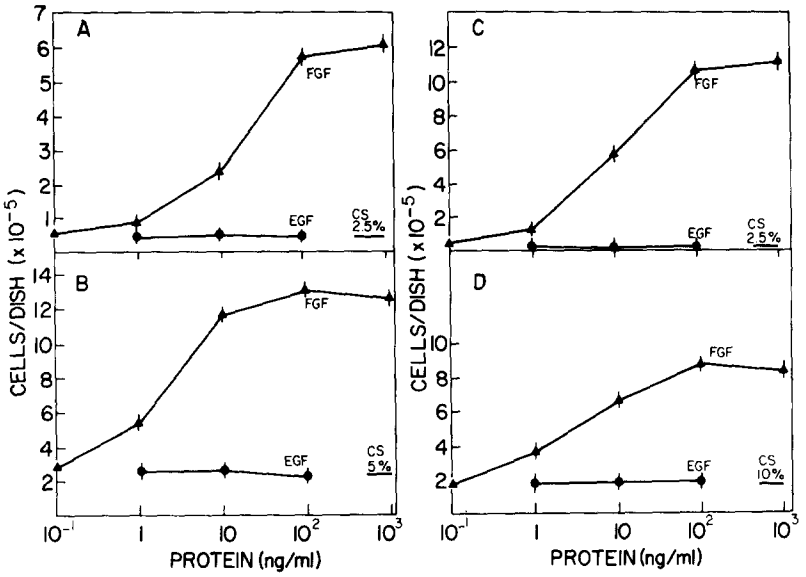

FIGURE 8 Effect of increasing concentrations of EGF and FGF on the proliferation of bovine vascular endothelial cells derived from the umbilical vein (A), the fetal aortic arch (B), the fetal heart (C), and the calf aortic arch (D). The cells were plated at 740 cells/cm² in a 6-cm dish in DME supplemented with calf serum. The vascular endothelial cells derived from the umbilical vein (A) and the fetal heart (C) were maintained in the presence of 2.5% calf serum, while the endothelial cells derived from the fetal aortic arch (B) or the calf aortic arch (D) were maintained in the presence of 5 and 10% calf serum, respectively. All cell strains were in their third passage; FGF or EGF was added at 100 ng/ml every other day. Cells from the umbilical vein were counted on day 7, and cells from the fetal heart, fetal aortic arch, and calf aortic arch were counted on day 8. The final cell densities reached in the absence of FGF or EGF for the cells derived from the umbilical vein and the fetal heart were 2,036 cells/cm² and 1,000 cells/cm², respectively, indicating a nearly total dependency upon FGF for proliferation. With both fetal and calf aortic arch, the densities reached were 8,150 cells/cm² and 6,500 cells/cm². The bar shows the standard deviation.

TABLE I

The Effect of Human and Calf Sera on the Response of Bovine Fetal Heart, Calf Aortic Arch, and Umbilical Vein Endothelial Cells to FGF, EGF, and Thrombin

	Calf serum			Human serum		
Endothelial cells	FGF	EGF	Thrombin	FGF	EGF	Thrombin
Fetal heart	160 ± 9.6*	4.9 ± 0.32	4.6 ± 0.27	152 ± 9.1	4.7 ± 0.28	4.6 ± 0.28
Calf aortic arch	221 ± 14.0	19.0 ± 1.1	17.8 ± 0.9	186 ± 9.2	14.5 ± 0.52	14.2 ± 0.86
Umbilical vein	197 ± 13.0	2.9 ± 0.17	3.2 ± 0.21	198 ± 12.0	2.8 ± 0.14	2.5 ± 0.14

The cells were plated at 740 cells/cm² in a 6-cm dish in DME supplemented with either calf or human serum. The vascular endothelial cells derived from the umbilical vein and the fetal heart (A and C) were maintained in the presence of 2.5% calf serum or 10% human serum, and the endothelial cells derived from the calf aortic arch were maintained in the presence of 10% calf serum or 10% human serum, respectively. All cell strains were in their fifth passage; FGF or EGF was added at 100 ng/ml, and thrombin was added at 2 μg/ml every other day. Triplicate cultures were counted on day 12.

* Cell number × 10^4 ± standard error.

cornea is maintained in organ culture, the same cell type will proliferate actively when exposed to EGF (19).

When the specific binding of EGF to bovine vascular endothelial cells derived from the bovine aortic arch or bovine umbilical vein was compared to that of human umbilical vein endothelial cells, it was observed that the human umbilical endothe-

lial cells maintained in the presence of either human or calf serum bound EGF, whereas the bovine cells maintained in the presence of either human or calf serum do not have receptors for EGF (Fig. 9). When the specific binding of ^{125}I-EGF to endothelial cells derived from human umbilical vein, bovine umbilical vein, and bovine aortic arch was compared (Fig. 9 A), it was found that only the human cells bound labeled EGF, and the the time-course of binding to the human vascular endothelial cells was similar to that obtained for binding of ^{125}I-EGF to 3T3 cells (unpublished observations of K. Brown) and to that published for NRK cells (33) or for human foreskin fibroblasts (5). The effect of EGF concentration on binding was studied by measuring the maximal binding after the addition of increasing quantities of ^{125}I-EGF to the cells (Fig. 9 B). No significant binding to the bovine endothelial cells was seen at any concentration tested, whereas binding to the human endothelial cells was shown to be a saturable process. A Scatchard plot of EGF-binding to these cells is shown in Fig. 9 C. Half-maximal binding capacity was 165 pg/10^6 cells. These values are similar to those found for EGF-binding to epithelial cells (17), to 3T3 cells (unpublished observations), and to human foreskin fibroblast cultures (5). These results demonstrate that bovine vascular endothelial cells in the presence of bovine or human serum differ from human umbilical vein endothelium exposed to human or bovine serum in their ability to bind EGF, and that these physiological differences are reflected in the growth responses of the cells maintained either in homologous or heterologous serum.

DISCUSSION

Our results demonstrate that by using FGF and thrombin one can develop cultures of human vascular endothelial cells capable of being passaged repeatedly while maintaining a high mitotic index. The stock cultures used for these studies are passed weekly with a split ratio of 1 to 10 and are currently in their 30th passage. These cultures are indistinguishable from earlier passages when examined for the presence of Weibel-Palade bodies or Factor VIII antigen. One could conclude that the use of FGF and thrombin can prevent the precocious senescence observed in most human endothelial cell cultures previously described (10, 24). This result is similar to results obtained earlier with the bovine vascular endothelial cells. Those cells have been shown to be easily main-

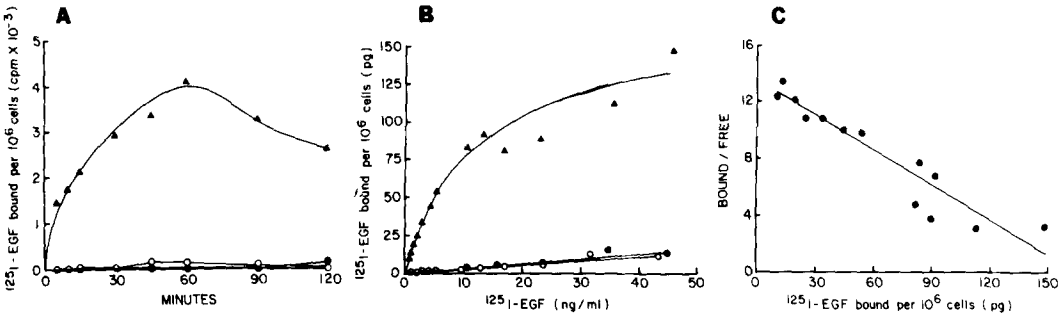

FIGURE 9 Comparison of the binding of ^{125}I-EGF to human endothelial cells and bovine endothelial cell cultures. (A) Time-course of specific ^{125}I-EGF binding to endothelial cells from human umbilical vein (▲) maintained in the presence of human sera, bovine umbilical vein (○), or bovine aortic arch-derived (●) endothelial cells maintained in bovine serum. 2 ml of binding medium, containing 7 ng/ml of ^{125}I-EGF (60,000 cpm/ng), were added to each dish and the dishes were incubated at 37°C. At the indicated time intervals, the cell-bound radioactivity was determined as described in Materials and Methods. For each time-point, nonspecific binding was measured in the presence of 500 ng/ml of unlabeled EGF and subtracted from the total bound. Identical results were obtained when the endothelial cell cultures were maintained in the presence of heterologous sera (human cells with bovine serum and bovine cells with human serum). Standard deviation did not exceed 10% of the mean. (B) Effect of ^{125}I-EGF concentration on binding to endothelial cells from human umbilical vein (▲), bovine umbilical vein (○), and bovine aortic arch (●). Indicated concentrations of ^{125}I-EGF (60,000 cpm/ng) in 2 ml of binding medium were added to dishes, and the cell-bound radioactivity was determined after 60 min. Incubation is described in Materials and Methods. Standard deviation did not exceed 10% of the mean. (C) Scatchard plot of binding data for human umbilical endothelial cells presented in Fig. 9 B. The line was fitted by a linear regression program without weighted factors.

tained in tissue culture for extended periods of time, provided FGF is present in the medium. Clonal lines of bovine vascular endothelial cells have been passaged weekly with a split ratio of 1 to 64 for over 65 wk (390 generations) without any changes in their karyotypes (2n = 60) and without loss of differentiated functions as expressed by their contact inhibition pattern at confluence and the presence of Factor VIII antigen.

The responses of bovine and human vascular endothelial cells to mitogens have been found to differ. Whereas human vascular endothelial cells respond to EGF as well as to FGF, bovine endothelial cells do not respond at all to EGF, even at concentrations as high as 1–10 μg/ml (unpublished data). Furthermore, binding studies indicate that the lack of response of the bovine endothelial cells is due to a lack of receptor sites for EGF. In contrast, the response of human vascular endothelial cells to EGF can be correlated with the presence of receptor sites for that mitogen. The lack of responsiveness of bovine endothelial cells to EGF is not specific to a given vascular territory, since it has been observed with bovine endothelial cell cultures obtained from the umbilical vein, as well as from the heart and aortic arch. Although it may be possible that only human umbilical vein endothelial cells have receptor sites for EGF, such conclusions will have to await the investigation of endothelial cells derived from other human vascular territories such as the heart or aorta.

Another discrepancy between the mitogenic responses of bovine and human vascular endothelial cells is that bovine cells respond optimally to FGF, whereas human cells require the potentiating effect of thrombin. This demonstrates that the proliferation of endothelial cells from different species can be under the control of different sets of factor(s). Likewise, endothelial cells from different species can also exhibit great differences when one considers their structural features. Weibel-Palade bodies, for example, which are prominent in human umbilical vein endothelium, have not been detected in our bovine endothelial cell cultures, even with cells derived from the bovine umbilical vein. Such a pronounced difference between the two species leads us to believe that other differences, as yet unknown, could also be present. Among those could be differences relating to lipid transport or to hormonal receptor sites.

One could argue that human endothelial cells derived from the umbilical vein are not the best human endothelial cell models, as those cells are derived from a fetal tissue usually discarded at birth. In late pregnancy, the earliest sign of degeneration involving the placenta always occurs in the umbilical vein, thus resulting in a diminished blood flow to the fetus and in cerebral damage. This could indicate that endothelial cells obtained from the umbilical vein, because they belong to a senescent territory, are preprogrammed to die, and that their requirements for proliferation could therefore be quite different from those of other fetal or adult vascular territories. This point will, however, be difficult to evaluate in view of the difficulties in obtaining human samples. However, if one refers to tissue of bovine origin (in which a comparison of the characteristics of different vascular territories can readily be done), we cannot observe any differences among endothelial cell cultures derived from the bovine umbilical vein, aortic arch, or heart in regard to their control of proliferation. This fact leads us to believe that the precocious senescence observed with human umbilical vein endothelial cell cultures in vitro reflects the inadequacy of the medium in which the cells are maintained rather than a preprogrammed fixed rate of mutation which leads to the death of the cells. Similar conclusions have been reached by others concerning the life in culture of either keratinocytes from different origins (26, 30) or granulosa cell cultures (14), as the addition of EGF and/or FGF to these cultures greatly extends their replicative lifespan.

The requirement for thrombin of cultured human vascular endothelial cells could explain why human cells, in contrast to bovine endothelial cells, are so difficult to maintain in human tissue culture. Gimbrone et al., for example, reported that one-third of their primary human umbilical vein cultures did not reach confluence, and that inocula of less than 1.2×10^4 viable cells/cm^2 usually failed to become established (10, 11). In contrast, Booyse et al. (2), as well as others (29, 34), have recently reported that adult bovine endothelial cells could be passaged when seeded at a density as low as 4×10^2 cells/cm^2. Because bovine endothelial cells do not require thrombin to be sensitized to the mitogenic effect of serum factors, one would expect that, when maintained in the presence of optimal concentrations of serum factors, they would proliferate rapidly. In contrast, human cells require thrombin in addition to serum factors. Although thrombin is a normal component of serum, it is rapidly inactivated and is usually present in low concentration in its active

form. It is therefore quite possible that human cells, although exposed to the same concentration of serum factors as bovine cells, will fail to proliferate because of inadequate thrombin concentration.

The ability of thrombin to stimulate cell proliferation or to potentiate the effects of other growth-promoting factors is a relatively recent addition to our knowledge concerning this enzyme (3, 6, 37). Although thrombin does not appear to be a primary mitogen for as wide a variety of cell types as does FGF or EGF (8, 15, 18, 21), its effects are of particular interest for those cell types that can be expected to encounter high concentrations of thrombin under physiological circumstances. Since vascular endothelial cells are in direct and continual contact with circulating fluids, they are very likely to be influenced by fluctuations in the levels of those proteases, such as thrombin, that are involved in hemostasis.

The mechanism by which thrombin potentiates the effect of other growth factors is unclear, but is probably mediated by action on the cells themselves and not by direct cleavage of the factors. The observation that thrombin can remove specific proteins from cell surfaces (7, 31, 35) suggests that the enzyme could act to expose new binding or transport sites for the other growth-effecting molecules. Whether thrombin is internalized by endothelial cells as it is by chick embryo fibroblasts is not yet known (36).

Our finding that thrombin allows cultured human endothelial cells to respond to concentrations of FGF or EGF that are by themselves insufficient gives an indication of the kind of situation in which thrombin might be expected to play a role in regulating cell proliferation. Although normal vascular endothelium represents a slowly renewing cell population in vivo, endothelial cell regeneration can be triggered by a variety of stimuli, especially intimal denudation, which is frequently followed by thrombus formation. The contents of secretory granules are released from platelets while, simultaneously, high, localized concentrations of thrombin are generated. The growth factors released from platelets (1, 27), along with growth factors normally present in plasma such as EGF (4, 22), could act in concert with thrombin to stimulate regeneration of the endothelium. Because these factors may be mitogenic for many cell types, an intriguing fail-safe mechanism could be provided if the growth factors were present in subsaturating concentrations and were active only in the presence of the high transient concentrations of thrombin found in such situations.

Because the continuity of the vascular endothelium is essential for the growth and survival of the organism, the elucidation of the factors involved in endothelial cell survival and proliferation is important. The development of endothelial cell lines is one of the first steps toward such an understanding, but there are practical implications which extend beyond the development of these cell lines. Until now, endothelial cell cultures have been developed primarily to study the complex functions of these cells, such as the transport of high and low density lipoproteins, the production and secretion of plasma protein, and that of the components of the basal lamina (for review, see reference 11). The presence of hormone receptor sites and the responsiveness of endothelial cells to different hormones (11) have also been investigated. One would expect that studies done with cell populations maintained in inadequate tissue culture conditions which give rise to senescent populations would lead to misleading conclusions as to what the cells are really capable of doing. The elucidation of the functions and mechanisms through which endothelial cells achieve these functions can only be achieved by first establishing the natural requirements for survival and proliferation of this cell type. These requirements are best reflected by long-term maintenance of endothelial cells at low density as well as by high mitotic index of the cultures. Such cell lines should provide an adequate model to study the metabolic function of the vascular endothelium.

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