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JOURNAL OF CELLULAR PHYSIOLOGY 146:8–17 (1991)

Macrovascular and Microvascular Endothelium During Long-Term Hypoxia: Alterations in Cell Growth, Monolayer Permeability, and Cell Surface Coagulant Properties

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In bovine aortic or capillary endothelial cells (ECs) incubated under hypoxic conditions, cell growth was slowed in a dose-dependent manner at lower oxygen concentrations, as progression into S phase from G_1 was inhibited, concomitant with decreased thymidine kinase activity. Monolayers grown to confluence in ambient air, wounded, and then transferred to hypoxia showed decreased ability to repair the wound, as a result of both decreased motility and cell division. Hypoxic ECs demonstrated a ≈3-fold increase in the total number of high-affinity fibroblast growth factor receptors, and levels of endogenous FGF were suppressed. Consistent with the presence of functional FGF receptors, addition of basic FGF overcame, at least in part, hypoxia-mediated suppression of EC growth, and enhanced wound repair in hypoxia, stimulating both motility and cell division. Despite slower growth in hypoxia, ECs could achieve confluence, and the monolayers consisted of larger cells with altered assembly of the actin-based cytoskeleton and small gaps between contiguous cells. The permeability of these hypoxic EC monolayers to macromolecules and lower molecular weight solutes was increased. Cell surface coagulant properties were also perturbed: the anticoagulant cofactor thrombomodulin was suppressed, and a novel Factor X activator appeared on the EC surface. These data indicate that micro- and macrovascular ECs can grow and be maintained at low oxygen tensions, but hypoxic endothelium exhibits a range of altered functional properties which can potentially contribute to the pathogenesis of vascular lesions.

Hypoxia often occurs during regeneration of endothelium, such as takes place after vascular injury, or during angiogenesis in wound repair or neovascularization of tumors. In each of these situations, endothelial cell (EC) growth, and formation of a monolayer regulating permeability and coagulation, must occur for blood flow to be initiated and maintained. These considerations led us to examine the effect of hypoxia on the proliferation and functional properties of endothelium.

Anoxia inhibits cell division in several types of cultured cells (Bedford and Mitchell, 1974; Fanburg and Lee, 1987; Peterson and Lindmo, 1983; Rice et al., 1985). Since complete anoxia is not a common occurrence in vascular lesions, we examined the effect of low concentrations of oxygen, i.e., concentrations which can be achieved in the intravascular space in pathophysiologic states (Hamer et al., 1981; Hultgren, 1978; Fanburg and Lee, 1987; Roca et al., 1990), on growth and other central homeostatic properties of EC during long-term exposure of cultures to hypoxia. The results indicate that hypoxia reversibly slows EC growth and the response to wounding, but this inhibition can be overcome by basic fibroblast growth factor (bFGF). Despite a decrease in the rate of cell division, a monolayer eventually forms under hypoxic conditions, but it is one with diminished barrier function and altered coagulant properties. These studies provide insights into EC-dependent mechanisms operative in the vascular response to hypoxemia.

MATERIALS AND METHODS

Culture of ECs under hypoxic conditions

Bovine aortic ECs were grown from aortas of newborn calves in minimal essential medium supplemented with penicillin-streptomycin (50 U/ml-5 μg/ml), HEPES (pH 7.4; 10 mM, glutamine and fetal calf serum

Received April 11, 1990; accepted September 7, 1990.

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(10%; Hyclone, Logan, Utah), as described (Schwartz, 1978; Gerlach et al., 1989). Bovine adrenal capillary cells were prepared and grown as described previously (Furie et al., 1984). Cultures were characterized as endothelial based on indirect immunofluorescence for thrombomodulin and von Willebrand factor, and morphologic criteria (Esmon, 1987; Jaffe et al., 1973; Schwartz, 1978). Cells were subcultured using trypsin/EDTA, and plated into tissue culture dishes (initial density $\approx 1.7 \times 10^4$ cells/cm^2) which were placed immediately into an incubator attached to the hypoxic chamber (Coy Laboratory Products, Ann Arbor, MI) (Ogawa et al., 1990). This apparatus established hypoxia by purging the chamber with a mixture containing the desired concentration of oxygen, 5% carbon dioxide, water vapor, and the balance as nitrogen. The oxygen content was monitored continuously and regulated using an oxygen analyzer which controlled an automated valve system. Throughout experiments, the concentration of oxygen dissolved in culture medium bathing the cells was analyzed (Model ABL-2 from Radiometer, Copenhagen, Denmark). Values shown in the figures are the oxygen pressure in the medium. Experiments were carried out in their entirety in the hypoxia chamber.

Cell viability was determined by trypan blue exclusion. Overall protein synthesis was assessed by measuring incorporation of ^{3}H-leucine (60 Ci/mmole; New England Nuclear) into trichloroacetic acid-precipitable material using the method described by Madri et al. (1988). Radioactive leucine was added 12 hr before the end of an experiment, and then cultures were harvested at the indicated times. Wounding of the EC monolayer was carried out using the narrow edge of a cell scraper (Corning, Baxter Scientific, NJ) (Gerlach et al., 1989). Migration of cells into the wound was determined as described by Tsuboi et al. (1990).

Endothelial cell growth, flow cytometry, and thymidine kinase activity

EC growth was assessed by counting cell number, using a Coulter counter (Model ZM; Coulter Electronics, Luton, England). Incorporation of ^{3}H-thymidine by growing cells was determined by incubating cultures during the last 7 hr of an experiment in the presence of ^{3}H(methyl)thymidine (New England Nuclear, Boston, MA; 50 Ci/mmol; 0.08 mCi/ml), washing cultures ten times in Hanks' balanced salt solution, lysing the cells with 2% SDS, and then counting the samples in a β-counter (RackBeta, LKB, Rockville, MD).

Flow cytometry to assess DNA content was performed as previously described by Dolbeare et al. (1990), using murine monoclonal antibody to bromodeoxyuridine (BrdUrd). Cultures were pulse-labeled with BrdUrd for the final 1 hr of a 48 hr incubation, removed from the growth surface with trypsin, and fixed in ethanol. DNA was thermally denatured at low ionic strength, and then incubated with the antibody to BrdUrd (Amersham, Arlington, IL). Samples were next incubated with fluorescein-conjugated goat anti-mouse IgG, the cells were resuspended in a solution of propidium iodide (10 μg/ml) in phosphate-buffered saline, and cell cycle analysis was performed on a FACScan flow cytometer (Becton Dickinson) using a 488 nm single laser system. A bivariate contour histogram of DNA content (X-axis) versus log BrdUrd (Y-axis) was generated from which fitted values for percentages of cells in G_1, S, and G_2+M were calculated.

Thymidine kinase activity was measured as previously described (Lieberman et al., 1988). In brief, cells were suspended in saline, pelleted, washed with hypotonic buffer (10 mM Tris HCl pH 8.0, 10 mM KCl, 1 mM $MgCl_2$ 1 mM β-mercaptoethanol, 50 mM Tdr), repelleted, and stored at −80°C or resuspended in 0.2 ml of fresh lysis buffer. The resuspended cells were then lysed by three rapid freeze/thaw cycles, and cell debris was pelleted by centrifugation at 16,000*g*. The supernatant (30 μl) was added to 20 μl of reaction cocktail (60 mM Tris HCl pH 8.0, 15 mM $MgCl_2$, 15 mM ATP, ^{3}H-TdR, 50 Ci/mmol, .08 mCi/ml), incubated at 37°C for 30 min, and the reaction was stopped by the addition of 30 μl of 50 mM EDTA (tubes were then stored at 4°C). The reaction mix was spotted on to DE81 filters (Whatman) and air-dried. Filters were then washed sequentially 3 times over 15 min with 10 mM ammonium formate, water, and ethanol, air dried and placed into scintillation vials containing 0.1 M HCl, 0.2 M KCl (1 ml). After 30 min, 5 ml of aqueous scintillation cocktail was added, and the vials were counted. Protein content in the supernatant was determined by the method of Lowry et al. (1951), and cell number was assessed using the Coulter counter. Thymidine kinase activity was calculated as ^{3}H-dTMP (^{3}H-thymidine monophosphate) formed/hr/cell.

Binding and production of FGF by hypoxic endothelial cells

Confluent monolayers of normoxic or hypoxic capillary ECs were washed twice with phosphate-buffered saline, equilibrated in serum-free Medium 199 containing gelatin (1.5 mg/ml) for 2 hr at 37°C, washed again, and then incubated with ^{125}I-bFGF alone (total binding) or in the presence excess unlabeled bFGF (50-fold molar excess; nonspecific binding) for 2 hr at 4°C. At the end of the incubation period, cultures were washed with high salt buffer (2 M NaCl), and cell-bound radioactivity was solubilized with Triton X-100 (1%). This method has been previously described in detail by Moscatelli (1987). Specific binding, total minus nonspecific binding, was then fit to the equilibrium binding equation of Klotz and Hunston (1984): $B = nKA/(1 + KA)$, where B is the amount of specifically bound ligand, n is the number of binding sites per cell, A is the concentration of free radioligand, and K is the association constant. A nonlinear least-squares program was used to obtain the best fit curve, to solve for n and K, and to determine the standard error. Radioiodinated bFGF was obtained from Amersham (specific radioactivity 6×10^4 cpm/ng), and unlabeled bFGF (purified, recombinant basic fibroblast growth factor) was generously provided by Farmitalia (Milan, Italy). FGF content of ECs was assessed by solubilizing EC monolayers with Triton X-100 as described (Tsuboi et al., 1990), preparing samples and running them on SDS-PAGE (Laemmli, 1970), and performing Western blotting (Towbin et al., 1979). After electrophoretic transfer of proteins to the nitrocellulose membrane, excess binding sites on the membrane were blocked (Johnson et al., 1984) and blots were reacted sequentially with a monospecific rabbit anti-human bFGF antibody and ^{125}I-

affinity-purified anti-rabbit IgG (1.2×10^5 cpm/ml). Dried blots were subjected to autoradiography. This protocol has been described in detail previously (Stern et al., 1986). Standard proteins were run simultaneously for molecular weight determination: ovalbumin (Mr ≈46 kDa), carbonic anhydrase (Mr ≈30 kDa), trypsin inhibitor (Mr ≈21.5 kDa), lysozyme (Mr ≈14.3 kDa), aprotonin (Mr ≈6.5 kDa), and insulin b-chain (Mr ≈3.4 kDa). FGF activity of hypoxic and normoxic endothelial cell supernatants was tested in the fibroblast growth assay using NIH 3T3 cells (Witte et al., 1989). The antibodies used in this study were those raised in rabbits and characterized as described in a previous publication (Joseph-Silverstein et al., 1988).

Assay of EC monolayer permeability

Permeability studies were performed as described previously (Albelda et al., 1988; Brett et al., 1989; Del Vecchio et al., 1987; Shasby and Roberts, 1987), using cultures grown on 6.5 mm diameter polycarbonate membranes (pore size, 0.4 μm), mounted on polystyrene inserts (Transwell plates, Costar, Cambridge, MA). Five days after ECs achieved confluence (confluence was defined at the time when cells were contiguous and the labeling index was <1%), permeability studies were carried out by washing them with Hanks' balanced salt solution, and then adding minimal essential medium containing fetal calf serum (8%) (all solutions were equilibrated with the gas mixture being used for that experiment and experiments were carried out completely in the hypoxia chamber). Medium was added to both the inner and outer chambers so that the oncotic and hydrostatic pressures in the two chambers remained equal. A radiolabeled marker was then added at trace concentrations, either ^{3}H-inulin (3 μg/ml; 271 mCi/g; New England Nuclear), ^{3}H-sorbitol (38 ng/ml; 24 Ci/mmole; New England Nuclear), or ^{125}I-bovine serum albumin (150 ng/ml; 5,000 cpm/ng) to the upper chamber, as described previously (Brett et al., 1989). Radioiodinated albumin was prepared by the lactoperoxidase method (David and Reisfeld, 1974), and the final product used for permeability studies was >95% precipitable in 10% trichloroacetic acid. Transport of tracers from the inner to outer chamber, i.e., across the endothelial monolayer, was determined by dividing radioactivity emerging in the outer well by radioactivity remaining in the inner well.

Assays of EC coagulant function

Endothelial thrombomodulin activity was assessed after exposing cultures to hypoxia, as described previously (Ogawa et al., 1990). In brief, functional assays for cell surface thrombomodulin were performed in a buffered salt solution by measuring endothelial cell-dependent thrombin-mediated protein C activation using a chromogenic substrate assay (Ogawa et al., 1990; Esmon, 1987).

Assays of endothelial procoagulant properties were performed on either intact monolayers (9.6 cm^2) or cells scraped with a rubber policeman to obtain a suspension (≈10^6 cells/ml). Cells in suspension were >90% viable, based on trypan blue exclusion. Following washing of the cultures three times with EDTA-containing salt solution, coagulant assays were performed in veronal buffer by adding citrated bovine plasma and calcium chloride, and determining the time required for the first visual evidence of a fibrin clot at 37°C, as described previously (Nawroth et al., 1985). The Factor Xa clotting assay was performed by incubating purified Factor X (1 μM) with hypoxic or normoxic ECs for the indicated times, removing aliquots, and adding them to Factor VII/X-deficient bovine plasma (Sigma), cephalin, and calcium chloride (Bajaj and Mann, 1973; Ogawa et al., 1990). The amidolytic assay for Factor Xa (van Dieijan, 1981) was performed similarly, except that samples from the endothelial reaction mixture were incubated with the chromogenic substrate methoxycarbonyl-D-cyclohexyl-Gly-Gly-Arg-nitroanilide (Spectrozyme FXa; American Diagnostica, NY). This assay was sensitive to 0.4–0.5 nM Factor Xa. In either the coagulant or amidolytic assays for Factor Xa, enzyme concentration of samples was determined by comparison with a standard curve made with known amounts of Factor Xa. Where indicated, after exposure to hypoxia, ECs were further incubated in the same hypoxic environment in the presence of either antibody to tissue factor (10 μg/ml; 1 hr at 37°C) (blocking anti-bovine tissue factor monoclonal antibody was generously provided by Dr. R. Bach, Mt. Sinai School of Medicine, NY) (Bach et al., 1981), anti-Factor IX/IXa antibody (10 μg/ml of purified IgG; 1 hr at 37°C) (blocking rabbit polyclonal IgG to bovine Factor IX/IXa was generously provided by Dr. W. Kisiel, Univ. of New Mexico School of Medicine, Albuquerque), mercury chloride (0.1 mM; 30 min at 37°C), or PMSF (2 mM; 30 min at room temperature) prior to carrying out the coagulant assay. Cultures were then washed extensively, and procoagulant properties were assessed as described above.

Purified bovine coagulation factors X, IX, and prothrombin for these studies were prepared as described previously (Fujikawa et al., 1972, 1973; Walker et al., 1979). The Factor X had no detectable Factor VII activity. Purified human recombinant tumor necrosis factor/cachectin (10^8 U/mg) was generously provided by Dr. P. Lomedico (Hoffman-LaRoche, Nutley, NJ).

Morphologic studies

For immunocytologic studies, cell monolayers grown on coverslips in normoxia or hypoxia were fixed in that atmosphere in phosphate-buffered saline, pH 7.2, containing 3.5% formalin and 0.1% NP-40 for 5 min and washed in phosphate-buffered saline. For visualization of F-actin, coverslips were incubated with rhodamine-conjugated phalloidin (Molecular Probes, Junction City, OR) for 30–45 min, washed in phosphate-buffered saline, and mounted in Gelvatol containing 1 mg/ml p-phenyl-enediamine. For visualization of thrombomodulin, monolayers were fixed as above and incubated with a monospecific rabbit anti-bovine thrombomodulin IgG (Gerlach et al., 1989). Localization of sites of binding of the first antibody were visualized with fluorescein-conjugated goat anti-rabbit IgG (Sigma). Mounted coverslips were examined in a Leitz Dialux 20 microscope with a 2.4 Ploempak filter block and water immersion fluorite objectives, and recorded on Kodak Tri-X film.

RESULTS

Endothelial cell growth and regenerative response to a wound under hypoxic conditions

Exposure to aortic or capillary ECs to low atmospheric concentrations of oxygen immediately after subculturing led to a dose-dependent decrease in cell growth, as reflected by a fall in ^{3}H-thymidine incorporation, once pO_2s below 47 mm Hg were reached (Fig. 1A,B). At the lowest pO_2 examined, 14 mm Hg, after 48 hr of incubation ^{3}H-thymidine incorporation was approximately one-third that of the normoxic controls. The decrease in cell growth at 48 hr postplating was paralleled by a similar decrement in cell number compared with equivalent normoxic controls. Alteration in EC growth in hypoxia was reversible; both the number of cells and ^{3}H-thymidine incorporation increased to levels in normoxic cells, once cells were restored to an ambient air pO_2 of 147 mm Hg (Fig. 1).

These data suggested that hypoxia reversibly decreased entry of cells into S phase, which was confirmed by flow cytometry studies (Fig. 2A–E): the percentage of capillary or aortic cells in S phase in hypoxia was half that of their normoxic counterparts. Thymidine kinase, a pyrimidine salvage enzyme whose levels increase in S phase of the cell cycle (Sherley and Kelly, 1988), was proportionately decreased in hypoxic endothelial cells (Fig. 2F).

Repair of the endothelial monolayer, which is dependent on both EC motility and growth, follows after wounding or focal denudation. Since vascular injury often occurs in the setting of hypoxia, it was important to understand the effect of hypoxia on the migration and growth of ECs into a wound. When confluent monolayers were wounded and then placed in hypoxia, wound repair was delayed compared to normoxic controls (Fig. 3A,B). This was due to both decreased motility, as indicated by the fall in the number of cells which migrated into the denuded area during a 24 hr period (Fig. 3D), and decreased cell growth, as shown by labeling indices at the margin of the hypoxic wound (data not shown).

The increased fraction of hypoxic endothelial cells in G_1 and delayed wound repair suggested that depressed motility and proliferation could reflect decreased activity of an autocrine growth factor, such as FGF (Sato and Rifkin, 1988; Rifkin and Moscatelli, 1989), under hypoxic conditions. When exogenous bFGF was added to hypoxic EC cultures, growth was stimulated in a dose-dependent manner, as assessed by ^{3}H-thymidine incorporation, with a maximal 12-fold increase above the unstimulated hypoxic controls (Fig. 4). FGF had a similar effect on the growth of hypoxic aortic ECs. Addition of bFGF to wounded hypoxic endothelial monolayers (Fig. 3C) increased mobility (also see Fig. 3D) and labeling index (data not shown) at a bFGF concentration of 5 ng/ml, enhancing the EC reparative response during hypoxia. In monolayers of wounded aortic ECs, FGF also stimulated EC growth (data not shown).

These findings led to the hypothesis that hypoxic ECs would still express high-affinity FGF receptors, enabling them to respond to exogenous FGF, but that their production of FGF would be suppressed. Binding studies with ^{125}I-FGF and hypoxic ECs demonstrated ≈300% fold increase in the number of high-affinity binding sites (the affinity of FGF binding was comparable on hypoxic and normoxic cultures) (Fig. 5A). In contrast to the increase in high-affinity receptors, the level of endothelial bFGF in hypoxic cultures was suppressed, as indicated by Western blotting (Fig. 5B). The pattern of immunoreactive bands in the normoxic capillary endothelium appeared to be quite similar to that described previously with major bands corresponding to approximately 18, 22, 24, and 26 kDa (Tsuboi et al., 1990). In contrast, under hypoxic conditions, there was only a very faint band visible corresponding to ≈18 kDa. These changes in levels of FGF and high-affinity FGF binding sites were reversible on replacement of the cells into an ambient atmosphere.

Although EC proliferation and motility were slowed by hypoxia (at pO_2 ≈14 mm Hg), cell attachment proceeded normally after subculture, and cell viability was intact, as assessed by trypan blue exclusion. A confluent monolayer formed at each of the pO_2s exam-

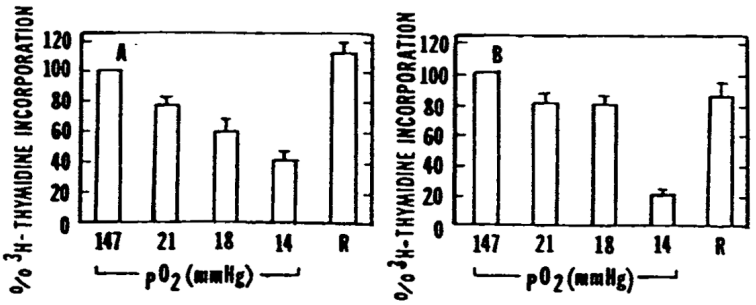

Fig. 1. Effect of oxygen tension on endothelial cell ^{3}H-thymidine incorporation. ECs, aortic (A) and capillary (B) were plated at a density of 3.4×10^4 cells/cm^2 and grown at the indicated oxygen tension for 48 hr. During the last 7 hr ^{3}H(methyl)-thymidine was added, and its incorporation into DNA was assessed as described in the text. In each case, ^{3}H-thymidine incorporation at a given oxygen tension is compared with that at 147 mm Hg (the latter was arbitrarily defined as 100%), and the mean ± SD is shown. R denotes ^{3}H-thymidine incorporation of cultures exposed to hypoxia (pO_2 ≈14 mm Hg) for 48 hr followed by replacement into ambient air 48 hr (radiolabel was added 7 hr prior to the end of the re-oxygenation period).

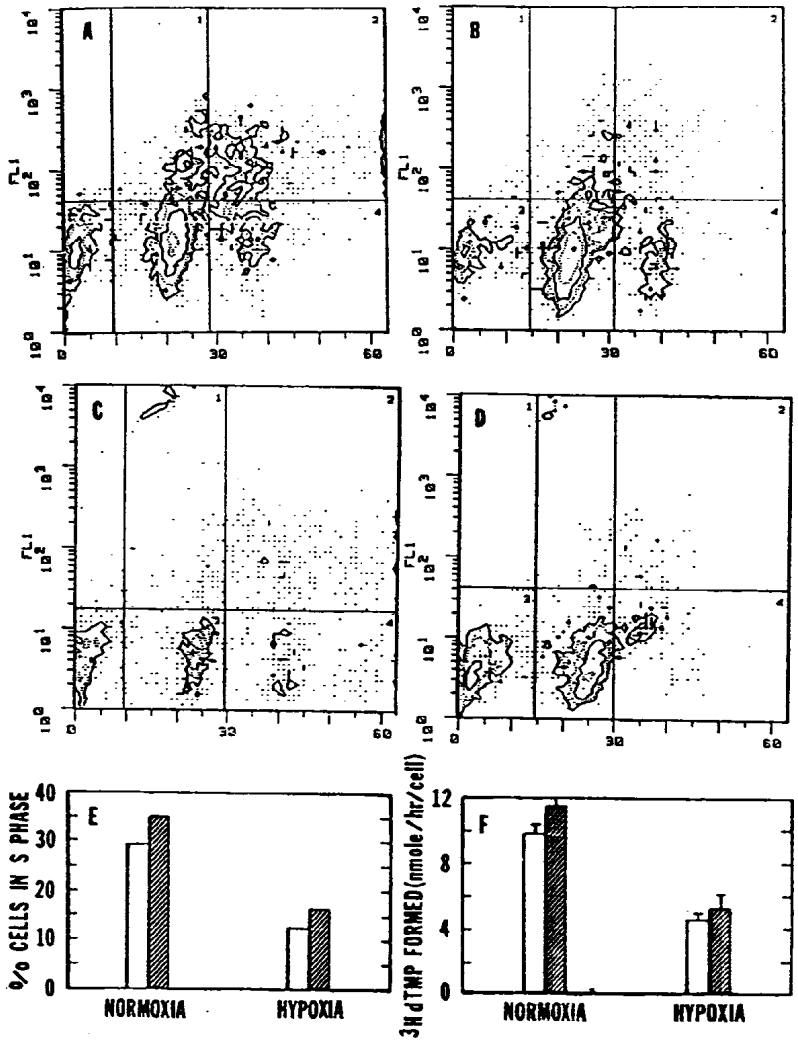

Fig. 2. Effect of hypoxia on the endothelial cell cycle. A–D: Bivariate contour histograms generated by flow cytometry (FACS analysis) of double-labeled aortic endothelial cells grown either under normoxic (pO_2 = 147 mm Hg) (A) or hypoxic (pO_2 = 14 mm Hg) (B) conditions, and capillary endothelial cells grown under either normoxic (C) or hypoxic (pO_2 = 14 mm Hg) (D) conditions. In each case, cultures were pulse-labeled with BrdUrd for the last hour of a 48 hour incubation, and flow cytometry was performed as described in the text. E: Summary of DNA analysis. Fitted values for cells in G_1, S, and G_2+M were calculated from the contour histograms in normoxic and hypoxic cultures, and then the percentage of cells in S phase was calculated in each subset: aortic endothelial cells (open bar) and capillary endothelial cells (hatched bar). F: Thymidine kinase activity of aortic endothelial cells (open bar) and capillary endothelial cells (hatched bar). Endothelial cultures were plated at a density of 3.5×10^4 cells/cm^2, and grown in normoxia or hypoxia (pO_2 = 14 mm Hg) for 48 hr. Then, thymidine kinase activity was assessed as described in the text: ^{3}H-dTMP/hr/cell (mean ± SD) of aortic (open bar) and capillary ECs (hatched bar) is shown.

ined, but at the lower oxygen tensions cytoskeletal assembly and cell shape were perturbed. For example, at $pO_2 \approx 14$ mm Hg, the aortic EC monolayer consisted of larger cells with altered arrangement of the actin-based cytoskeleton (central actin arrays were reduced and altered, circumferential stress fiber bands attenuated as were the actin networks on the cytoplasmic face of apposed cellular margins) (Fig. 6A) compared with normoxic controls (Fig. 6B). In addition, elliptical gaps became apparent between contiguous cells in hypoxic cultures (Fig. 6A). Following restoration of a normal air atmosphere, within 48 hr, the appearance of previously hypoxic cultures closely resembled normoxic controls (Fig. 6C). Similar results were observed with hypoxic capillary ECs.

Barrier and coagulant function of EC monolayers grown to confluence under hypoxic conditions

Structural studies showing intercellular gaps in the EC monolayer under hypoxia suggested that these monolayers would have augmented permeability to

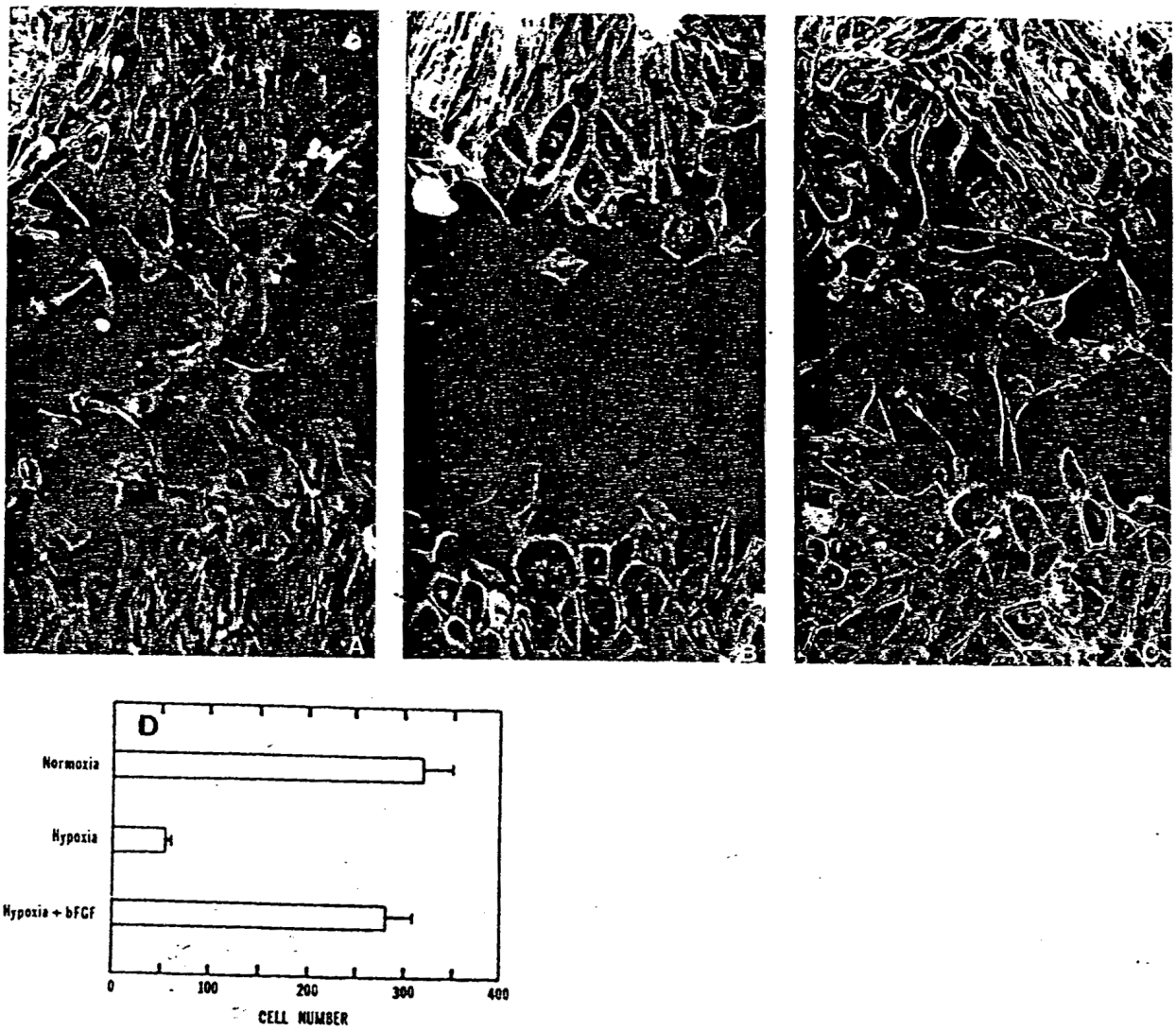

Fig. 3. Effect of hypoxia on the reparative response of aortic endothelial cells to wounding. Confluent monolayers of aortic ECs were wounded using a cell scraper and incubated for 24 hr in either normoxia or hypoxia (pO_2 ≈14 mm Hg) in the presence or absence of FGF. Cells were then fixed and visualized using rhodamine phalloidin (A–C). A: Cells maintained in normoxia after wounding. B: Cells maintained in hypoxia after wounding. C: Cells maintained in hypoxia after wounding with the addition of bFGF, 5 ng/ml. D: Effect of hypoxia on EC migration. Confluent monolayers of aortic ECs were wounded using a cell scraper and incubated overnight either in normoxia or hypoxia (pO_2 ≈14 mm Hg). The number of cells that migrated into the denuded area was determined as described in the text. The mean ± SD is shown, based on examining five fields. Normoxia = cultures maintained in normoxia after wounding; hypoxia = cultures maintained in hypoxia after wounding; hypoxia + bFGF = cultures maintained in hypoxia after wounding with the addition of bFGF, 5 ng/ml.

macromolecules and lower molecular weight solutes. To assess this, capillary and aortic ECs were grown in a controlled oxygen atmosphere on polycarbonate membranes until confluence was achieved, and the passage of macromolecular and lower molecular weight tracers (^{3}H-sorbitol, 500 Da, ^{3}H-inulin, 5,000 Da, and ^{125}I-bovine serum albumin, 67,000 Da) was studied. Increased diffusional passage of these tracers across postconfluent monolayers of aortic endothelium was observed: there was a 300–500% increase at pO_2 ≈14 mm Hg, and a 150–200% increase at higher oxygen concentrations, pO_2s of 18 and 21 mm Hg, respectively, compared with cultures maintained in an ambient air atmosphere. Hypoxia induced similar changes in permeability in the micro- and macrovascular endothelial cultures, although the capillary cells appeared somewhat more resistant to hypoxia-induced perturbation of barrier function. The increase in permeability of hypoxic EC monolayers was reversible, even in cultures maintained at pO_2 ≈14 mm Hg, with diffusional pas-

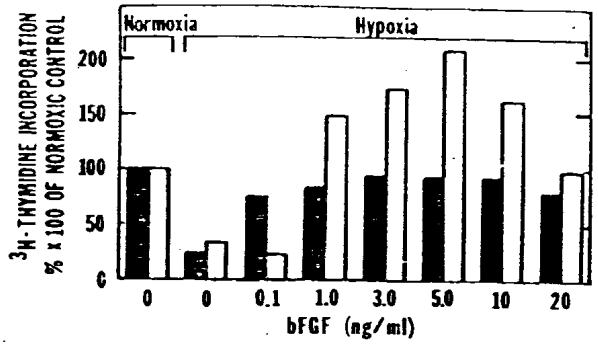

Fig. 4. Effect of bFGF on ^{3}H(methyl)thymidine incorporation by aortic (shaded bars) and capillary (open bars) endothelial cultures grown in hypoxia (pO_2 = 14 mm Hg). ECs were plated (3.5 x 10^4 cells/cm^2) and incubated for 24 hr under hypoxic condition before addition of the indicated concentrations of bFGF. The incubation was continued for 48 hr, and during the last 7 hr ^{3}H(methyl)thymidine was added. Incorporation of radiolabel was assessed as described in the text and is expressed as fold increase (mean ± SD) compared with that observed in cultures maintained in hypoxia without added bFGF, the normoxic control (the latter was arbitrarily defined as 1).

sage of the tracers returning to levels observed in normoxia after 48 hr (data not shown). This coincided with disappearance of the gaps between cells present in hypoxic monolayers, suggesting that a paracellular pathway was responsible for increased permeability of the cultures at lower oxygen tensions. These findings parallel our earlier results in which acute exposure of endothelial cultures to hypoxia reversibly decreased endothelial monolayer barrier function (Ogawa et al., 1990).

The induction of multiple changes in EC physiology by hypoxia suggested that cell surface coagulant function might be altered as well. In normal homeostasis, the action of mechanisms promoting blood fluidity, the anticoagulant pathways, are dominant (Ryan, 1987). This led us to examine the integrity of the protein C/protein S anticoagulant mechanism (Esmon, 1987) on the EC surface by studying the effect of hypoxia on the expression of thrombomodulin. In intact EC monolayers grown to confluence under hypoxic conditions, thrombomodulin activity was reduced only at the lowest oxygen tensions examined: for aortic ECs thrombomodulin activity was reduced at pO_2s of 18 and 14 mm Hg, whereas for capillary ECs thrombomodulin activity was reduced only at pO_2 ≈14 mm Hg. The fall in thrombomodulin activity was paralleled by a fall in total thrombomodulin-associated antigen, measured in detergent extracts of ECs. Following placement of cultures into an ambient air atmosphere, thrombomodulin levels returned to the baseline.

In addition to suppressing the anticoagulant cofactor thrombomodulin, hypoxia induced EC surface procoagulant activity. In a previous study, we found that ECs grown to confluence in normoxia and subsequently exposed to hypoxia expressed a cell surface Factor X activator (Ogawa et al., 1990). Aortic and capillary ECs grown to confluence in hypoxia also activated Factor X, in direct proportion to the degree of hypoxia to which the cultures had been exposed. Activation of Factor X

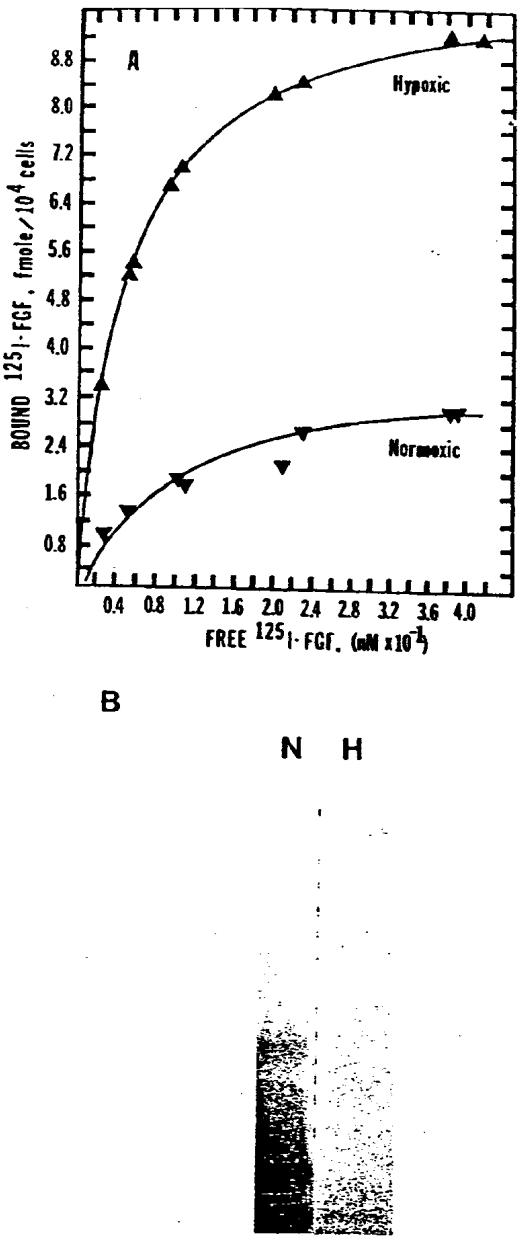

Fig. 5. Effect of hypoxia on high-affinity FGF binding sites and FGF levels in hypoxic capillary endothelial cells. A: Expression of high-affinity FGF binding sites by hypoxic capillary ECs. Capillary ECs were grown to confluence either under normoxia (solid upside down triangles; pO_2 147 mm Hg) or hypoxia (solid triangles; pO_2 ≈14 mm Hg), and then a radioligand binding assay using ^{125}I-FGF was performed as described in the text. Data were analyzed by the nonlinear least squares program and the curve indicates the best fit line: for hypoxic cultures (Hypoxic; Kd = 47 ± 5 pM; ≈6.1 ± 10^4 sites/cell) and for normoxic cultures (Normoxic; Kd = 55 ± 6 pM; 2.0 ± 0.3 x 10^4 sites/cell). B: Western blotting of hypoxic (lane N) and normoxic (lane H) cultures of ECs. Cultures were grown to confluence either under normoxia or hypoxia (pO_2 ≈14 mm Hg) and cell lysates were prepared for SDS-PAGE (15%) as described in the text. Each lane was loaded with 200 μg of total protein. After electrophoresis, proteins were transferred to nitrocellulose paper, and FGF was visualized using anti-FGF antibody as described in the text. N = sample obtained from normoxic culture; H = culture obtained from hypoxic culture.

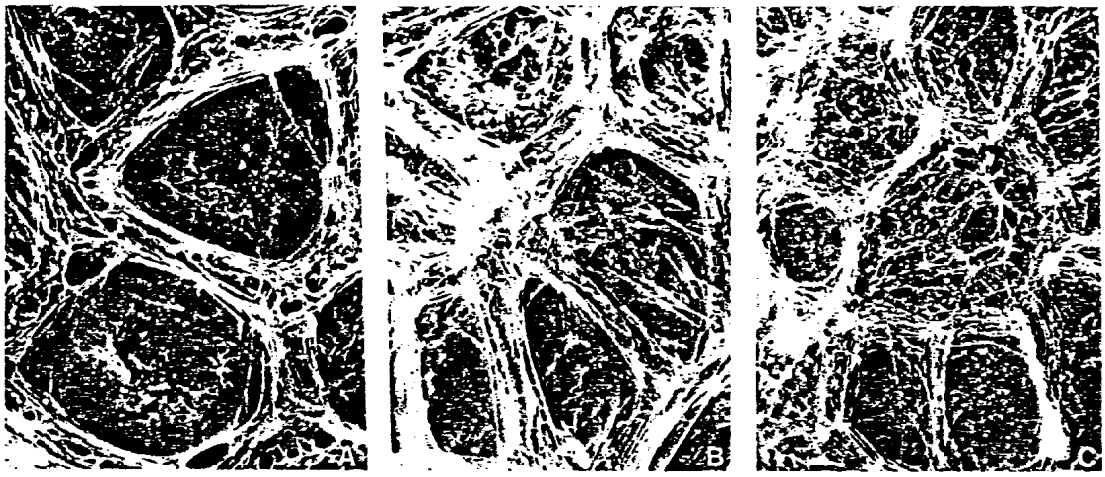

Fig. 6. Effect of hypoxia on the organization of the actin-based cytoskeleton of endothelial monolayers. A: Aortic endothelial monolayers were grown to confluence in hypoxia (pO_2 ≈14 mm Hg), and the actin-based cytoskeleton was visualized by rhodamine-phalloidin staining. Note the presence of numerous gaps between cells. B: Aortic endothelial cells were grown to confluence in normoxia and the actin-based cytoskeleton was visualized as described in A. C: Aortic endothelial cells were grown to confluence in hypoxia, then they were restored to normoxia for 48 hr and the actin-based cytoskeleton was visualized as described in A. Magnification: ×650.

was not affected by the presence of blocking antibody to tissue factor or Factor IX/IXa, as described previously (Ogawa et al., 1990). Hypoxia-induced activation of Factor X by ECs could be inhibited by mercury chloride, an inhibitor of cysteine proteases, but not by PMSF, an inhibitor of serine proteases (Table 1). In addition, expression of this Factor X-activating capacity in hypoxic endothelium could be blocked by inclusion of cycloheximide in culture medium simultaneously with placement of ECs in hypoxia, suggesting that protein synthesis was involved (Table 1). In contrast, exposure of cultures to a warfarin derivative had no effect (Table 1). Further evidence of a role for protein synthesis in expression of the hypoxia-induced Factor X activator of endothelium is that neither the supernatants nor membrane pellets of normoxic EC lysates had comparable Factor X-activating capacity. In hypoxic endothelial cells, Factor X-activating capacity was concentrated in the membrane fractions: it was not observed in the supernatants of intact hypoxic cultures and was present only to a limited extent in soluble fractions of cell lysates. These findings suggested that Factor X activation by hypoxic cultures was due to the synthesis and expression of a membrane-associated protease, distinct from the classical intrinsic and extrinsic pathways of coagulation, in which enzyme a sulfhydryl group was essential for activity. These changes in endothelial coagulant properties of cultures grown in hypoxia were similar to those observed when cultures grown to confluence in normoxia were exposed for shorter times to hypoxia (Ogawa et al., 1990).

DISCUSSION

As the cells constituting the primary interface between blood and tissues, ECs must be capable of responding to a range of environmental stimuli while continuing to perform many functions vital to homeostasis. Hypoxemia is an ubiquitous feature of many vascular disorders, especially those associated with ischemia. Growth of new blood vessels in a wound or tumor requires capillary EC migration and cell division in a hypoxic milieu (Folkman and Klagsburn, 1986; Rifkin and Moscatelli, 1989). The current studies indicate that ECs can grow and be maintained in hypoxia, but their functional properties are altered. Proliferation of capillary and aortic ECs in hypoxia is suppressed, as has previously been shown with pulmonary ECs (Fanburg and Lee, 1987), with an apparent decrease in the fraction of cells in S phase. Decreased ability to divide, along with hypoxia-mediated suppression of cell motility, contributes to a slower response of hypoxic ECs to repair of the monolayer after wounding. This may be due, at least in part, to a fall in levels of endogenous endothelial FGF in hypoxia. However, enhanced expression of what appear to be functional, high-affinity cell surface receptors for FGF in hypoxia allows endothelium to respond to bFGF released from

TABLE 1. Characterization of the Factor X activator of endothelium grown in hypoxia[1]

Condition	Relative factor Xa formation	
	Aortic, %	Capillary, %
Normoxia	5 ± 1.2	4.8 ± 0.9
Hypoxia	100	100
+ PMSF	95 ± 2.3	98 ± 4.8
+ $HgCl_2$	42 ± 4.9	52 ± 3.9
+ cycloheximide	31 ± 4.4	26 ± 3.1
+ warfarin	102 ± 5.3	98 ± 1.8

[1] Aortic and capillary ECs were grown to confluence in normoxia or hypoxia (pO_2 ≈ 14 mm Hg). Where indicated, cycloheximide (0.1 μg/ml) or the warfarin derivative 3(α-acetonylbenzyl)-4-hydroxycoumarin (1 μg/ml), was added for 24 hr before the assay was performed. Then, cultures were incubated with either medium alone or medium containing PMSF (2 mM), or $HgCl_2$ (0.1 mM) for 30 min to inactivate sensitive proteases. After washing the cells extensively, Factor X activation was assessed during a 30 min incubation period with Factor X (1 μM) at 37°C, as described in the text. Factor X activation in a given experimental condition (mean ± SD) is reported as a percent of the Factor Xa formed in cultures maintained in hypoxia alone (defined as 100%, ≈ 120 ng/ml per 5×10^5 cells).

damaged cells. FGF release from cells could occur acutely, following hypoxic injury to other cells, such as cardiac myocytes, which are more sensitive to hypoxia (and thus could undergo lysis) and have been shown to contain FGF (Weiner and Swain, 1989; Heathers et al., 1987). Alternatively, mitogenic factors could be selectively elaborated by hypoxic cells, such as macrophages. In this context, low oxygen tensions have been shown to induce macrophage production of active angiogenesis factor(s) (Knighton et al., 1983).

In a previous study, we found that aortic ECs grown to confluence in normoxia and then subjected to hypoxia maintained their viability, but demonstrated reversible changes in barrier function and cell surface coagulant properties (Ogawa et al., 1990). This led us to perform the current studies in which ECs were grown and maintained for prolonged times in hypoxia. Although the response of capillary and aortic ECs to hypoxia was similar, with respect to suppression of cell growth, decreased barrier function, and a shift in coagulant properties with attenuation of thrombomodulin and induction of procoagulant activity, it appeared that capillary ECs were more resistant to hypoxia-induced modulation of cellular properties. This could reflect the greater ability of capillary ECs to function effectively in environments with a range of lower oxygen tensions, as can often occur in the microcirculation. For example, monolayers of capillary ECs only displayed significantly increased permeability at pO_2 ≈14 mm Hg, and even at that oxygen tension barrier function was only decreased by about one-third compared with aortic EC monolayers which showed increased permeability at higher levels of oxygen and a change in permeability of greater magnitude pO_2 ≈14 mm Hg. Similarly, a more severe suppression of thrombomodulin was seen at higher oxygen concentrations with aortic than capillary ECs. Suppression of thrombomodulin appeared to be due to selective inhibition of its expression (up to 75% inhibition on aortic endothelium), while general EC protein synthesis was decreased only by ≈20%.

Expression of an apparently novel activator of Factor X by hypoxic cultures appeared to be similar in aortic and capillary ECs. This Factor X activator was distinct from tissue factor or Factor IXa, and could only be detected if protein synthesis was intact. Previously, we have observed expression of a comparable novel procoagulant activity in confluent aortic ECs grown in normoxia following their exposure to hypoxia (Ogawa et al., 1990). Production of this apparently novel procoagulant by both aortic and capillary ECs leads us to speculate that it could be a marker of hypoxemic vessel wall injury. These data contribute to an emerging picture in which the EC response to hypoxia results from a spectrum of changes in cellular physiology and biosynthetic pathways; some of these may eventually be exploitable to allow for detection of hypoxemic vascular injury at a reversible stage.

ACKNOWLEDGMENTS

We would like to thank Mr. Samuel Rover for his generous contribution and Ms. Carmen Viana for her generous efforts on behalf of our laboratory. We are grateful to Dr. Peter Caldwell (Dept. of Medicine) for his critique of the manuscript and Dr. Gabriel Godman (Dept. of Pathology) for his suggestions during the course of these studies. We are grateful to Ms. Siegrun Rannucci for expert technical assistance in the preparation of endothelial cultures. D. Stern completed this work during the tenure of a Genentech-AHA Established Investigator Award.

This work was supported by grants from USPHS (HL34625, HL42833, HL42507, HL2271), Council for Tobacco Research (1971 and 2101R1), American Heart Association, and New York Lung Association (Research Award).

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