Alterations in Endothelial Superoxide Dismutase Levels as a Function of Growth State In Vitro

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Damage to the retinal vasculature in retinopathy of prematurity is primarily at the level of the growing neovascular front. This clinical observation in combination with experimental observations that correlate the induction of superoxide dismutase (SOD) activity with differentiation led to the hypothesis that the basis for the relative vulnerability to oxygen lies in the relative “undifferentiated” nature of proliferating endothelial cells (EC). To test this hypothesis, an in vitro model of microvascular EC was used and levels of SOD activity were assayed as a function of growth state and differentiation. The SOD activity was elevated significantly in EC that stopped growing as a contact-inhibited monolayer compared with its activity in cells in their log phase of growth. In addition, SOD levels were elevated in microvascular cells that stopped growing and were organized into a network of capillary-like tubes, the ultimate differentiated state of microvascular EC. To understand the mechanism of increases in SOD activity with differentiation, the effect of extracellular matrix synthesized by a confluent monolayer of EC was examined as was artificial growth arrest caused by mitomycin. Both treatments led to an increase in SOD activity over control cells. Thus, it is possible that SOD activity in cells is modulated by information provided from the extracellular matrix and “intracellular” signals that indicate cessation of cell growth. These data support the hypothesis that the growing front of retinal microvessels is more vulnerable to effects of oxygen-induced damage because of their relatively undifferentiated state with respect to the oxygen radical-scavenging enzyme system of SOD. Invest Ophthalmol Vis Sci 33:36–41, 1992

In the pathogenesis of retinopathy of prematurity (ROP), relative hyperoxia appears to be particularly deleterious to the endothelial cells (EC) of the less developed retinal vasculature. The vanguard of the differentiating capillary network in the peripheral retina is damaged or obliterated; more mature central retinal microvessels usually are spared. The damage in ROP is statistically correlated with both hyperoxia and prematurity.1,2 Although oxygen-induced damage to vascular EC is well established,3,4 little is known of vascular immaturity as a factor in oxygen-induced damage. With respect to this possibility, we propose that less mature vascular EC may be damaged preferentially because they are poorly protected against oxygen radicals.

Although cells have many mechanisms of such protection, superoxide dismutase (SOD) is a key enzyme in the defense against oxygen-induced toxicity. Increased SOD activity confers protection against hyperoxic damage in a number of eukaryotic and prokaryotic cells.5 There is much evidence indicating that developing organisms have large increases in SOD activity, whereas changes in other antioxidant defenses are correlated poorly with development.6–9 Changes in SOD also are associated with cell state transitions, such as malignant transformation or dedifferentiation. Tumor cells are associated with low manganese SOD (Mn SOD) activity10 compared with their untransformed counterparts. Augmentation of cultured EC with SOD from liposomes11 prevents oxygen-induced injury.

Therefore, we hypothesized that mature EC might be spared in ROP, at least in part, because of a relative increase in the level of SOD activity in mature versus immature vascular EC. To test the hypothesis that SOD levels increase as a function of the decreased proliferation and/or more differentiation, we examined SOD activity in an in vitro system of bovine vascular EC. In their log phase of growth, EC were assumed to represent the most immature endothelium, whereas confluent EC and cells induced to form a system of capillary-like branching tubes were used to model mature endothelium.12 In both model systems, the activities of copper–zinc SOD (Cu/Zn SOD) and the cytoplasmic isozyme, Mn SOD, were examined as a function of cell "differentiation."
Materials and Methods

Tissue Culture and Cell Preparation

Capillary endothelial cells (BCE) from bovine adrenal cortex and bovine aortic EC (BAE) were isolated and cultured as previously described. The BCE were maintained on gelatin-coated plastic in Dulbecco's modified Eagle's medium with 10% calf serum (DMEM/10 CS; Hyclone, Logan, UT) with crude retinal factor (5 μg/ml) and were used between passages 9 and 14. The BCE were maintained in DMEM/10 CS and were used between passages 3 and 15. An atmosphere of 95% room air 5% CO₂ was maintained.

For analysis of subconfluent cells, EC were plated at 2 × 10⁴ cells in 60-mm tissue culture plates. To obtain cells at confluence, EC were plated at 1.6 × 10⁵ cells per 60-mm plate. After 5 days' growth, the cell number of a parallel plate was determined using a Coulter counter (Hialeah, FL). Subconfluent plates yielded approximately 6 × 10⁵ cells per 60-mm dish, whereas cells at confluent density yielded approximately 2 × 10⁶ cells per 60-mm dish. The cells of the remaining dishes were rinsed with phosphate-buffered saline (PBS), scraped into 1 ml of PBS, and then transferred to a test tube. The tissue culture dishes were rinsed twice, the rinse was added to the original 1 ml of scraped cells, and the cells were pelleted by centrifugation for 10 min at 1000 rpm. The supernatant was aspirated, and the cells were resuspended in 0.5 ml 0.05% Triton, 50 mM phosphate buffer, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8, before sonication on ice with three 20-sec bursts. The cell lysates were clarified by centrifugation at 1000 rpm for 10 min, and the supernatant was analyzed for SOD activity according to a previously described method.

To arrest EC growth, the cells were treated with mitomycin C (10 μg/ml) for 2 hr, trypsinized, and plated. They were cultured for another 5 days before scraping and analysis for SOD as described. Because the cells were totally growth inhibited, they did not divide and remained at a sparse density.

The formation of tube-like structures was induced according to earlier methods. The growth media of the BCE was changed to DMEM with 2.5% CS without retinal extract 2 days before plating. The cells then were plated at a confluent density (1.0 – 1.5 × 10⁶ cells per 60-mm plate) onto plates coated with gelatin (1.5% in PBS) or fibronectin (0.25–10 μg per plate in carbonate buffer). All cells were plated in defined media: DMEM, 1% bovine serum albumin, basic fibroblast growth factor (2 ng/ml of bFGF), high-density lipoprotein (10 μg/ml), and transferrin (5 μg/ml). Tube-like structures began to form within 24 hr and were stable for up to 48 hr.

Preparation of BAE-Derived Extracellular Matrices

To prepare a native extracellular matrix, BAE were grown to confluence on 60-mm tissue culture dishes in media as described. Three days after the cells reached confluence, the cell monolayers were incubated in 10⁻⁴ M ethylene glycol tetraacetic acid (in PBS) to remove the cells but leave the matrix intact. The BAE then were replated onto the matrix at 2 × 10⁴ cells per plate. After 5 days, the cells were removed by scraping, and the SOD levels were analyzed. Matrices alone were scraped and analyzed for SOD activity.

Time Course of SOD Activity

Subconfluent BAE were harvested and either replated at subconfluence (2 × 10⁴ per 60-mm dish) or at a confluent density (10⁵ cells per 60-mm dish) as described. Subconfluent cells were allowed to attach and grow for 24 hr. The confluent cells were allowed to reestablish confluence, a process that required a minimum of 12 hr. Triplicate plates of confluent cells were harvested by scraping 12 hr, 24 hr, and 96 hr after plating. These cells and subconfluent cells that served as controls were analyzed for SOD activity. A parallel study was conducted in which confluent cultures of BAE were used for plating.

Assay of SOD Activity

The SOD activity was assayed by the xanthine oxidase–cytochrome c method in a reaction mixture containing 50 mM potassium phosphate, 0.1 mM EDTA, 50 μM xanthine, and 10 μM ferri cytochrome c (Sigma, St. Louis, MO), pH 7.8, at 25°C in a total volume of 1 ml. The reduction of cytochrome c was followed spectrophotometrically at 550 nM. Sufficient milk xanthine oxidase was added to yield a change in absorption of 0.025/min at 550 nM. One unit of Cu/Zn SOD halves the rate of cytochrome c reduction. The activity of Mn SOD was assayed in the presence of a final concentration of 3 mM NaCN which inhibits Cu/Zn SOD activity.

All SOD activities we found represent total activity in extracts of BCE or BAE. The activity of Mn SOD was at the limit of detectability and therefore could not be quantified accurately despite the manipulation of SOD assay conditions to enhance its sensitivity. Although low levels of Mn SOD activity were found in extracts of confluent EC, there was often no measurable Mn SOD activity in subconfluent cell extracts.

Results

SOD Activity in Subconfluent and Confluent EC

The SOD activity per cell was significantly greater in confluent than in subconfluent cells for both BCE
and BAE (Fig. 1). There was a greater than fourfold increase in SOD activity from subconfluence to confluence in BCE and a 30% increase in SOD activity in confluent compared with subconfluent BAE. There was little difference in the total SOD activity per cell between confluent BAE and BCE, but subconfluent BAE had consistently higher SOD activity than subconfluent BCE. Similar results were found when SOD activity was expressed per unit protein (data not shown).

When subconfluent EC were trypsinized and replated at a high density, the cells reached visual confluence within about 12 hr. When the level of SOD activity in these newly confluent cells was compared with SOD levels in subconfluent cells, an increase in SOD activity from 0.5-1.25 units/10^6 cells was seen by 12 hr, the first time where measurement was possible. The SOD levels remained constant for up to 96 hr. Similar results were obtained in studies in which flasks of confluent cells (versus subconfluent used in these studies) were used to seed the dishes.

**Influence of Native BAE-Derived Matrices on Level of SOD Activity**

Because extracellular matrix has been shown in many systems to induce differentiated phenotypes, we investigated the possibility that the matrix also might influence levels of SOD in EC. The level of SOD activity of BAE plated and harvested at subconfluent densities on BAE matrix-coated dishes was found to be higher than the activity of parallel cultures of cells grown on gelatin-coated dishes (Fig. 2).

(Similar results were obtained for BCE plated onto BAE-derived matrices.) The SOD activity of the matrix material itself was measured to determine whether the increase in SOD was intracellular or extracellular. No measurable SOD activity was found in the BAE-synthesized matrices. The BAE cell growth on matrix was the same as or greater than that on gelatin.

**SOD Activity of BCE Organized Into Capillary-Like Tubes**

When BCE were grown in serum-free media on gelatin- or fibronectin-coated plates,12 cells frequently formed tube-like structures (Fig. 3). In some experiments, however, under identical conditions, the cells remained as confluent monolayers, and these cells were used as controls. The SOD activity of BCE that had organized into tube-like structures was significantly greater than SOD of cells of confluent monolayers; this, in turn, was greater than the SOD activity of subconfluent cells (Fig. 4). There was no significant difference in SOD levels whether tubes were formed on fibronectin- or gelatin-coated substrates. Subconfluent cells expressed approximately 1 unit of SOD activity per 10^6 cells. This increased by about 50% in cells at confluence. However, BCE organized into
Fig. 3. Morphology of BCE during various stages of growth. This micrograph illustrates the morphology of BCE at: (a) subconfluence, (b) confluence, and (c) organized into tube-like structures. (Original magnification, 125×.)

tubes had SOD levels 250% higher than subconfluent cells and about 65% higher than their confluent counterparts.

Effect of Mitomycin-Induced Growth Arrest on SOD Activity

To determine whether the increases in SOD activity in confluent EC or in EC organized into tubes was a function of the cessation of growth or whether intercellular contacts were important, we arrested the growth of BCE with mitomycin C before plating. This procedure allowed us to assay SOD in cells that were not growing but were still subconfluent (and therefore had not formed intercellular contacts). The cells were plated at a sparse density and then allowed to incubate for 5 days before the evaluation of SOD levels. To be sure that mitomycin did not influence the cells' ability to recover from trypsinization, other cells were plated first, then treated with mitomycin, before being allowed to incubate for 7 days. The SOD activity in these growth-arrested cells was compared with SOD levels in cells that reached confluence during the same period and SOD in cells plated at densities that permitted them to remain subconfluent during the 5-day course. The SOD in mitomycin-treated subconfluent cells was evaluated as a function of protein and per cell because we observed that mitomycin treatment caused significant increases in spread area and presumably in cell volume. Subconfluent mitomycin-treated cells had more than a 15-fold increase in levels of SOD activity compared with SOD activity in untreated subconfluent cells and a fourfold increase over SOD in untreated confluent cells (Fig. 5). These increases were comparable when SOD was expressed as a function of cell number or protein. Similar results were obtained in experiments using BAE.

Discussion

The increase in SOD activity we observed in EC concomitant with the cessation of growth at confluence or their organization into capillary-like tubes was consistent with the elevations in SOD activity as-
This process, SOD activity increases 46-fold.618 Likewise, total SOD activity increases 3.5-fold during development in *Physarum polycephalum* the syncytial slime mold differentiates from mitotically active microplasmodia to mitotically inactive microsclerotia. Parallel with this process, SOD activity increases 46-fold.618 Likewise, total SOD activity increases 3.5-fold during development in *Drosophila melanogaster,* and a greater than fivefold elevation is measured between human adult and fetal lung tissue.19

The stimulus for the SOD increase with differentiation is not clear. It is attractive to speculate that the induction of SOD with birth occurs as a result of exposure to a relatively hyperoxic environment. Although this may be a factor, it appears that it is not the only cause because SOD activity can be measured before birth in many tissues. That other factors are involved is evident in our cell culture model, where SOD activity increases with "differentiation" under constant oxygen conditions.

Differentiation is usually correlated with a termination of division. There is little evidence to indicate whether lack of growth is a prerequisite for differentiation or if growth ceases as a result of differentiation. Our results after arresting growth with mitomycin suggest that inhibition of cell proliferation (independent of confluence) is sufficient. However, because the effects of mitomycin are not well characterized, it is also possible that the elevation of SOD is caused by other activities of mitomycin and is unrelated to the lack of cell division. Mitomycin is capable of redox cycling with production of superoxide radical and hydrogen peroxide. Elevation of SOD activity might reflect, in part, an adaptive induction of SOD to augmented oxidative stress.

The extracellular matrix can influence cell shape, proliferation, and differentiation in many systems.20–22 In our studies, growth of subconfluent EC on extracellular matrix synthesized by confluent EC induced an elevation of SOD activity in subconfluent cells to levels approaching those of confluent cells. These results suggest that the extracellular matrix somehow signals EC differentiation and agree with other studies showing that extracellular matrix and matrix molecules can induce cultured EC to take on a more differentiated phenotype. For example, growth of adrenal cortical-derived EC on matrices derived from kidney epithelial line MDCK induced EC to form fenestrations, a characteristic not seen in cells plated on gelatin or uncoated tissue culture plastic.23 Similarly, EC plated into particular matrices organize into three dimensional capillary-like structures.24 Because EC on the matrices grew as well as or better than EC on plastic, "differentiation" in this case would appear not to be linked to inhibition of proliferation. Although we have not systematically examined this, we suspect that the cell population on matrix is a mix of both stimulated and inhibited cells because the matrix contains both stimuli such as bFGF25 and components that block cell growth, such as laminin.24

The use of cultured cells for these studies has allowed us to isolate relevant and interesting parameters. Although the relatively hyperoxic nature of standard culture conditions may affect the baseline level of SOD activity,26 we observed an increase in SOD activity over baseline levels concomitant with the cessation of cell growth. Our experiments suggest that, in vascular EC, there is a correlation between "differentiation" and increased SOD activity. The lack of protection against superoxide radicals, resulting from inadequate SOD expression, could account, in part, for the increased susceptibility to oxidative stress of the underdifferentiated vasculature in the retina of premature infants.

**Key words:** retinopathy of prematurity, oxygen radicals, capillaries, neovascularization, hyperoxia, endothelial cells

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