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Effect of Cell Substrate on Antioxidant Enzyme Activities in Cultured Renal Glomerular Epithelium

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The activities of three antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase, were monitored in isolated guinea pig glomeruli and primary or subcultured glomerular epithelial cells. Cell injury was assessed by morphologic studies and by measurement of cellular lipid peroxidation (levels of malondialdehyde). Antioxidant enzyme activities were very different in cultured cells than in parent glomeruli. The possible effect of culture substrates (tissue culture plastic, bovine corneal endothelial [BCE] cell basement membrane, and PF-HR-9 endodermal cell basement membrane) on antioxidant enzyme status, cell morphology, and lipid peroxidation was also assessed. Glomerular epithelial cells cultured on the BCE cell basement membrane substrate survived longer and showed less lipid peroxidation than cells cultured on plastic or the HR-9 substrate. Cells cultured on a plastic substrate had substantially less glutathione peroxidase activity than cells cultured on either BCE or HR-9 basement membranes. (Am J Pathol 1988, 130:616–628)

IT IS well-established that reactive oxygen species are constantly generated intracellularly in aerobic organisms[1–3] and are released extracellularly during the respiratory burst of phagocytes.[2] These highly reactive oxygen species, which include superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2), organic peroxide radicals ($ROO\cdot$), and singlet molecular oxygen ($^1O_2$), are thought to be the major mediators of oxygen cytotoxicity.[4–6] The discovery of an intracellular system that functions to detoxify reactive oxygen species in aerobic cells suggested that an oxidant–antioxidant equilibrium existed in these cells.[7,8] Although the entire antioxidant system has not been completely elucidated, major known constituents include 1) certain enzyme systems (eg, superoxide dismutase-catalase-peroxidase and glutathione peroxidase-glutathione reductase-glucose-6-phosphate dehydrogenase); 2) nonenzymatic cellular components (eg, vitamins E, C, and A); 3) low-molecular-weight reducing agents (glutathione and other thiols—cysteamine, cysteine, etc.); and 4) polyunsaturated fatty acids.[7–11]

Although early research found that the kidney could be damaged by prolonged exposure of laboratory animals to high oxygen partial pressure, the exact pathology was not clear.[12] Several recent studies have suggested that reactive oxygen metabolites damage the kidney.[13–15] Shah et al showed that reactive oxygen metabolism was quite active in normal glomerular tissue.[16] They demonstrated that glomeruli, but not tubules, were able to generate reactive oxygen species *in vitro*. The use of perfused glomerular preparations ruled out the possibility that reactive oxygen species were produced by leukocytes trapped in the glomerular microcirculation. However, this study did not conclude which type(s) of glomerular cell was responsible for the generation of these species. The

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histochemical demonstration of a specific type of peroxidase activity in glomeruli but not in renal tubules further supported Shah et al's results and implied that glomerular cells might have a unique metabolism for reactive oxygen species.[17,18] Instead of using whole glomeruli, more recent studies have used purified glomerular cells for the investigation of reactive oxygen metabolism.[19] Hydrogen peroxide and O_2^- are produced by cultured mesangial cells through the activation of arachidonic acid metabolism.[20–22] A recent study has suggested a role for reactive oxygen metabolites in the toxicity to glomerular visceral epithelial cells caused by the aminonucleoside of puromycin.[23]

Our laboratory has recently reported studies on reactive oxygen metabolism in primary kidney glomerular explants grown in chemically defined media in tissue culture plastic flasks.[24] Because the kidney glomerulus is composed of several cell types, we have recently developed techniques to selectively grow homogenous populations of different glomerular cell types (epithelial and mesangial cells).[25] The present study presents correlative data on cultured kidney glomerular epithelial cells. We demonstrate that the cell substrate affects culture lifespan and levels of antioxidant enzymes, especially the enzyme glutathione peroxidase. Lipid peroxidation, a nonspecific indicator of cell injury, was elevated in cells grown on a plastic substrate. The results are discussed in terms of developing a model for oxidant injury in culture.

Materials and Methods

Preparation of Basement Membrane-Coated Flasks

Preparation of HR-9 basement membrane-coated flasks was based on a modified procedure of Gospodarowicz[26] as described previously.[25] Flasks coated with bovine corneal endothelial (BCE) cell basement membrane (ExtraCell) were purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). All prepared flasks were stored at −20 C. Flasks were thawed at room temperature and rinsed three times with sodium phosphate buffer, pH 7.4. Before seeding the cells, flasks were preincubated with the medium to be used for culture and kept in a culture incubator (37 C, 5% CO_2) for at least 5 hours.

Preparation of Primary Glomerular Explants

Glomeruli were isolated by a screening technique as previously described[25] with modifications. Five- to 6-week-old Hartley guinea pigs (O'Brien, Oregon, WI) weighing 300–400 g each were anesthetized with Metofane (Pittman-Moore, Washington Crossing, NJ), and kidneys were removed under sterile conditions. Kidneys were first rinsed with sterile phosphate-buffered saline (PBS) (10 mM sodium phosphate, 133 mM NaCl, pH 7.4, 37 C), that contained penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO, Grand Island, NY). Pericapsular fat and capsule were removed, and the cortex was separated from medulla. The isolated cortex was minced and ground with a pestle while being washed with PBS through a 450-μ nylon sieve (Tetko, Elmsford, NY). The homogenate was further passed through one layer each of 132-μ (HC-3-132, Tetko) and 73-μ (HD-3-73, Tetko) nylon sieves. Glomeruli were collected from the 73-μm sieve.

For biochemical assay, freshly isolated glomeruli were immediately washed twice with PBS (37 C) and disrupted with a glass tissue homogenizer (Wheaton, Type A) in the presence of ice-cold 50 mM potassium phosphate buffer (pH 7.4) and 1 mM diethylenetriaminepentaacetic acid (DETAPAC). The homogenate was further sonicated as described below.

For culturing glomerular epithelial cells, freshly isolated glomeruli were directly distributed into plastic culture flasks (75 sq cm, Corning, NY) that had been preincubated with initial culture medium for 2 hours as described below.

Culture Conditions

The chemically defined culture medium used in this series of experiments was enriched Waymouth's medium (EW) as described previously,[27] with slight modification. This medium was based on Waymouth's MB752/1 from GIBCO (Grand Island, NY) and was supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml) (premixed package, Collaborative Research, Waltham, Mass), triiodothyronine (7 μg/ml), hydrocortisone (0.18 μg/ml) (Sigma Chemical Co., St. Louis, Mo), epidermal growth factor (50 ng/ml) (Collaborative Research), penicillin (100 U/ml), and streptomycin (100 μg/ml) (GIBCO). All cultured cells were maintained at 37 C in an unhumidified incubator equilibrated with 95% air and 5% CO_2. Because exposure to light generates oxygen free radicals in cell culture media,[28] cells were exposed to light for only brief periods for microscopic examination; cells were otherwise maintained in a darkened incubator.

Primary glomerular cultures were initiated on a plastic substrate (75-sq cm flasks, Corning) and in EW medium supplemented with human plasma fibronectin (8 ng/ml; a gift from Dr. Deane Mosher, University of Wisconsin). Glomeruli were seeded at a density of about 600/sq cm. Unattached glomeruli were re-

moved by aspirating the medium supernatant when primary glomerular cultures reached 80–90% confluency (Day 10); attached glomeruli and glomerular epithelial cells in cell outgrowths were harvested by exposing monolayers to trypsin–EDTA (0.25% trypsin and 0.1% EDTA in PBS, 37 C).[27] The outgrowth glomerular epithelial cells were further purified from trypsinized cell mixtures by passing through a 34-μ pore nylon screen (HD-3-34, Tetko) to separate all parent glomerular explants from resultant cell outgrowths of glomerular epithelial cells. Glomerular epithelial cells were recovered by centrifugation at 800 g for 5 minutes with a model CL clinical centrifuge (International Equipment Co., Needham, Mass). For growing glomerular epithelial cells on different culture substrates, cell pellets were resuspended in EW medium and distributed into either basement membrane-coated flasks or plastic flasks. Plastic flasks were preincubated for at least 2 hours with fibronectin (8 ng/ml) containing EW medium and washed twice with sterile PBS before use. Cells were seeded at a saturation density of 10^6/25 sq cm per flask and maintained in EW medium for more than 20 days. Medium was changed every other day. Cell samples for biochemical assay and ultrastructural analysis were obtained from Day 10 cultures of primary explants and Day 9 cultures of trypsinized secondary cultures.

DNA Assay

To avoid overestimating total cellular protein content caused by contaminating basement membrane substrates, antioxidant enzyme activities and malondialdehyde (MDA) levels were normalized by total DNA content. Total DNA was determined by the method of Downs and Wilfinger[29] as previously described.[24]

Measurement of Antioxidant Enzymes and Lipid Peroxidation

Cell lysates for enzyme assay and MDA measurement were prepared from either freshly isolated glomeruli or cultured cells. Cultured cells were first washed with PBS (37 C) and then suspended in ice-cold potassium phosphate buffer (50 mM, pH 7.4) containing 1 mM DETAPAC. Both cell suspensions and primary glomerular homogenates were then sonicated as described previously.[25] After brief centrifugation to remove cell debris,[26] sonicated cell homogenates were divided into small aliquots and frozen at −60 C for further analysis. All samples were covered with aluminum foil and kept in a dark chamber or freezer to prevent exposure to light.

Total superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured with a luminometer (Picolite, Model 6112, Packard Instruments, Downers Grove, Ill; detecting range, 300–600 nm) as described previously, with slight modifications.[27,30–32] This method utilized xanthine oxidase acting upon hypoxanthine as a source of O_2^- radicals, and suppression of O_2^--mediated luminol chemiluminescence (maximum emission wavelength, 430 nm) as an indicator of SOD activity.[33] For the assay, a freshly prepared mixture containing 50 mM potassium phosphate buffer (pH 7.8, air-saturated), 1 mM DETAPAC, 0.125 mM hypoxanthine, catalase (1 U/ml), 0.1 mM luminol, and 0.1 mg/ml bovine serum albumin was maintained in the dark at 37 C. Part of this mixture (800 μl) and 100 μl of sample or phosphate buffer (as a blank) were pipetted into a cuvette and placed into a thermostated sample chamber of the luminometer at 37 C. For each assay, photoemission was monitored by counting 20 intervals (30 seconds each) immediately after injection of 100 μl xanthine oxidase (0.001 U/ml, Sigma) into the sample chamber. The peak of photoemission (PE_{15}) was reached between 420 and 450 seconds (15th interval) in our system. The percentage of maximum photoemission (PE_{max}) was defined as follows:

$$(PE_{15} \text{ of unknown sample} / PE_{15} \text{ of blank sample}) \times 100\%$$

Each sample was serially diluted into six concentrations: 1:5, 1:20, 1:80, 1:320, 1:640, and 1:1280. Inhibition curves were plotted as PE_{max} versus ln(DNA μg). One unit of SOD activity was defined as the amount of enzyme that gives 50% of maximal photoemission (blank). Total SOD activity was calculated and expressed as units per microgram DNA, with the use of the straight linear portion of the inhibition curve.

Catalase (CAT) (EC 1.11.1.6) activity was determined with a biologic oxygen monitor equipped with Clark-type polarographic electrodes (YSI, Model 53) as described previously.[27] This method is based on measurement of CAT-mediated oxygen release in an oxygen-free assay system.[34] The monitor was set to 100% oxygen when the electrode was placed in an air-saturated assay buffer (4 ml, 50 mM potassium phosphate buffer with 1 mM DETAPAC, pH 7.4) at 30 C. The assay buffer was then deoxygenated by bubbling nitrogen gas into the sample chamber. One hundred microliters of hydrogen peroxide (H_2O_2) (300 mM) was first introduced into the sample chamber as reaction substrate, and the nonenzymatic deposition of H_2O_2 was recorded for 30 seconds. Seri-

ally diluted samples (100 μl) with CAT activity were subsequently introduced, and the change in oxygen level was monitored for up to 120 seconds. The change in oxygen level caused by CAT activity was calculated from initial velocity (percent oxygen released per minute) of the reaction curve and corrected by subtracting the part contributed by spontaneous decomposition of H_2O_2. One unit of CAT was defined as the amount of enzyme that gives a rate of oxygen release of 1%/min. CAT activity was expressed as units per microgram DNA.

Glutathione peroxidase (GPX) (EC 1.11.1.9) activity was measured according to the method of Gunzler et al[35] as previously described.[27] Eight hundred microliters of potassium phosphate buffer (50 mM, pH 7.4, with 1 mM DETAPAC), 20 μl glutathione (0.1 M), and 20 μl glutathione reductase (50 U/ml, Sigma) were added to a 1.5-ml cuvette. The initial NADPH concentration was followed at 340 nm in a spectrophotometer (Beckman 24) at 30 C. Spontaneous oxidation of NADPH in the assay buffer was determined. A 200-μl sample of phosphate buffer (blank) or cell lysate was added to the assay mixture, followed by 20 μl t-butyl hydroperoxide (t-BOOH). Both total and nonenzymatic (t-BOOH-dependent) oxidation rates of NADPH were determined. Oxidation of NADPH caused by GPX activity was determined by subtracting the spontaneous and t-BOOH-dependent NADPH oxidation from total NADPH oxidation. The rate of NADPH oxidation was calculated using a molar absorbance coefficient of 6200 $M^{-1}cm^{-1}$. One unit of GPX was defined as the amount of enzyme that causes oxidation of 1 nmol GSH/min. GPX activity was expressed as U/mg DNA.

Cellular MDA was determined as previously described.[27] The thiobarbituric acid (TBA) assay was performed by mixing the sample with freshly prepared TBA reagent consisting of 0.375% TBA, 15% trichloroacetic acid, and 0.002% butylated hydroxytoluene in 0.25 N HCl. The reaction mixtures were boiled, cooled, and centrifuged, and the absorbance of the supernatants was followed at 532 nm. A molar absorbance coefficient of 1.5×10^5 $M^{-1}cm^{-1}$ for MDA was obtained using a standard of MDA tetraethylacetal under the conditions described above. Cellular MDA was normalized by DNA content and expressed as nanomoles MDA per milligram DNA.

Gel Electrophoresis

Electrophoresis and localization of SOD on polyacrylamide gels were based on the method of Beauchamp and Fridovich[36] as described previously.[27] Manganese-SOD (Mn-SOD) and copper-zinc SOD (CuZn-SOD) were separated in nondissociating polyacrylamide slab gels without sodium dodecyl sulfate, which were constructed with a 2.5% photopolymerized stacking gel and a 10% chemically polymerized resolving gel. Electrophoresis was performed at 4 C with constant voltage (200 V) for 1 hour. Purified CuZn-SOD from human erythrocytes (Sigma) and liver homogenate from guinea pig were applied to each gel as standards. Gels were made in duplicate and stained by soaking in 2.45×10^{-3} M nitroblue tetrazolium (Sigma) for 40 minutes and then immersed in a solution containing 0.028 M tetramethylethylenediamine (Bio-Rad, Richmond, Calif), 2.8×10^{-5} M riboflavin (Sigma), and 0.036 M potassium phosphate at pH 7.8 for 40 minutes in a dark chamber. Gels were then illuminated under a cold fluorescent light until optimal contrast was achieved. Parallel gels preincubated with 6 mM sodium cyanide for 1 hour before staining confirmed that the faster moving band was CuZn-SOD because cyanide abolished its activity.

Morphologic Studies

The morphology of primary and secondary cultures of glomerular epithelium was closely observed and recorded using a phase-contrast microscope (Diavert, Leitz, Weitleir, FRG) equipped with a micrographic camera (Olympus, Tokyo, Japan, OM-2). Cultures were ultrastructurally examined as previously described,[25] with modification. Ultrathin sections, cut at either 0° (primary cultures) or 3° (secondary cultures) to the plastic cell interface, were double-stained with lead and uranyl acetate and viewed with a Hitachi H-500 electron microscope.

Statistical Analysis

Data were obtained from two duplicate experiments. Graphic data were expressed as columns (sample means) with error bars (standard errors). Sample means were compared by means of the Student t test. Values of $P < 0.05$ (95% levels) were considered significant. In the Results section, the phrase "significantly different" indicates that a statistical analysis was performed and the P values obtained were less than 0.05.

Results

Morphologic Analysis of Primary Glomerular Outgrowths *in Vitro*

Extensive morphologic studies (using both light- and electron-microscopic examination) of primary glomerular explants and their outgrowths in serum-

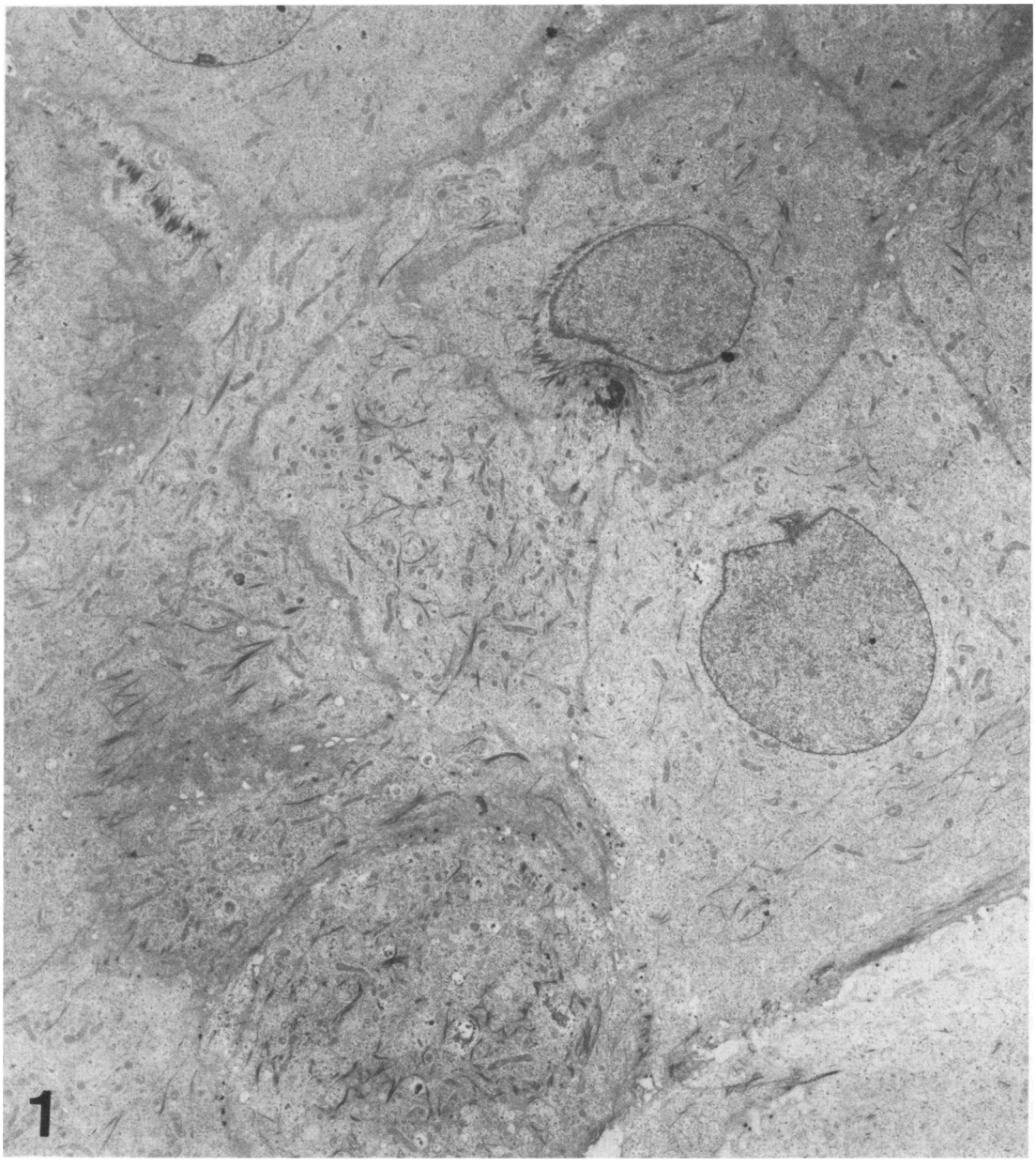


Figure 1—Transmission electron micrograph of primary glomerular culture maintained on a plastic substrate and in chemically defined medium (EW). Horizontal section (0° to plastic surface) shows glomerular epithelial cells in glomerular outgrowth on Day 10. Polygonal epithelial cells form closely packed monolayers and contain a moderate number of mitochondria and abundant rough endoplasmic reticulum. There is no evidence of cell damage. GBM, glomerular basement membrane. (×3008)

free cultures have been previously reported.[37,38] Only ultrastructural observations of Day 10 primary glomerular cultures that had been submitted for biochemical analysis in this series of experiments are reported here. Although pronounced morphologic changes occurred after glomerular epithelial cells had spread on a plastic substrate (loss of foot processes), individual glomerular epithelial cells in monolayers (outgrowths) did not show significant cell damage (myelin figures or lipofuscin) (Figure 1, horizontal section). All attached glomerular epithelial cells showed large nuclei with one or two prominent nucleoli. Cell surface membranes were smooth, with few microvilli. The cytoplasm contained abundant rough endoplasmic reticulum and a small amount of smooth endoplasmic reticulum. A moderate amount of mitochondria, usually oval or elongated, were evenly distributed in the cytoplasm. There were no significant intracytoplasmic accumulations of multivesicular bodies or lysosomal organelles.

Morphologic Analysis of Glomerular Epithelial Cells in Secondary Cultures

Glomerular epithelial cells purified from primary cultures were transferred to secondary culture and maintained on different substrates as described in Materials and Methods. Continuous light-microscopic monitoring of morphology of monolayers on bovine corneal endothelial basement membrane (BCE), HR-9 basement membrane (HR-9), and a plastic substrate is depicted in Figure 2. Cells were plated at a saturation density of 10^6 cells/25-sq cm flask. One hour after seeding cells, subcultured glomerular epithelial cells had spread on the BCE substrate and occupied all culture surfaces, whereas most cells on the plastic substrate had not yet spread out to form confluent monolayers. The degree of attachment and spread of cells on the HR-9 substrate was intermediate to that of cells cultured on the BCE and plastic substrates. Rapid formation of a closely packed monolayer on BCE within 1 hour suggests that the BCE substrate greatly enhanced attachment and spread of the cells. After 24 hours, confluent monolayers with closely packed polygonal cells were observed on all three types of substrates. Confluent epithelial monolayers could be maintained on the plastic substrate for about 5 days without significant morphologic changes (evident by light microscopy), whereas monolayers could be maintained on BCE up to 16 days. Cells on the HR-9 substrate exhibited higher viability than cells on the plastic substrate. However, progressive degeneration of epithelial monolayers on the HR-9 substrate with progressive detachment of cells was observed after 10 days in culture. At the end of the third week in culture, only about 10% of the cells remained on the plastic substrate; most showed senescent changes (cytoplasmic vacuolization and extreme flattening of cells). Significant degeneration of epithelial monolayers on the BCE substrate was also observed at this time. However, degeneration was much slower on BCE than on the HR-9 and plastic substrates.

Ultrastructural examination of subcultured glomerular epithelial cells on the BCE substrate showed individual cells with abundant oval or longitudinal mitochondria and lysosomal organelles filled with electron-dense material (residual bodies) (Figure 3a). Some residual bodies contained cytomembrane remnants, suggesting that these organelles might represent cytolysosomes. Structures resembling cell junctions were also observed (Figure 3c). Sections (3°) cut tangentially through both cells and underlying BCE basement membrane revealed a close relationship between microfilaments and BCE basement membrane. Figure 3b, which shows a high magnification of the cell–substrate interface shown in Figure 3a, suggests transmembrane colinearity between BCE fibrils and microfilaments (between arrowheads). The morphology of cells on the HR-9 and plastic substrates was similar to that of cells on the BCE substrate. However, although quantitative analysis was not performed, there appeared to be greater accumulation of lysosomal organelles on the plastic substrate.

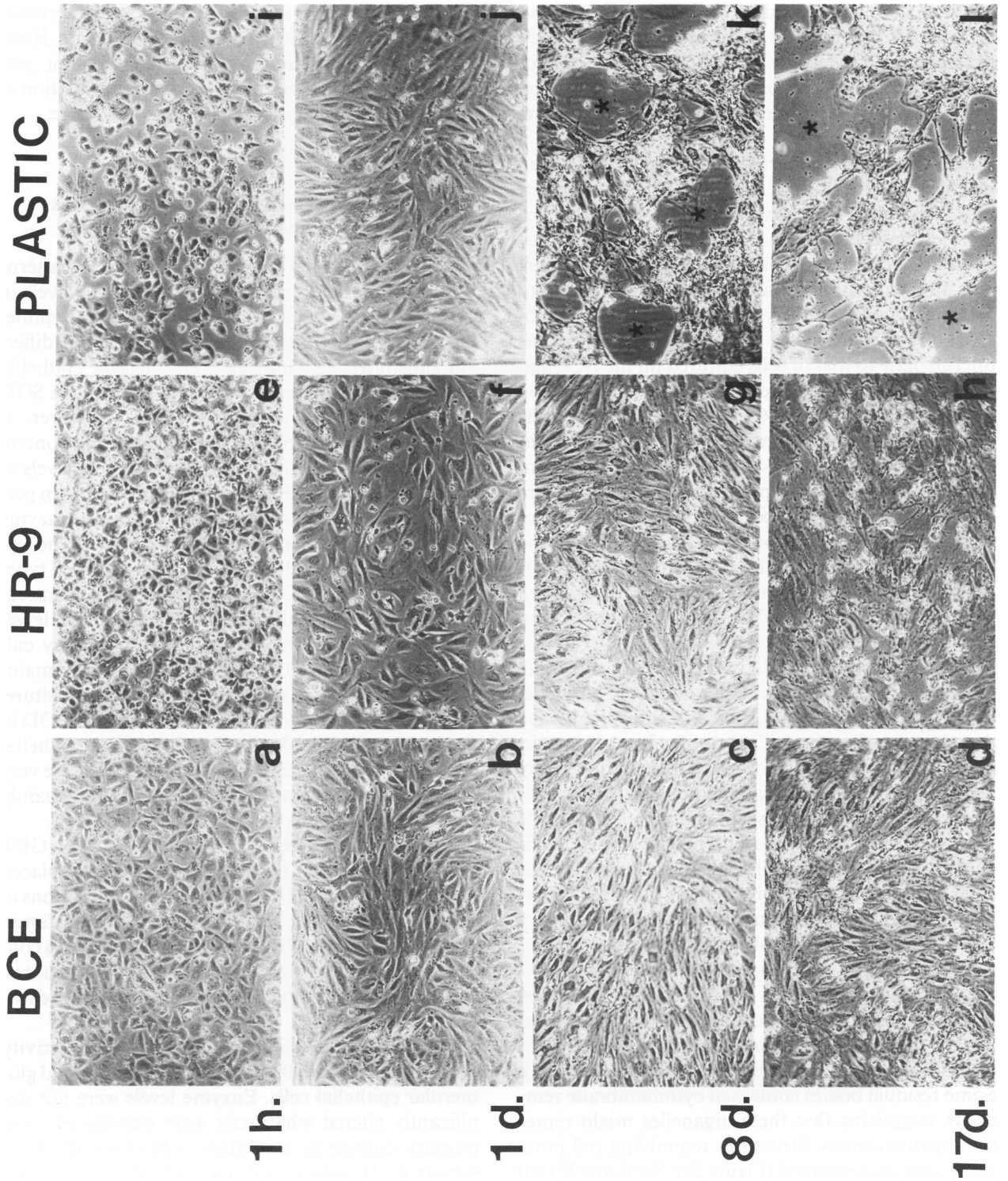
Quantitative Analysis of Antioxidant Enzymes and Lipid Peroxidation

Total SOD activity and SOD isoenzyme patterns are shown in Figures 4 and 5a and b. SOD levels in parental glomerular explants and glomerular epithelial cells in primary and secondary cultures on different substrates are compared. Glomerular epithelial cells in culture showed a significant increase in SOD activity compared to parent tissue. However, it should be emphasized that parent glomeruli contain many types of cells that might have varying levels of SOD activity. Thus, SOD activity measured from parent glomeruli might not represent the *in situ* enzyme activity of glomerular epithelial cells, because the glomerular epithelial cell is only one of several cell types in the glomerulus.[39] On all substrates tested, glomerular epithelial cells had significantly higher SOD levels in secondary cultures, compared with primary cultures. The highest SOD level was found in cells maintained on the BCE substrate in secondary culture. Electrophoretic analysis (Figures 5a and b) of SOD in parent glomeruli and cultured glomerular epithelial cells suggests that CuZn-SOD levels in cells were very low and that the alterations of SOD levels were mainly caused by alterations of Mn-SOD levels.

GPX activities were also measured (Figure 6). GPX did not significantly increase when cells were placed in primary culture. However, significant elevations of GPX levels were observed when cells were transferred and maintained on HR-9 and BCE substrates. In contrast to the cells grown on HR-9 and BCE substrates, cells maintained on the plastic substrate exhibited low GPX activity.

CAT profiles are shown in Figure 7. CAT activity was higher in parental glomeruli than in cultured glomerular epithelial cells. Enzyme levels were not significantly altered when cells were transferred from primary culture to secondary culture on the BCE substrate. However, enzyme activity slightly decreased when cells were transferred to HR-9 and plastic substrates.

As an indicator of cell injury, lipid peroxidation


BCE
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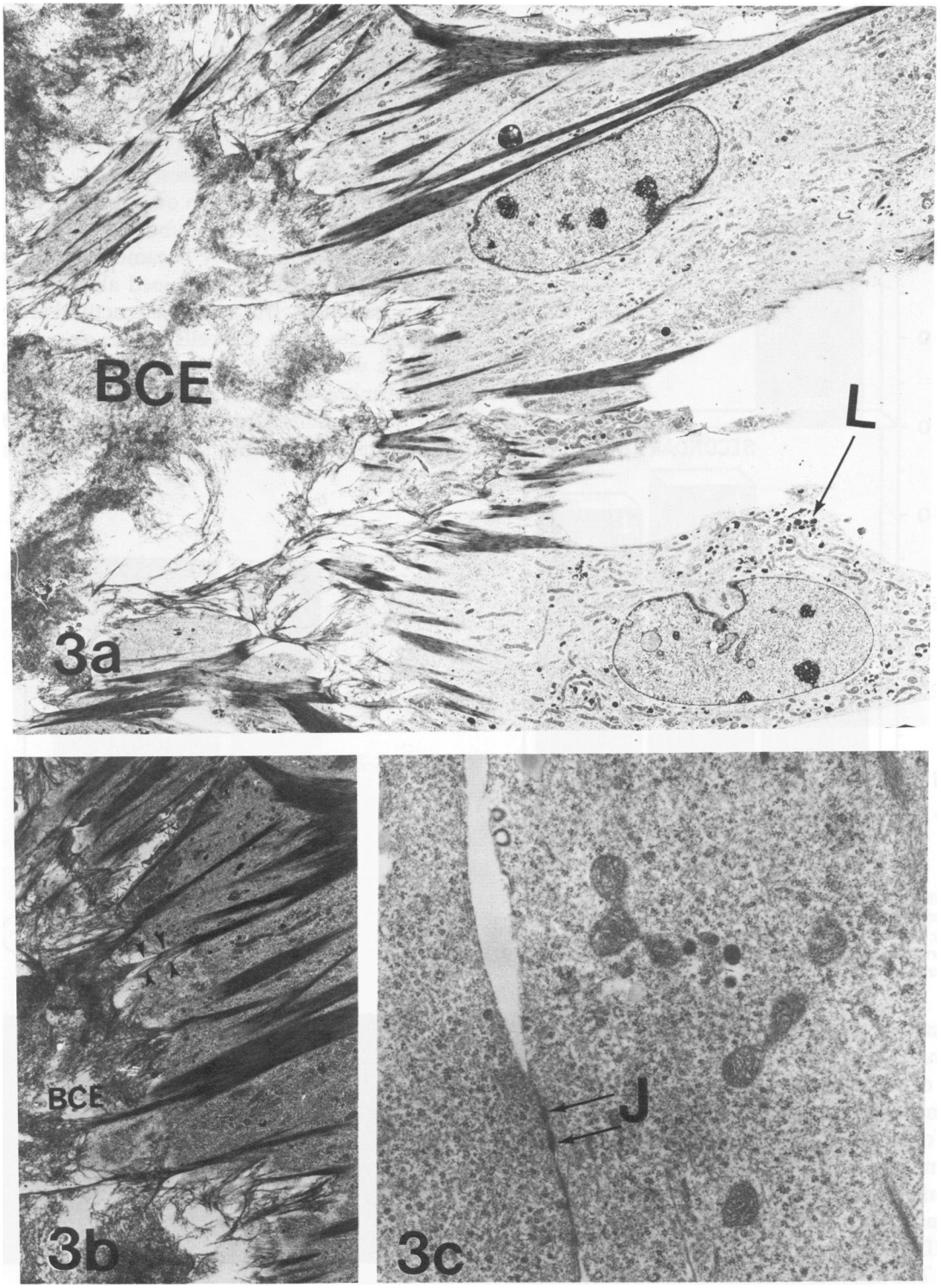

Figure 3—Electron micrograph of glomerular epithelial cells in secondary culture on the BCE substrate. **a**—Oblique section (3° to plastic surface) shows glomerular epithelial cells and underlying BCE substrate. Cells contain abundant mitochondria and a small to moderate amount of lysosomal organelles (*L*). **b**—High magnification of the cell-substrate interface demonstrates transmembrane colinearity between cytoplasmic microfilaments and BCE substrate fibrils (*between arrowheads*) **(c)**—Cell junctions (*J*) are frequently seen between epithelial cells. Cell surfaces also showed a few microvilli. (**a**, ×2000; **b**, ×4440; **c**, ×20,000)

Figure 2—Phase-contrast micrographs of secondary glomerular epithelial cell cultures. Glomerular epithelial cells were transferred from primary glomerular cultures and maintained on either plastic or biosynthetic substrates (BCE and HR-9 basement membrane) as described in Materials and Methods. Sequential morphologic changes of epithelial monolayers on the BCE substrate (**a–d**), the HR-9 substrate (**e–h**), and the plastic substrate (**i–l**) are shown in this composite micrograph. Labels on the left side of the micrograph indicate culture periods after plating cells. Note early and progressive detachment of monolayers on the plastic substrate, resulting in exposure of culture plastic (*asterisks* in **k** and **l**). (×80)

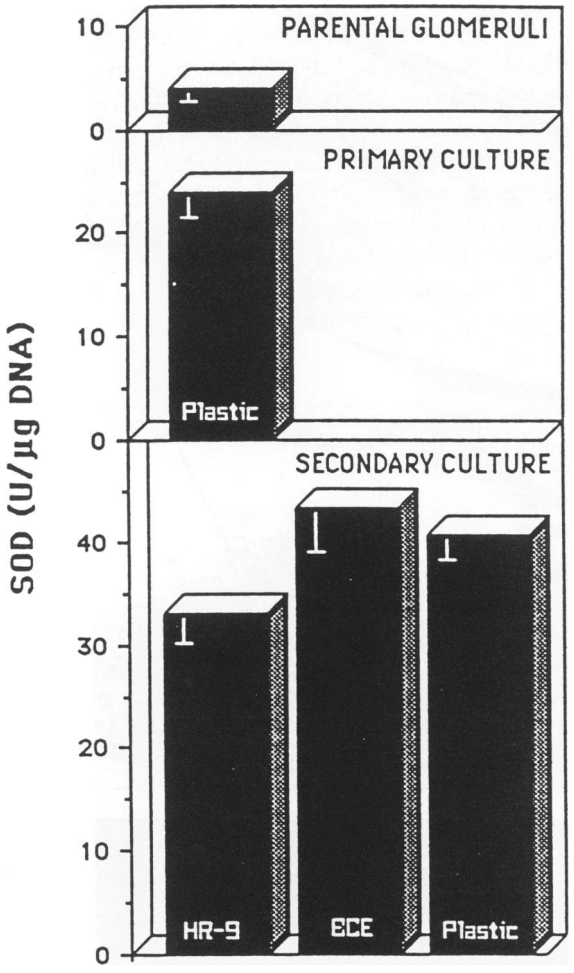

Figure 4—Effect of culture conditions on total SOD activity. Culture conditions are described in Materials and Methods. *Parent*, fresh parental glomerular explants; *primary*, primary cultures on Day 10 after explant; *HR-9*, *BCE*, *plastic*, secondary cultures on HR-9, BCE, and plastic substrates on Day 9 after trypsinization.

was assessed by measuring MDA in cell lysates. MDA was normalized by DNA content and is herein used as an index of cellular injury from peroxidation of cytomembranes. The results are compatible with the morphologic observations of decreased viability after subculture and show that cells in secondary cultures had significantly higher levels of MDA than parent glomeruli and cells in primary cultures (Figure 8). The lowest MDA level was observed in parent glomerular explants. The MDA level of glomerular epithelial cells in primary culture was only slightly higher than that in parent glomerular explants. Although all secondary cultures had high levels of MDA, MDA was significantly lower in cells maintained on the BCE substrate.

Discussion

The present study shows that antioxidant enzyme activities are significantly different in parent glomeruli and primary or subcultured glomerular epithelial cells. Subcultured glomerular cells show an increase in SOD activity (primarily Mn-SOD), a decrease in CAT activity, and variable GPX activity, depending on the cell substrate. The present study confirms and extends a previous study in our laboratory that measured reactive oxygen metabolism of primary glomerular explants rapidly became necrotic *in vitro:* lar explants originally contained all glomerular cell types, morphometric and ultrastructural analysis[37,38] demonstrated that the cells in the glomerular outgrowths were almost all glomerular epithelial cells. Furthermore, the other cell types present in the glomerular explants rapidly became necrotic *in vitro.* Therefore, our previous study was in reality a study

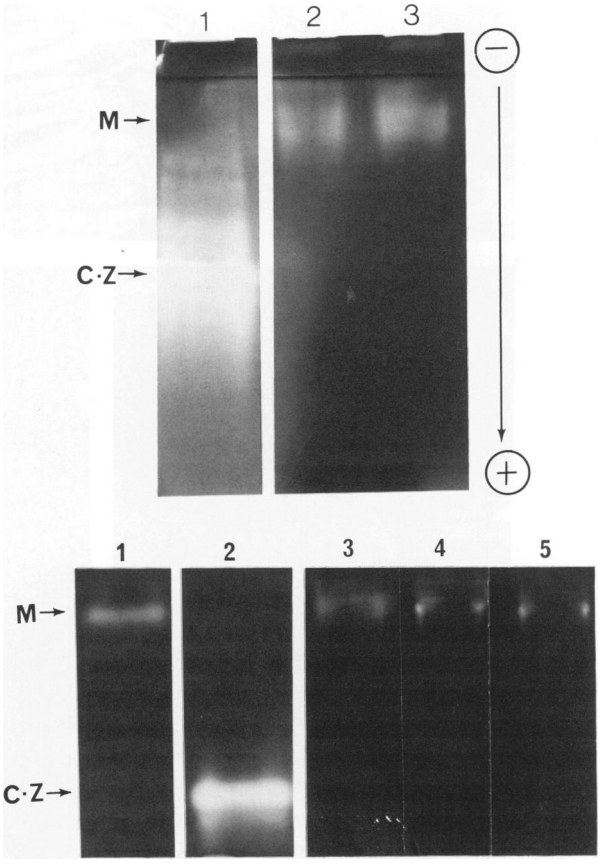

Figure 5—Polyacrylamide gel electrophoresis of SOD isoenzymes in parental glomerular explant and primary culture (**a**) and secondary culture (**b**). *M*, manganese SOD (Mn-SOD); C-Z, copper-zinc SOD (CuZn-SOD). Track 1 (sample, 10 μg protein) in **a** and Track 2 (sample, 1 μg protein) in **b** represent standard CuZn-SOD purified from human erythrocytes (Sigma). In **b**, Track 1 (sample, 1 μg DNA) represents standard Mn-SOD from guinea pig liver homogenate; CuZn-SOD band was inhibited by incubation of the gel with sodium cyanide prior to staining. In **a**, Track 2 (sample, 24.2 μg DNA) represents fresh parental glomerular explants, and Track 3 (sample, 11.8 μg DNA) represents glomerular epithelial cells in primary culture. In **b**, Tracks 3, 4, and 5 represent glomerular epithelial cells in secondary cultures (Track 3, BCE substrate—sample, 2.5 μg DNA; Track 4, HR-9 substrate—sample, 2.0 μg DNA; Track 5, plastic substrate—sample, 2.0 μg DNA).

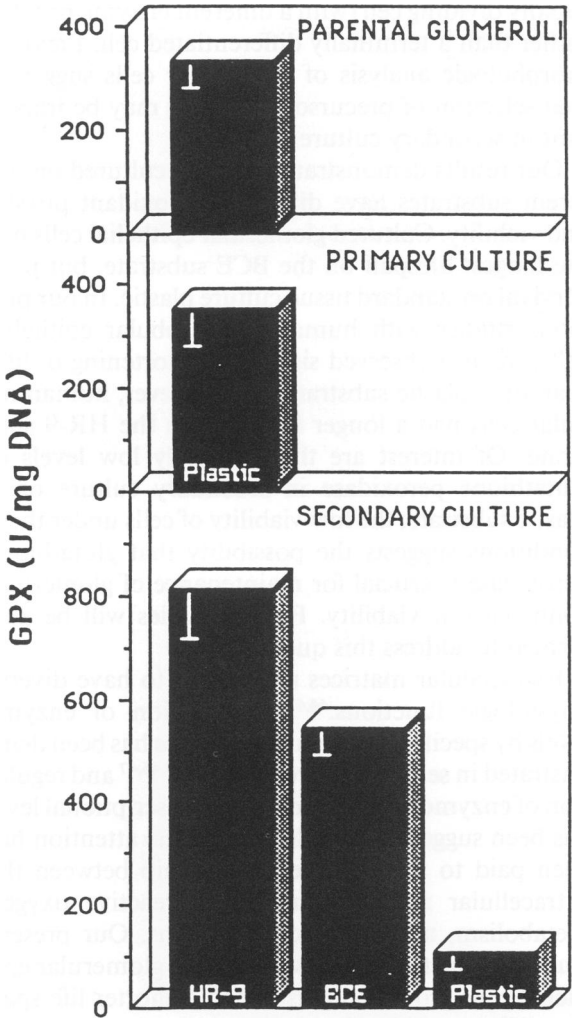

Figure 6—Effect of culture conditions on GPX activity. Culture conditions are described in Materials and Methods. *Parent,* fresh parental glomerular explants; *primary,* primary cultures on Day 10 after explant; *HR-9, BCE, plastic,* secondary cultures on HR-9, BCE, and plastic substrates on Day 9 after trypsinization.

primarily of reactive oxygen metabolism in cultured glomerular epithelial cells; in this previous study, Mn-SOD increased, CAT decreased, and H_2O_2 increased after the cells had been in culture for prolonged periods. The present study confirmed these results by using purified *homogeneous* populations of glomerular epithelial cells. We further demonstrated that lipid peroxidation increased with the age of the culture. Our experiments were not able to distinguish whether the lipid peroxidation observed was secondary to cell injury or the result of some more specific oxidant stress.

A comparison of our results from the study reported and our previous study[24] shows similar values of total SOD and CAT activities for parent glomeruli, even though completely different methodologies were used. However, in the previous study, which utilized cyanide inhibition of the NBT spectrophotometric assay for analysis of Mn-SOD activity, we concluded that the major component of SOD activity was the CuZn form of SOD. In the present study, which utilized electrophoretic analysis of SOD isoenzymes, we concluded that Mn-SOD is the primary form of the enzyme in glomerular cells. The reason for the discrepancy between these two studies is not clear, but may depend on the use of different assays used or the methods of cell preparation. However, in our present

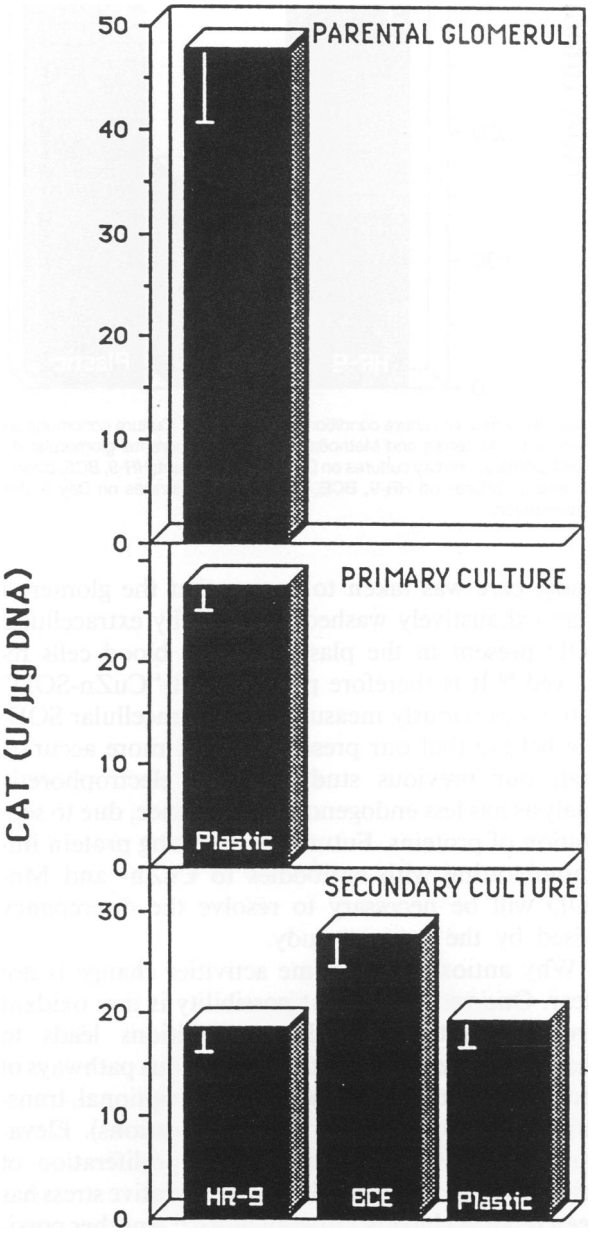

Figure 7—Effect of culture conditions on CAT activity. Culture conditions are described in Materials and Methods. *Parent,* fresh parental glomerular explants: *primary,* primary cultures on Day 10 after explant; *HR-9 BCE, plastic,* secondary cultures on HR-9, BCE, and plastic substrates on Day 9 after trypsinization.

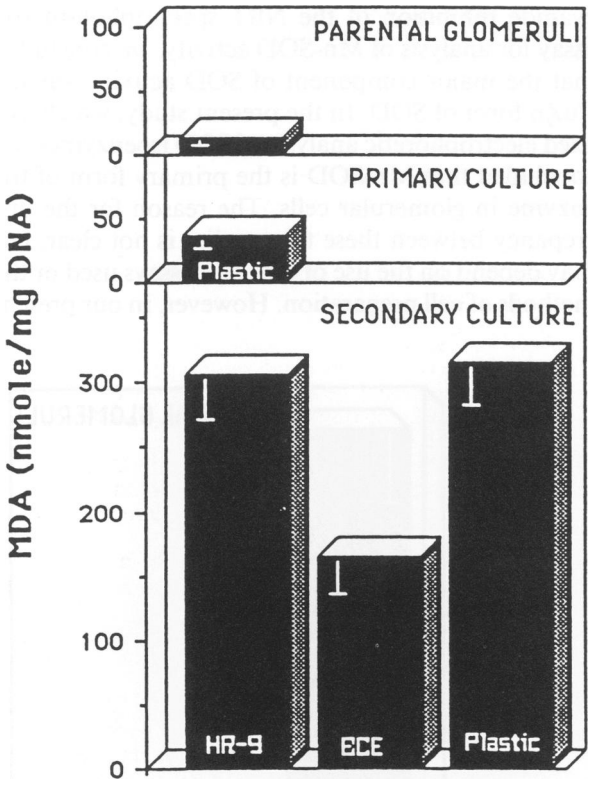

Figure 8—Effect of culture conditions on MDA level. Culture conditions are described in Materials and Methods. *Parent*, fresh parental glomerular explants; *primary*, primary cultures on Day 10 after explant; *HR-9, BCE, plastic*, secondary cultures on HR-9, BCE, and plastic substrates on Day 9 after trypsinization.

study, care was taken to ensure that the glomeruli were exhaustively washed and thereby extracellular SOD present in the plasma and/or blood cells removed.[40] It is therefore possible that "CuZn-SOD" activity previously measured was extracellular SOD. We believe that our present study is more accurate than our previous study because electrophoretic analysis has less endogenous interference, due to separation of proteins. Future studies using protein immunoblotting with antibodies to CuZn- and Mn-SOD will be necessary to resolve the discrepancy raised by the present study.

Why antioxidant enzyme activities change is not clear. One very important possibility is that oxidant stress generated by culture conditions leads to changes in enzyme activities (via various pathways of biochemical regulation such as transcriptional, translational, or posttranslational modifications). Elevation of Mn-SOD accompanied by proliferation of mitochondria under conditions of oxidative stress has been reported by other investigators.[41] Another possibility is that cells in culture may undergo dedifferentiation. Finally, selection of precursor cell types may occur such that the predominant cell in culture is a rapidly dividing cell (with a different enzyme profile), rather than a terminally differentiated cell. Previous morphologic analysis of glomerular cells suggested that selection of precursor cell types may be important in secondary culture.[42]

Our results demonstrate that cells cultured on different substrates have different antioxidant profiles and viability. Cultured glomerular epithelial cells had the longest lifespan on the BCE substrate, but poor survival on standard tissue culture plastic. In our previous studies with human renal tubular epithelial cells, we also observed significant shortening of lifespan on a plastic substrate.[27,43] However, human tubular cells had a longer life span on the HR-9 substrate. Of interest are the extremely low levels of glutathione peroxidase in secondary culture on a plastic substrate; the low viability of cells under these conditions suggests the possibility that glutathione peroxidase is crucial for maintenance of glomerular epithelial cell viability. Future studies will be performed to address this question.

Extracellular matrices are known to have diverse physiologic functions.[44,45] Modulation of enzyme levels by specific extracellular matrices has been demonstrated in several culture systems,[43,46,47] and regulation of enzyme production at the transcriptional level has been suggested.[46,47] However, little attention has been paid to the possible relationship between the extracellular matrix, intracellular reactive oxygen metabolism, and antioxidant systems. Our present study demonstrated that subcultured glomerular epithelial cells grown on plastic had a shorter life span and different levels of antioxidant enzymes (eg, glutathione peroxidase), compared with cells grown on the BCE or HR-9 substrates. These results emphasize that results from tissue culture models of oxidant stress will depend on the culture environment (probably both substrate *and* medium), and care must be taken in extrapolating the results to the *in vivo* situation. Bishop et al[48] recently demonstrated that the culture medium dramatically affects levels of reactive oxygen metabolites and antioxidant enzymes in cultured endothelial and fibroblast cells, whereas our laboratory confirmed this result in cultured human renal tubular cells.[27] Future studies must develop culture environments that are as close to the *in vivo* situation as possible.

The recent suggestion that the nephrotoxicity of the aminonucleoside of puromycin is mediated by reactive oxygen metabolites[23] underscores the importance of understanding reactive oxygen metabolism in glomerular epithelial cells. It is important that a tissue culture model precisely duplicate the *in vivo* situation; as our study clearly documents, the activities of an-

tioxidant enzymes are different *in vitro* than *in vivo*. It is hoped that expanded knowledge of tissue culture methodologies will eventually allow us to duplicate the *in vivo* situation.

Compared with human[27] and guinea pig kidney proximal tubules (unpublished observations), kidney glomeruli have very low levels of SOD and glutathione peroxidase. It is intriguing to hypothesize that these low levels of enzymes make kidney glomeruli susceptible to oxidant injury. The high level of chemiluminescence observed in glomerular cells, compared with tubular cells, may reflect lower antioxidant enzyme activities in the glomerular cells.[16] We will direct future studies in our laboratory toward analyzing whether antioxidant enzymes can be induced in glomerular cells, because these enzymes may then be used to protect glomerular cells against oxidant injury.

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