Cultured Rat Mesothelial Cells Generate Hydrogen Peroxide: A New Player in Peritoneal Defense?¹

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ABSTRACT

This study was designed to examine whether rat peritoneal mesothelial cells in culture could generate hydrogen peroxide in different experimental conditions. Mesothelial cells, incubated in M-199, spontaneously released hydrogen peroxide. This process was significantly increased by addition of phorbol myristate acetate, as well as of superoxide dismutase to the medium, whereas it was substantially inhibited by catalase. Exposure of mesothelial cells to modified M-199 medium with 1.5% glucose concentration-lactated peritoneal dialysis solution did not seem to interfere either with the spontaneous release of hydrogen peroxide, or with that induced by phorbol myristate acetate. Furthermore, exposure of mesothelial cells to the glucose (4.25%) peritoneal dialysis solution in Medium M-199, was coincident with increased hydrogen peroxide generation, which was significantly higher than the spontaneous release, and not far from that observed with phorbol myristate acetate and superoxide dismutase. So far, it can be inferred from this evidence that peritoneal mesothelial cells in culture are not only endowed with the capability of producing hydrogen peroxide, but they can also be activated to do so in a way comparable to that observed in neutrophils and macrophages. This attribute is one more indication that mesothelial cells play a relevant role in the peritoneal mechanism of defense against infection. On the other hand, continuous exposure of mesothelial cells to glucose-enriched fluids, as occurs in clinical continuous ambulatory peritoneal dialysis, may well also be at the origin of a process of continuous injury, resulting from an increased hydrogen peroxide generation.

Key Words: Cultured mesothelium, peritoneal dialysis, oxygen free radicals, peritoneal dialysis fluids, mesothelial injury

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The mesothelium represents the fifth resistance encountered by water and solutes in their pathway between the intravascular compartment and the peritoneal dialysis fluid (1). Its participation in the defense mechanisms against infection is, at this moment, still a challenge under intensive research (2-7).

In 1990, Mac Callum (8) showed that peritoneal mesothelial cells are endowed with phagocytic capability. This observation was confirmed by other investigators (9-12).

In other cells, like polymorphonuclear (PMN) neutrophils, the phenomenon of phagocytosis coincides with increased oxygen uptake (13) and initiates a process of ROS (reactive oxygen species) production, which is involved in the killing and destruction of bacteria (14). On the other hand, severe oxidative stress in an area of acute inflammation can also compromise vital functions and even viability of the various cellular components making up the affected tissue (15). ROS generation was also observed, under different experimental conditions, in peritoneal macrophages (16), human mast cells and basophils (17), monocytes (11,18), cultured glomerular mesangial cells (19), arterial smooth muscle cells in culture (20), rat pleural mesothelial cells (21), calf pulmonary artery smooth muscle (22), pulmonary endothelial cells in situ (23), and fibroblasts (18).

ROS production, also called respiratory burst, requires contact between the exposed cells and the stimulating agent (24). This reaction can also be induced by phorbol myristate acetate (PMA) (25,26), a nonspecific membrane activator (27).

One of the ROS products, hydrogen peroxide (H₂O₂), has a remarkable bactericidal activity by itself, which can become even stronger by a combined reaction including H₂O₂ with myeloperoxidase and halide (18).

ROS production by PMN leukocytes plays a pivotal role at the time of their participation in the acute inflammatory response, namely the oxidative microbial mechanism, the local production of vasoactive and immunoregulatory arachidonic acids, as well as in the secondary local tissue injury (28,29). The fact that Czybowksi et al. (30,31) detected the presence of antioxidant capabilities in human mesothelial cells, in addition to the demonstration that rat pleural cells in vitro can liberate H₂O₂ (32), prompted us to investigate whether cultured rat peritoneal mesothelial cells could eventually produce H₂O₂ after being stimulated by PMA. In this sense, a positive answer to this hypothesis would eventually imply that, in addition to cytokine and prostaglandin synthesis (3,4,5,7), the mesothelial monolayer also participates in the defense mechanisms of the peritoneal cavity against infection through the activation of the oxidative burst.
MATERIAL AND METHODS

Cell Harvesting and Primary Culture

This procedure was performed with a modification of the technique reported by Satoh and Prescott (33). Albino rats weighing 160 to 200 g were intraperitoneally injected with 40 mL Hanks' balanced salt solution, without phenol red, and 0.125% trypsin. After a dwell time of 30 min, the fluid was drained and centrifuged at 1500 rpm for 10 min. All animal experimentation described here was conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals. The pellet was washed three times with Medium M-199 and 20% fetal calf serum (FCS), and centrifuged again at 1500 rpm for another 10 min. The harvested mesothelial cells were plated in 25-cm² tissue culture flasks (Corning Glass Works, Corning, NY), and 60 min later the plates were washed with medium and incubated at a temperature of 37°C in Medium M-199, supplemented with 20% FCS, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B, in an atmosphere of 5% CO₂ until confluence. Refeeding was done every 3 days.

Identification of Cells

Cells were identified as mesothelial, based on their cobblestone appearance when observed by optical microscopy, as well as by immunohistochemical staining performed with monoclonal antibodies to rat pancytokeratin, cytokeratin 8, vimentin, monocytes/macrophages (Chemicon, Temecula, CA), and Factor VIII (Biomakor, Rechovot, Israel), and by using fluorescein isothiocyanate goat antimouse IgG (Zymed Laboratories Co., San Francisco, CA). All cells were positively stained for cytokeratins and vimentin, whereas reactions to Factor VIII antigen and monocytes/macrophages antigen were not detected.

Hydrogen Peroxide: Preparatory Experiments

Mesothelial cells growing to a confluent monolayer were harvested with 0.25% trypsin and 0.05% EDTA, resuspended in M-199 fresh medium without phenol red, supplemented with 20% FCS, centrifuged twice at 1500 rpm for 5 min, and seeded into 96-well tissue culture flat bottom plates (Corning Glass Works, Corning, NY) in a final volume of 100 μL at various cell concentrations (8.75 × 10⁵ to 8.8 × 10⁶/mL).

H₂O₂ assay production. H₂O₂ production of mesothelial cells was evaluated with the micro assay modification of Freund and Pick (34), based on the horseradish peroxidase-phenol red assay. The mesothelial cell monolayer growing on 96-well tissue culture flat bottom plates was rinsed three times with warm phenol red free Earle's Salt Solution ( Biological Industries, Kibbutz Beit Haemek, Israel), and covered with 100 μL/well of the horseradish peroxidase-phenol red solution (HRP) (Sigma Chemical Co., St. Louis, MO; P-8250, P-5530). In all experiments, reagents were dissolved in phenol red-free Earle's Salt Solution, and H₂O₂ assays were performed in the presence of 10 μg HRP for each well. Plates were incubated for 1 h at 37°C, and the amount of oxidized phenol red in each well was measured in an automatic enzyme immunoassay photometer (Spectra, SLT, Labinstruments, Salzburg, Austria) fitted with a 610-nm interference filter. Mean absorbance values from eight replicate wells were calculated, and results were expressed as nanomoles of H₂O₂ produced per milligram of protein in each well during 60 min, by use of standard curves generated with H₂O₂ solution of known concentration (30% H₂O₂, Aldrich Chemical Co., Milwaukee, WI). (Concentrations ranged between 1 and 5 μmol.) Eight experiments were done for each study.

Cell protein determination. Protein content of each well was determined in parallel with the H₂O₂ assay according to the methodology described by Shibahara and Eng (35). The mesothelial cell monolayer was rinsed with Tris buffer saline twice; plates were incubated for 15 min at 37°C with 50 μL of 0.1 N NaOH and exposed to 200 μL of diluted dye reagent (one part Bio-Rad dye reagent, three parts distilled water) for 30 min.

Measurements were done with an automatic enzyme immunoassay photometer (Spectra, SLT, Labinstruments) fitted with a 630-nm interference filter and reference wavelength at 405 nm. Mean absorbance values from eight replicate wells were calculated, and results are expressed as micrograms of cell protein per well. A standard curve was prepared from bovine serum albumin in a range of 0 to 10 μg. Eight experiments for each study were performed.

Cell viability. Cell viability was evaluated with the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-thiazolylblue (MTT) test (Sigma M-5655). This technique is a very sensitive assay of mitochondrial function, based on the reduction of MTT to form blue formazan particles, and is an indicator of the activity of mitochondrial dehydrogenases, as initially described by Mosmann (36) and modified by Reitman (37). MTT was prepared as a 5 mg/mL solution in phosphate-buffered saline (PBS). After the medium was removed, 10 μL of 5 mg/mL MTT solution were added to 100 μL of fresh culture medium in each well. The plates were gently shaken and incubated for 4 h at 37°C, in a 5% CO₂ incubator. Then, the medium was discarded, and the blue formazan precipitates were resuspended in 140 μL of acid isopropanol solution (0.04 N HCl in isopropanol).

Measurements were done within 1 h with an automatic enzyme immunoassay photometer (Spectra, SLT, Labinstruments), fitted with a 570-nm interference filter and reference wavelength at 630 nm. Mean absorbance values from eight replicate wells were estimated. Results are expressed as absorbance (A). During the preparatory experiments, absorbance values of MTT showed a close correlation with a large range of cell numbers (Figure 1).

![Graph](image-url)

Figure 1. Number of cells plated per well, after 60 min of incubation, are plotted against the observed values of MTT presented as absorbance (A). The two-tailed coefficient of regression (0.99) was significant at the P < 0.001 level (N = 40).
Cell growth and density. A colorimetric microtiter assay was used for the quantitation of the mesothelial cell monolayer. The method is based on the staining of cells with a kit (Merck, Darmstadt, Germany) holding three solutions: (1) one solution containing methanol for fixation; (2) another solution with a xanthine dye (orange color); and (3) a thiazine solution containing a mixture of Azure I dyes and methanol blue (37). So, supernatant of the monolayers cultured in 96-well flat bottom plates (Corning Glass Works, Corning, NY) was removed, and the cells were quickly dried for approximately 60 s in the air. The monolayers were then fixed with methanol (50 μL/well) for another 60 s. The plates were rinsed quickly with tap water three times; were filled again with water; and, 5 minutes later after removal of the water, were dried out extensively. Evaluation of staining was performed after stain extraction with 0.5% sodium dodecyl sulfate (Sigma, L-5750) dissolved in PBS (0.2 mL/well) for at least 90 min and measured at 600-nm wavelength with automated microplate reader (Spectra, SLT, Labinstruments, Austria). Mean absorbance values from eight replicate wells were calculated, and results were expressed as absorbance (A).

Cell counts were performed as follows: after washing the plates with PBS, cells were exposed to 0.25% trypsin and 0.05% EDTA for 3 min, verifying by microscopy that all cells were detached from the plates. Cell suspensions were repeatedly passed through a fine Pasteur pipette to disrupt clumps and counted in a hemocytometer. Preparatory experiments showed a close correlation between absorbance and cell counts (Figure 2).

Experimental Protocols

Stimulation of H2O2 production. PMA (Sigma P-8139), a very weak tumor promoter and membrane activator, has been found to be the most potent activator of H2O2 release by peritoneal macrophages (25,27). In this study, PMA was used as a mesothelial cell stimulant. One milligram of PMA, dissolved in dimethyl sulfoxide (20 nmol/well) (Sigma D-2650) was added to the HRP-phenol red solution and to the modified medium Dianea (glucose, 1.5%, 4.25%) for 60 min, and H2O2 production was measured as described previously. A significant correlation between spontaneous release of H2O2 (nanomoles per milligram of protein) and cell concentration was observed in preparatory experiments in which mesothelial cells were incubated in M-199 (Figure 3).

Effect of catalase on H2O2 production by mesothelial cells. Catalase (from bovine liver, 1540 U/mg, Sigma C-6665) is a H2O2 scavenger which can directly degrade H2O2 to H2O and O2. Twenty micromoles per well of enzyme were added to HRP-phenol red as well as to the modified medium, prepared by addition of commercially available lactate-buffered, heat-sterilized solution for peritoneal dialysis, containing glucose in concentrations of 1.5% and 4.25% (Dianea, Travenol, Ashdod) to M-199. H2O2 production was evaluated as previously described.

SOD and catalase activities were assayed with the xanthine/xanthine oxidase system (Sigma) in a standard assay mixture containing 1.5% glucose, 50 mM HEPES, pH 7.4, 0.1 mM EDTA, 0.25 mM xanthine, and 50 mM xanthine oxidase. The reaction was started by the addition of catalase (Sigma) and stopped after 2 min by the addition of 1.5% ascorbic acid.

Statistical Analysis

Data are presented as means ± SD of at least eight experiments. Each experiment, in turn, represents the mean
of eight wells taken from each sample. Therefore, sample size for each experimental group is N = 64.

As stated in the text, differences between groups were evaluated by using the two-tailed t test or the two-tailed Bonferroni method of multiple comparison against a single control. The level of significance was set at P < 0.05. For each paired comparison, the t test was calculated only after analysis of data showed that the number of cases was within the minimal required sample size, according to \( \alpha = \beta = 0.05 \). Linear regression was calculated as \( \mu = A + B(X - \bar{X}) \), where A and B are parameters, \( X \) is any given value and \( \bar{X} \) is the mean of the chosen \( X \) values of the individuals in the sample.

RESULTS

Mesothelial cells incubated in M-199 for 60 min spontaneously released \( H_2O_2 \), at a mean value of 1.30 ± 0.54 nmol/mg protein (Figure 4). This value, which was used as control for the experimental incubations reported in this study, was not significantly different from that observed in the preparatory study (1.29 ± 0.55 nmol/mg protein). Indeed, release of \( H_2O_2 \) by cells exposed to PMA for 60 min, significantly increased to a mean of 1.952 ± 0.690 nmol/mg protein (P < 0.001) (Figure 4).

Exposure of mesothelial cells to SOD was coincident with a significantly higher concentration (P < 0.001) of \( H_2O_2 \) (1.84 ± 0.36 nmol/mg protein after 60 min of incubation), compared with the estimation made in control, unstimulated mesothelial cells (Figure 5).

\( H_2O_2 \) production by cells incubated in the presence of catalase (1.04 ± 0.44 nmol/mg protein) was significantly lower (P < 0.01) than that seen in the control group (1.30 ± 0.54 nmol/mg protein) (Figure 6), as well as that detected in cells incubated in M-199 with PMA (1.97 ± 0.76 nmol/mg protein; P < 0.001).

\( H_2O_2 \) release by mesothelial cells exposed to modified medium Dianeal (glucose, 1.5%) was 1.56 ± 0.50 nmol/mg protein per 60 min, a value not significantly different from the spontaneous release (1.30 ± 0.54 nmol/mg protein) (Figure 7). Addition of PMA to the modified medium 1.5% Dianeal glucose increased the \( H_2O_2 \) production to 1.86 ± 0.50 nmol/mg protein per 60 min, which is significantly different from the spontaneous release (1.30 ± 0.54 nmol/mg protein; P < 0.01), but not far from that seen in cells incubated in M-199 with PMA (1.95 ± 0.70 nmol/mg protein; P > 0.05).

Exposure of mesothelial cells to modified M-199 medium with 4.25% Dianeal glucose increased production of \( H_2O_2 \) to 1.70 ± 0.54 nmol/mg protein per 60 min, a value significantly higher than the spontaneous release (1.30 ± 0.54 nmol/mg protein; P < 0.01).

Addition of PMA to this modified M-199 increased even more \( H_2O_2 \) production (2.15 ± 0.34 nmol/mg protein per 60 min, P < 0.001, compared with spontaneous release), even though this level was not significantly different from that obtained after incubation.
of cells in Medium M-199 with PMA (1.87 ± 0.56 nmol/mg protein) (Figure 8).

After 60 min of exposure, H₂O₂ production by mesothelial cells exposed to modified medium 4.25% Dianeal glucose was higher than that observed with the 1.5% glucose fluid, even though the difference did not reach statistical significance (1.70 ± 0.46 versus 1.56 ± 0.50 nmol/mg protein).

As shown in Table 1, cell viability evaluated with the MTT test was not reduced in any experimental group.

**DISCUSSION**

Hydrogen peroxide is a diffusible reactive oxygen metabolite formed by either enzyme-catalyzed or spontaneous dismutation of superoxide anion. It belongs to the group of metabolites called ROS, generated in the process of oxidative burst. Exposure of granulocytes to bacteria initiates a process of superoxide production involved in the killing of the microorganisms which results in the oxidative burst (14,15,38,39). This observation, in addition to the known phagocytic capabilities of peritoneal mesothelium (8–12), prompted us to investigate in the in vitro setup whether cultured mesothelial cells could eventually produce and liberate H₂O₂. Indeed, the experiments reported here showed spontaneous release of H₂O₂ when cells were incubated in Medium M-199. This process was substantially increased by addition of PMA, as well as of SOD to the medium, whereas it was significantly inhibited by catalase.

So far, it can be inferred from this evidence that peritoneal mesothelial cells in culture are not only endowed with the capability of producing H₂O₂, but they can also be activated to do so in a way similar to that observed in neutrophils and macrophages (13–16), even though in considerably lower concentrations. It is not clear, however, whether the enhanced presence of H₂O₂ in the experimental condition described in this study resulted only from increased generation, or from a coincidence of higher production and a simultaneously reduced degradation (30,31). It should be noticed that exposure of cells to the lactated peritoneal dialysis fluid in both glucose concentrations did not seem to interfere either with the spontaneous release of H₂O₂ or with that induced by PMA. Indeed, our experiments point to the dose-related effect of glucose concentration on the generation of H₂O₂.

The relevance of the participation of mesothelial cells in the defense mechanisms against infection is obvious. On the other hand, a large body of evidence supports the concept that H₂O₂ is an important me-

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**Figure 7.** H₂O₂ generation by cells exposed to 1.5% glucose-modified solution with PMA and to PMA alone are not far from each other. However, both means are significantly higher than values observed in the control group, as well as from those seen in cells treated only with 1.5% glucose-modified solution without PMA (**, P < 0.01).**

**Figure 8.** Mesothelial cells exposed to PMA, to 4.25% glucose-modified solution alone, and with PMA show levels of H₂O₂ production significantly higher than the spontaneous release observed in the control group (**, P < 0.01; ***, P < 0.001).

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**TABLE 1.** Cell viability was estimated with the MTT test.

<table>
<thead>
<tr>
<th>Cell Group</th>
<th>MTT Test, absorbance (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.057 ± 0.0069</td>
</tr>
<tr>
<td>Control ± PMA</td>
<td>0.0634 ± 0.0051</td>
</tr>
<tr>
<td>Modified Medium Dianeal, 1.5%</td>
<td>0.0566 ± 0.0089</td>
</tr>
<tr>
<td>Modified Medium Dianeal, 4.25%</td>
<td>0.0625 ± 0.01</td>
</tr>
<tr>
<td>Modified Medium Dianeal, 1.5% ± PMA</td>
<td>0.0624 ± 0.0087</td>
</tr>
<tr>
<td>Modified Medium Dianeal, 4.25% ± PMA</td>
<td>0.0661 ± 0.01</td>
</tr>
<tr>
<td>Control ± SOD</td>
<td>0.061 ± 0.0041</td>
</tr>
<tr>
<td>Control ± Catalase</td>
<td>0.0571 ± 0.0039</td>
</tr>
</tbody>
</table>

a Comparison of the means ± SD of each experimental group with control, with the Bonferroni test of multiple comparisons against single control, fail to show significant statistical differences.
diator of tissue injury (15,40–43). Actually, generation of free radicals, liberated during the oxidative burst, coincides with the remarkable injury of mesothelial cells in culture (44), which is maintained as long as the reactive electron is passed from molecule to molecule. Involvement of nucleic acids contributes to DNA damage. Experimental studies have shown that \( \text{H}_2\text{O}_2 \) induced DNA strand breaks in a variety of human target cells exposed for not more than 30 s (45,46). This evidence may well imply that generation of free radicals by mesothelial exposed to peritoneal dialysis solutions could be, at least in part, at the origin of the atypical mesothelial cells observed in effluent of patients in long-term peritoneal dialysis (47,48).

\( \text{H}_2\text{O}_2 \) is also the primary product of the highly specialized NADPH oxidase system present in the plasmalemma of cells involved in the mechanisms of acute inflammation, including neutrophils, eosinophils, monocytes, and macrophages. When activated, this NADPH oxidase mechanism catalyzes the reduct- tion of oxygen to \( \text{H}_2\text{O}_2 \) and superoxide anion resulting, again, in the respiratory burst (49).

In this context, it is interesting to remark that human PMN exposed to a low-pH, high-glucose concentration, lactate-buffered solution for 30 min showed a significant inhibition of their capability to generate oxidative free radicals (50,51). This behavior of PMN, opposed to that observed in this study in mesothelium, most probably may be explained by the different formulation of the incubation fluid. Indeed, at variance with the studies by Liberek et al. (50,51), our experiments were performed incubating cells in peritoneal dialysis fluids prepared in Medium M-199. Living cells depend absolutely on the medium in which they are immersed. Consequently, the extracellular environment used in this study is closer to the optimal chemical composition required to preserve their functional capabilities in vitro and, in this sense, obviously more adequate than the unmodified dialysis solution.

The normal background level of \( \text{H}_2\text{O}_2 \) production may serve as a still unrecognized physiologic function of mesothelial cells. Production of \( \text{H}_2\text{O}_2 \) in the peritoneal cavity by the mesothelial monolayer may be an important adaptive biologic response that, combined with endocytotic processes (1–5,13,29,35–37), could function as a host defense against transient bacterial growth in the peritoneal cavity. \( \text{H}_2\text{O}_2 \) could also act as a chemoattractant drawing leukocytes to the site of damaged mesothelium, initiating the adaptive host response. It should be noticed, however, that this superoxide ion and \( \text{H}_2\text{O}_2 \) production can be cytotoxic, and that its major toxicity occurs through a secondary generation of potent oxidants like HOCl. In this context, activation of mesothelial cells can also have the potential risk of damaging the peritoneal membrane and even of increasing its permeability, at least to macromolecules, as observed in lung alveolar epithelium and capillary endothelium (52). Furthermore, increased oxidative stress is considered to play a substantial role in the development of some microvascular complications derived from diabetic hyperglycemia (53–56). Also, endothelial cells cultured in high-glucose medium showed \( \text{H}_2\text{O}_2 \) cytotoxic effects derived from increased generation and/or reduced degradation of \( \text{H}_2\text{O}_2 \) (57–59). Oxidative stress has also been widely postulated to play a causal role in the aging process (60–62). Both situations are associated with thickening and layering of the subendothelial basement membrane in different microvascular beds (63–67). Indeed, these ultrastructural alterations were observed in peritoneal blood capillaries and submesothelial basement membrane of elderly, nondiabetic patients receiving long-term peritoneal dialysis (68), as well as in those obtained from streptozotocin diabetic rats (69).

So far, the evidence presented here shows that peritoneal mesothelial cells have the capability of generating \( \text{H}_2\text{O}_2 \). Consequently, this attribute, in addition to the production of interleukin-6, interleukin-8, and prostaglandins (3–6), indicates that the mesothelial monolayer plays a relevant role in the peritoneal mechanisms of defense against infection. On the other hand, the fact that short in vitro incubation of human mesothelium with free radicals induces severe cell injury (44) supports the view that the continuous exposure of mesothelial cells to glucose-enriched fluids for peritoneal dialysis, as occurs in clinical continuous ambulatory peritoneal dialysis, may well also be at the origin of a process of continuous injury resulting from an increased \( \text{H}_2\text{O}_2 \) generation coupled, perhaps, to an eventual reduced degradation.

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