Reactive Oxygen Species Induce Apoptosis in Cultured Human Mesangial Cells

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ABSTRACT

Apoptosis is a distinct form of cell death that is observed under various physiologic and pathologic conditions, and it is thought to be important in regulating the number of glomerular cells. This study investigated the possible role of reactive oxygen species in the induction of apoptosis in cultured human mesangial cells. Fragmented nuclei with condensed chromatin, a morphologic characteristic of apoptosis, were observed by electron microscopy in mesangial cells exposed to 0.02 mM hydrogen peroxide for 4 h. Nuclear DNA extracted from mesangial cells that had been incubated with hydrogen peroxide (2 to 20 mM) or with xanthine (0.05 mM) and xanthine oxidase (5 to 100 mU/mL) showed the ladder pattern on electrophoresis that is a biochemical marker for apoptosis. Hydrogen peroxide (0.02 to 20 mM) decreased the number of viable cells, as determined by trypan blue exclusion, in a dose-dependent manner. Hydrogen peroxide or xanthine and xanthine oxidase increased the lactate dehydrogenase release from mesangial cells in a dose- and time-dependent manner. The release of lactate dehydrogenase was prevented by treatment with a free radical scavenger, catalase. Hydrogen peroxide (2 mM) also significantly increased the number of mesangial cells with fragmented DNA as detected by in situ nick end-labeling. Results indicate that reactive oxygen species induce apoptosis in cultured human mesangial cells. Furthermore, apoptosis of mesangial cells induced by reactive oxygen species may contribute to the loss of such cells observed in glomerular disease.

A possible contribution of apoptosis to renal disease has been investigated mainly with regard to tubular injuries. For example, cell deletion by apoptosis occurs in tubular atrophy in experimental hydronephrosis (14), and renal ischemia and subsequent reperfusion initiate apoptosis in tubular cells (15). But for the past few years, wide-ranging and energetic research has been conducted in the association of apoptosis in renal diseases (16). Both infiltrative neutrophils (17) and the resident mesangial cells (MC) undergo apoptosis in glomerular disorders (18). Apoptosis is the major mechanism for cell clearance and mediates the resolution of glomerular hypercellularity in experimental mesangial proliferative glomerulonephritis (18, 19). Apoptosis may also be responsible for cell loss in glomerulosclerosis (20). Elucidation of the mechanism of apoptosis in glomerular cells, especially in MC, is therefore important in developing new approaches to treating glomerular disease.

Reactive oxygen species (ROS) are responsible for causing tissue injury in various glomerular diseases (21–24). Because ROS induce apoptosis in various types of cells (25), we investigated the role of ROS in the induction of apoptosis in cultured MC.
MC Preparation and Culture

Human renal glomeruli were isolated and MC were obtained as described previously (26,27). Portions of macroscopically normal renal cortex were obtained from human kidneys immediately after surgical nephrectomy performed for renal cell carcinoma. Glomeruli were isolated by differential sieving. The final preparation consisted of glomeruli without tubular fragments. All the steps of the isolation procedure were performed with saline. Isolated glomeruli were plated in plastic culture flasks (Costar, Cambridge, MA) and incubated at 37°C in a humidified 5% CO₂ incubator. The culture medium consisted of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Armour. Kankakee, IL), penicillin (100 U/mL) (Meiji, Tokyo, Japan), and streptomycin sulfate (50 µg/mL) (Meiji). Cellular outgrowth was observed 2 to 3 days after seeding, with confluence usually achieved after 10 to 14 days. Actively proliferating cells in confluent cultures were subcultured after detachment with 0.15% trypsin (Difco, Detroit, MI) in phosphate-buffered saline (PBS). For the morphologic and cell viability studies, MC were subcultured in chamber slides (Nunc, Naperville, IL), and 24- or 96-well plastic plates (Costar), respectively. Experiments were performed with subconfluent cells of the eighth to 20th passage. The identity of the cells was confirmed by standard criteria (28–29).

Treatment of MC with ROS

For all experiments, subconfluent MC were washed twice with PBS and then incubated for 1 to 4 h at 37°C in RPMI 1640 containing either various concentrations of hydrogen peroxide (H₂O₂) (20 µM to 20 mM) or xanthine (Xa) (50 µM) plus xanthine oxidase (XaO) (0.5 to 100 mU/mL) as a source of ROS (29). Control cells were incubated in RPMI 1640 in the absence or presence of FBS. For the scavenger experiments, either catalase (100 U/mL, Sigma Chemical Co., St. Louis, MO) or superoxide dismutase (100 U/mL, Sigma) was included in the medium with ROS.

Morphology of Cultured MC

Cells were examined by electron microscopy as described previously (30). After incubation with ROS, adherent MC on chamber slides were fixed in 2.5% glutaraldehyde at 4°C and postfixed with 1% osmium tetroxide. Slides were then processed for routine dehydration and epon embedding, and examined with an electron microscope (Hitachi, Tokyo, Japan). A cell was considered morphologically apoptotic if it displayed loss of cell volume, chromatin condensation along the nuclear membrane with intensely basophilic staining, or nuclear fragmentation into spherical structures containing condensed chromatin, but was still surrounded by the cell membrane (31). MC were also examined by fluorescent microscopy. After treatment with reagents, MC on chamber slides were fixed with 2% glutaraldehyde for 1 h and stained with 8 µg/mL Hoechst dye No. 33258 (Sigma) to visualize the localization of DNA (32).

Detection of Fragmented DNA by Electrophoresis and Southern Analysis

Oligonucleosomal DNA fragmentation associated with apoptosis was examined by DNA electrophoresis and Southern analysis (33,34). After incubation with reagents, MC were detached with trypsin in PBS and centrifuged together with cells present in the culture medium. Cells were lysed in a solution containing 10 mM Tris-HCl (pH 8.0), 0.1 M ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate, and ribonuclease (20 µg/mL, deoxyribonuclease free) for 1 h at 37°C and then incubated with proteinase K (2 mg/mL) (Boehringer Mannheim/Yamanouchi, Tokyo, Japan) for 1 h at 50°C. The lysates were extracted twice with 1:1 (vol/vol) phenol and precipitated with 0.2 M ammonium acetate and 2 vol of ethanol. The nucleic acid from each lysate was resuspended in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and its concentration was determined by absorbance at 260 nm. The same amount of nucleic acid from each sample (30 µg) was subjected to electrophoresis on a 1.8% agarose (Funakoshi, Tokyo, Japan) gel.

The DNA in the gel was denatured with 0.2 M NaOH and 0.6 M NaCl, sequentially neutralized in 0.24 M Tris-HCl (pH 7.5) and 0.6 M NaCl for 30-min intervals, and then transferred to a nylon filter (Hybond N; Amersham, Little Chalfont, Buckinghamshire, UK) by capillary blotting. The DNA was fixed to the filter by exposure to ultraviolet light for 150 s. Total genomic DNA that had been extracted from cultured human MC, digested with EcoRI and PstI restriction enzymes, and labeled with [α-32P]dCTP (Amersham) by random priming method (Multiprime labeling kit; Amersham) was used as a probe. After prehybridization in 5× sodium citrate-sodium chloride (1× SSC, 0.015 M sodium citrate, 0.15 M sodium chloride) containing 1% sarcosyl and salmon sperm DNA (50 µg/mL), the filter was hybridized overnight to probe DNA. After hybridization, unannealed labeled DNA was removed from the blot by sequential washes with decreasing concentrations of SSC buffer. The filter was then exposed to x-ray film (Amersham) for autoradiography.

Cell Viability Determination

Cell viability was assessed by trypan blue exclusion and by measuring lactate dehydrogenase (LDH) activity released in the medium (35). After incubation for 1 h with various concentrations (20 µM to 2 mM) of H₂O₂, adherent MC subcultured in 24-well plates were detached with trypsin and centrifuged together with cells present in the culture medium. Cell viability was assessed by trypan blue exclusion for triplicate wells. At least 400 cells were counted for each determination and the number of viable cells was expressed as a percentage of total cells. For LDH assay, the cells were seeded into 96-well microwell culture plates at a density of 5 × 10³ cells/well with 100 µL RPMI 1640 containing FBS, and were preincubated for 3 days at 37°C. Then the plates were washed twice with PBS and incubated with RPMI or appropriate concentrations of ROS in the absence or presence of FBS for an appropriate time. Fifty microliters of supernatant were transferred to the corresponding wells of 96-well plate. Then 50 µL of substrate and nitroblue blue mixture (nitroblue blue, NAD, diaphorase, ω-lithium lactate, and Tris buffer, Kyokuto Pharmaceutical Industries, Co., Tokyo, Japan) was added to each well. After 30-min incubation at 37°C, 100 µL of 1 M HCl was added to each well to stop enzyme reaction. The absorbance of each sample was measured at 590 nm.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End Labeling for Detection of Fragmented DNA

DNA fragmentation associated with apoptosis was detected by the addition of biotinylated nucleotides to free 3'-hydroxyl

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groups in DNA according to the method of Gavrieli et al. (36). After incubation for 4 h with 2 mM H$_2$O$_2$, the chamber slides were air dried, washed in PBS, immersed in transferase buffer (Gibco-BRL, Gaithersburg, MD), and then incubated with terminal deoxynucleotidyl transferase (0.3 U/ml) (Gibco-BRL) and 0.04 mM biotinylated dUTP (Boehringer Mannheim/Yamanouchi) in a humid atmosphere at 37°C for 90 min. The reaction was terminated by transferring the slides to TB buffer (30 mM sodium citrate, 300 mM sodium chloride) for 30 min at room temperature. The sections were rinsed with PBS, incubated with fluorescein isothiocyanate-labeled avidin (Seikagakukogyo, Tokyo, Japan) for 30 min, washed in PBS, and mounted in glycerol medium (Immunocon, Pittsburgh, PA). The number of fluorescent positive cells was counted in ten fields for triplicate wells with the use of an Olympus immunofluorescence microscope (Tokyo, Japan). The data are expressed as the number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells per microscopic field.

**Statistical Analysis**

Results are expressed as means ± SE and were evaluated by the Mann-Whitney U test. We considered $P$ values smaller than 0.05 as statistically significant.

**RESULTS**

**Effects of ROS on the Morphology of MC**

We first performed microscopic examination of whether occurrence of apoptosis after treatment with ROS was also available to cultured human MC or not. Because of the absence of definitive molecular markers, morphologic examination, particularly electron microscopy, is the only way to be sure that cells are undergoing apoptosis (12). After exposure to 20 μM H$_2$O$_2$ for 4 h, MC cultured on chamber slides showed a typical apoptotic morphology, with marked chromatin condensation, nuclear fragmentation into spherical structures, and cytoplasmic compacting, but with preservation of the cell membrane (Figure 1A). Such cells also exhibited condensed chromatin and nuclear fragmentation (Figure 1C) after staining with Hoechst dye in contrast to the control (Figure 1B).

**Effects of ROS on Internucleosomal DNA Fragmentation**

A characteristic of apoptosis is the cleavage of DNA into oligonucleosome-length fragments by activated endonucleases (37,38). We therefore examined the effects of ROS on DNA fragmentation in cultured human MC by gel electrophoresis. In addition, to increase the sensitivity for detection of fragmented DNA (34), we carried out Southern analysis of DNA from ROS-treated MC with radioactive probes derived from total genomic DNA of MC. We exposed MC to various concentrations of either H$_2$O$_2$ or Xa plus XaO, which generated all the members of ROS (29). A pattern of internucleosomal DNA cleavage into a ladder of regular subunits of about 200 base pairs was apparent after incubation of cells for 4 h with H$_2$O$_2$ at concentrations of 2, 10, and 20 mM, or with 0.05 mM Xa plus XaO at concentrations of 5, 50, or 100 mU/mL (Figure 2). Studies have shown that such fragmentation is characteristic of endonuclease cleavage of DNA in apoptotic cells and is not an artifact of the process of lysis (37,38). These results indicated that ROS induced apoptosis in cultured human MC.

**Effects of ROS on Mesangial Cell Viability**

Southern analysis sensitively detected apoptotic internucleosomal DNA cleavage, but it was difficult to
Mesangial Cell Apoptosis

Figure 2. Effects of ROS on DNA fragmentation in cultured human MC. Cells were incubated for 4 h in RPMI 1640 containing either various concentrations of H$_2$O$_2$ (A) or Xa plus XaO (B) as indicated. Then DNA extracted from MC were electrophoresed on an agarose gel, transferred to a nylon filter, and probed with 32P-labeled human DNA. The resulting blot was exposed to an x-ray film. Autoradiograms (A, B) show the presence of apoptotic DNA fragments as well as high molecular weight bands. The positions of molecular size markers are indicated.

quantify apoptotic cell death in MC. Therefore to quantify the effect of ROS on MC death, we incubated cells with various concentrations of H$_2$O$_2$ for 1 h and then measured cell viability by trypan blue dye exclusion test. The cell death induced by H$_2$O$_2$ increased in a concentration-dependent manner (Figure 3A). The incubation of MC with ROS resulted in loss of viability and cytolysis with release of LDH into the medium. The cell death induced by either H$_2$O$_2$ or Xa/XaO significantly increased in concentration (Figure 3B, C) and time (Figure 3D)-dependent manners.

TUNEL Determination of ROS-Induced DNA Fragmentation

We quantified ROS-induced apoptotic cell death in MC by TUNEL determination of DNA fragmentation (36). The number of TUNEL-positive cells was significantly increased by incubation of cells for 4 h either in the presence of 2 mM H$_2$O$_2$ or the absence of FBS (Figure 4 and 5). Significantly increased number of TUNEL-positive cells indicated the occurrence of DNA fragmentation after incubation of MC with H$_2$O$_2$. Few positive cells were cultured in the presence of 20% FBS (Figures 4 and 5), and this was a consistent background level of apoptosis.

Effects of Free Radical Scavengers on ROS-Induced Apoptosis

The survival of cells requires an appropriate balance between oxidants and antioxidants. Catalase, an enzyme that hydrolyzes H$_2$O$_2$, significantly inhibited the release of LDH both in H$_2$O$_2$- and in Xa/XaO-treated MC to the control level. But superoxide dismutase did not inhibit the LDH release in ROS-treated MC (Figure 6). These results suggest that ROS derived from H$_2$O$_2$ may mediate MC apoptosis.

DISCUSSION

ROS have been implicated in the pathophysiology of inflammatory and ischemic diseases (39–41) as well as in tissue injury associated with a variety of renal diseases (21–23). Infiltrative leukocytes produce ROS and cause proteinuria in many types of experimental glomerulonephritis, including antiglomerular basement membrane antibody-mediated nephritis (42) and immune-complex glomerulonephritis (43). In these models, inhibitors or scavengers of ROS reduce the amount of tissue injury and proteinuria (24,44). ROS are thought to cause proteinuria by inducing the breakdown or inhibiting the production of glomerular basement membrane proteins (29). Tubular injury

Figure 3. Effects of ROS on the viability of cultured human MC. Cells were incubated for 1 h (A, B) or 2 h (C) with reagents and cell viability was then determined by trypan blue exclusion (A) or LDH assay (B, C). The time course of LDH release is shown in Panel D. *P < 0.05; **P < 0.01 versus control.

Figure 4. TUNEL determination of DNA fragmentation in cultured human MC. Fluorescence micrographs correspond to cells incubated for 4 h in medium containing 2 mM H$_2$O$_2$ (A), medium alone (B), or medium containing 20% FBS (C). Original magnification, x200.
Many studies suggest that ROS are important mediators of apoptosis (25). Exposure to low concentrations of H$_2$O$_2$ induces apoptosis in a variety of cell types (25). Depletion of cellular antioxidants also can result in apoptosis in central neural cells (50). In cultured tubular epithelial cells, H$_2$O$_2$ induces endonuclease activation and subsequent DNA damage and cell death (51). Expression of the Bcl-2 protein prevents apoptosis induced by oxidative stress (52). ROS appear to act as common mediators of apoptosis (25).

Stimulation by TNF results in a rapid increase in the intracellular concentration of ROS in various cells (53). TNF-mediated apoptosis can also be inhibited by thioredoxin, an intracellular thiol reductant and free radical scavenger (54). Fas antigen-induced apoptosis is also blocked by thioredoxin and superoxide dismutase, an antioxidant enzyme (54,55). Thus, ROS both induce apoptosis and mediate apoptotic signals within cells. Factors such as Bcl-2 that inhibit apoptosis have been proposed to act as antioxidants (52).

We have now demonstrated that ROS induce apoptosis in cultured MC. Both H$_2$O$_2$ and ROS generated by Xa plus XaO induced apoptosis in MC. The effective concentration of H$_2$O$_2$ in our study is consistent with that described for other cell types (51). Higher concentrations of ROS may induce necrosis in other cells. Although the demonstration of DNA fragmentation by electrophoresis is not sufficient evidence of apoptosis, we also demonstrated the morphologic characteristics of apoptosis in MC by electron microscopy, which is the surest way to confirm apoptosis (12). Thus, ROS, at the concentrations used in our study, induced apoptosis, rather than necrosis, in MC.

Recently, Baker et al. (18) and Shimizu et al. (19) demonstrated that apoptosis was the major mechanism for resolution of glomerular hypercellularity in Thy 1.1 nephritis. They showed that MC apoptosis was beneficial in this model of self-limited mesangial proliferation. Apoptosis appears to play a role in maintaining intraglomerular cell number. However, apoptosis also may have a pathologic role as in other tissues. Thus, we have previously shown that apoptosis may contribute to cell loss in the progression of glomerulosclerosis in a rat remnant kidney model (20). In this model, transitory glomerular cell proliferation occurred at an early stage; however, after cell proliferation ceased and extracellular matrix began to accumulate, apoptosis was apparent during the period in which glomerular cell number decreased.

Whether ROS-induced MC apoptosis in vitro serves a beneficial function by maintaining glomerular cell number or acts in a harmful manner by decreasing the number of glomerular cells is not known. ROS that are produced during nonenzymatic glycation in diabetic glomerulopathy in the absence of MC proliferation might induce MC apoptosis and promote the development of glomerulosclerosis. Further investigation is required to connect the data between in vitro and in vivo for ROS-induced apoptosis in MC. We can observe glomerular endothelial and epithelial cell pro-
liferation in some glomerular diseases and all kinds of resident glomerular cells decrease in the process of glomerular scarring. Therefore we should study the mechanism for death of these cells as well. ROS may be one of regulatory factors to control glomerular cell numbers by apoptosis, and not only ROS but also some other factors involved in glomerular apoptosis should be investigated.

Persistent MC proliferation results in glomerular sclerosis and renal failure. Cell number may be maintained by a balance between cell proliferation and apoptosis. Although many studies have examined cell proliferation in glomerular diseases, the mechanisms of cell death have not been investigated sufficiently. The mechanism of glomerular cell apoptosis requires further study to gain new insights into the treatment of proliferative glomerulonephritis and the prevention of subsequent glomerular scarring.

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