Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via P2Z/P2X7 receptors

ECKHARD SCHULZE-LOHOFF, CHRISTIAN HUGO, SYLVIA ROST, SUSANNE ARNOLD, ANGELA GRUBER, BERNHARD BRÜNE, AND RALF BERND STERZEL
Nephrologisches Labor, Medizinische Klinik IV, Universität Erlangen-Nürnberg, D-91054 Erlangen, Germany

Schulze-Lohoff, Eckhard, Christian Hugo, Sylvia Rost, Susanne Arnold, Angela Gruber, Bernhard Brüne, and Ralf Bernd Sterzel. Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via P2Z/P2X7 receptors. Am. J. Physiol. 275 (Renal Physiol. 44): F962–F971, 1998.—Mesangial cells undergo cell death both by apoptosis and necrosis during glomerular disease. Since nucleotides are released from injured and destroyed cells in the glomerulus, we examined whether extracellular ATP and its receptors may regulate cell death of cultured mesangial cells. Addition of extracellular ATP (300 µM to 5 mM) to cultured rat mesangial cells for 90 min caused a 5.8-fold increase in DNA fragmentation (terminal deoxynucleotidyl transferase assay) and a 4.2-fold increase in protein levels of the tumor suppressor p53, which is thought to regulate apoptosis. Apoptotic DNA fragmentation was confirmed by the diphenylamine assay and by staining with the DNA-specific fluorochrome Hoechst 33258. The necrotic markers, release of lactate dehydrogenase and uptake of trypan blue, were not positive before 3 h of ATP addition. The effects of ATP on DNA fragmentation and p53 expression were reproduced by the purinergic P2Z/P2X7 receptor agonist, 3′-O-(4-benzoylbenzoyl)-ATP, and inhibited by the P2Z/P2X7 receptor blocker, oxidized ATP. Transcripts encoding the P2Z/P2X7 receptor were expressed by cultured mesangial cells as determined by Northern blot analysis. P2Z/P2X7 receptor-associated pore formation in the plasma membrane was demonstrated by the Lucifer yellow assay. We conclude that activation of P2Z/P2X7 receptors by extracellular ATP causes apoptosis and necrosis of cultured mesangial cells. Activation of purinergic P2Z/P2X7 receptors may play a role in causing death of mesangial cells during glomerular disease.

purinergic receptors; nucleotides; tumor suppressor p53

Glomerular disease is a common cause of progressive renal failure, ultimately requiring dialysis and kidney transplantation (19, 45). Both glomerular hypercellularity and a decreased glomerular cell number, which may be associated with glomerulosclerosis, are observed in various types of glomerular diseases. Therefore, it is important to study the factors that regulate cell growth and cell death of glomerular cells (1, 36, 37, 42). Among the different glomerular cell types, the pericyte-like mesangial cells play a central role in the development of hypercellularity and sclerosis in the glomerulus (1, 41, 42). Although multiple mitogens are known that promote mesangial cell growth and replication (1, 42), only a limited number of endogenously produced mediators have been identified that cause death of mesangial cells (41).

Two forms of cell death are distinguished in eukaryotic cells: apoptosis and necrosis (23, 25, 36, 37). Apoptosis is considered to be an ordered, genetically controlled cell suicide program that results in DNA fragmentation, condensation of nuclear chromatin, cell shrinkage, and activation of a number of specific biochemical pathways (16, 22, 23, 25). These include activation of aspartic acid-specific cysteine proteases (caspases), release of cytochrome c from mitochondria, and activation of the tumor suppressor protein p53 (14, 15, 35). By contrast, necrosis is characterized as an uncoordinated collapse of cellular homeostasis, resulting in an early damage of the plasma membrane and, consequently, loss of the integrity of the cell (22, 23, 25). Recently, it has been proposed to consider apoptosis and necrosis as different types of cell death resulting from more or less complete execution of the internal death program, since many common molecular events have been identified in the two forms of cell death (22). Apoptosis and necrosis play an important role in the pathogenesis of glomerular disease (36, 37, 45). Loss of glomerular cells as a consequence of immune injury due to activation of the terminal complement component C5b-9 is considered to cause necrosis of the attacked glomerular cells (45). Apoptosis has been demonstrated in association with the early damaging events as well as the resolution phase of anti-Thy-1 glomerulonephritis in rats (2, 29). Furthermore, loss of glomerular cells by apoptosis has been implicated in the pathogenesis of glomerulosclerosis (43).

Since nucleotides are assumed to be released into the extracellular space upon injury of glomerular cells and thrombocyte aggregation, these molecules are implicated in regulating turnover of glomerular cells (34, 39). Nucleotides bind to cell surface receptors, which are designated as purinergic P2 receptors and expressed by many cell types. Several P2 receptors have recently been cloned and are currently divided into two groups (4, 13): 1) P2X receptors are ATP-regulated ion channels, and 2) P2Y receptors are G protein-coupled heptahelial receptors. Each group is composed of seven members, which are designated as P2X1–P2X7 and P2Y1–P2Y12, respectively (4, 13).

Although it has been shown previously that extracellular ATP stimulates cell replication of mesangial cells via specific P2Y receptors (18, 40), it was noted in the original report that ATP at higher concentration (millimolar range) inhibited DNA synthesis of mesangial
cells (40). Therefore, we hypothesized that extracellular nucleotides cause cell death of cultured mesangial cells via a different purinergic P2 receptor type that is stimulated by high concentration of extracellular ATP. We found that cultured mesangial cells express mRNA encoding P2Z/P2X7 receptors, which are known to create pores in the plasma membrane (9). Our results indicate that activation of P2Z/P2X7 receptors by extracellular ATP causes apoptosis and necrosis of cultured mesangial cells. Mesangial cells as well as other cell types may utilize this endogenous receptor to initiate a cell death program under specific pathophysiological conditions.

MATERIALS AND METHODS

Materials. DMEM was obtained from Biochrom (Berlin, Germany), FCS was from Boehringer (Mannheim, Germany), bovine insulin was from Sigma (Deisenhofen, Germany), and all plastic articles were from Becton-Dickinson (Heidelberg, Germany). ATP, 3'-O-(4-benzoylbenzoyl)-ATP (Bz-ATP), adenosine 5'-O-(3-thiotriphosphate) (ATP S), α,β-methyleneadenosine 5'-triphosphate (APCP2), UTP, UDP, oxidized ATP (ox-ATP), and adenosine came from Sigma. 2-Methylthio-ATP (MeS-ATP) was delivered by Research Biochemicals (Natick, MA).

Mesangial cell culture. Culture of rat glomerular mesangial cells was performed as previously described (38). Male Sprague-Dawley rats weighing 160–200 g were killed, and the kidneys were removed. The cortex was separated from the medulla and homogenized with razor blades under sterile conditions. The homogenate was passed over three sieves with pore sizes of 106, 180, and 75 μm, respectively. Glomeruli were collected on the 75-μm sieve, washed three times with PBS, and seeded in culture flasks in DMEM, 20% FCS, 5 μg/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin at high density (30,000 glomeruli/75 cm² flask). Primary outgrowth consisted predominantly of mesangial cells and epithelial cells. After 3–4 wk in primary culture, glomerular outgrowths were passed three to four times, and pure mesangial cell populations were obtained. These cells exhibited the typical stellate appearance of mesangial cells and were characterized by the following criteria: positive immunocytochemical staining with antibodies against Thy-1.1 (Dako Diagnostika, Hamburg, Germany) and smooth muscle actin (Dako). The presence of other contaminating cell types was excluded by negative staining for the following antigens: endothelial cells, factor VIII (Dako); podocytes, antigen pp44 (antibody donated by W. Kriz, Heidelberg, Germany); parietal epithelial cells, cytokeratin 5/8 (Progen Biotechnik, Heidelberg, Germany); and macrophages, antigen ED-1 (Serotec, Kidlington, UK). Mesangial cells were frozen in liquid nitrogen at passage 4 and used for experiments at passages 5–30. At passages 5–6, the concentration of FCS in the culture medium was reduced to 10%.

In situ detection of DNA fragmentation in mesangial cells. In situ detection of 3'-OH-fragmented DNA in cultured mesangial cells was performed using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method. Adherent cultured mesangial cells were grown on 8-well chamber slides, washed twice with PBS, and fixed in 1% paraformaldehyde for 15 min on ice. After a brief wash with PBS, the cells were incubated in ice-cold 70% ethanol for 1 h. The fixed cells were washed again using 0.1% BSA/PBS and incubated with 50 μl enzyme buffer containing 0.135 U/ml TdT, 1 mM CoCl₂, and 0.0044 nmol/ml biotin-deoxy-UTP. The chamber slides were placed in a moist chamber for 30 min at 37°C. After two washes with 0.1% BSA/PBS, the cells were incubated for 30 min in a dark, moist chamber at room temperature with 50 μl of streptavidin-rhodamine (10 μg/ml) in 4× SSC, 0.1% Triton X-100, and 5% nonfat dry milk. Finally, the cell layer was mounted and examined by fluorescence microscopy. For each experimental dish, TUNEL-positive nuclei were counted in five microscopic fields of subconfluent mesangial cells (magnification, ×250).

Analysis of nuclear chromatin condensation in mesangial cells. Cultured mesangial cells grown on 8-well chamber slides were washed twice using PBS and subsequently incubated in Carnoy’s solution (75% methanol, 25% acetic acid) for 15 min at room temperature. The cell monolayer was then washed with PBS and incubated in PBS containing 1 μg/ml of the DNA-specific fluorochrome Hoechst 33258 for 30 min in a dark chamber. After removal of the solution by inversion, the monolayer was washed three times with PBS and analyzed by fluorescence microscopy. For each experimental dish, condensed and fragmented nuclei were counted in five microscopic fields of subconfluent mesangial cells (magnification, ×250).

Quantification of DNA fragmentation. DNA fragmentation was determined using the diphenylamine assay as previously described (27). After stimulation with agonists or PBS as control, mesangial cells were scraped off the culture plates, resuspended in 250 μl 10 mM Tris and 1 mM EDTA, pH 8.0 (TE buffer), and incubated with one additional volume of lysis buffer (5 mM Tris, 20 mM EDTA, and 0.5% Triton X-100, pH 8.0) for 30 min at 4°C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 13,000 g. Pellets were resuspended in 500 μl TE buffer, and samples were precipitated by adding 500 μl 10% trichloroacetic acid at 4°C. Samples were pelleted at 4,000 rpm for 10 min, and the supernatant was removed. After addition of 300 μl 5% trichloroacetic acid, samples were boiled for 10 min. DNA content was quantitated using the diphenylamine reagent (27). The percentage of fragmented DNA was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

Immunoprecipitation and Western blot analysis. The tumor suppressor protein p53 was counted in mesangial cells using immunoprecipitation followed by Western blot analysis as previously described (26). Subconfluent mesangial cells in 10-cm petri dishes were stimulated with agonists or the solute PBS as control for indicated time periods. At the end of the incubation period, the cells were scrapped off and lysed for 15 min in 700 μl lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride, pH 8.0). Lysed cells were sonicated for 10 s using a Branson sonicator (duty cycle 100%, output control 1). After centrifugation for 5 min at 13,000 g, non-specific absorbants were removed from the resulting supernatant by incubation with 40 μl 50% (vol/vol) protein A-Sepharose for 10 min at 4°C, followed by centrifugation for 15 min at 13,000 g. p53 was immunoprecipitated overnight at 4°C by adding 200 μl hybridoma supernatant (clone PAb 122; from Prof. Hans Stahl, Hamburg/Saar, Germany) and 50 μl 50% protein A-Sepharose. Immune complexes were spun down at 13,000 g for 60 s and washed three times with 500 μl SNNTE (5% sucrose, 1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris, and 5 mM EDTA, pH 7.4). Finally, the samples were resuspended in 20 µl sample buffer (125 mM Tris, 2% SDS, 10% glycerin, 1 mM dithiothreitol, and 0.002% bromophenol blue, pH 6.9) and boiled for 5 min. The samples were electrophoretically separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes by electroblotting.
F964 ATP-INDUCED CELL DEATH IN MESANGIAL CELLS

The blots were preincubated overnight at 4°C in PBS containing 0.1% Tween 20 and 5% nonfat dry milk powder to block nonspecific binding, washed three times in PBS containing 0.1% Tween 20, and incubated overnight at 4°C with the anti-p53-antibody (hybridoma supernatant of clone PAb 122, diluted 1:6 in PBS/0.1% Tween 20/5% nonfat dry milk powder). After three washes with PBS/0.1% Tween 20, the bound primary anti-p53-antibody was detected with a secondary antibody as described in the enhanced chemiluminescence protocol (Amersham, Braunschweig, Germany) using horse-radish peroxidase-conjugated anti-mouse IgG (Dako).

Northern blot analysis. Subconfluent mesangial cells in petri dishes were growth-arrested for 24 h in DMEM medium without FCS. RNA was isolated from mesangial cells by the guanidinium thiocyanate/phenol/chloroform extraction method according to Chomczynski and Sacchi (7). The RNA samples (20 µg per lane) were separated by agarose gel electrophoresis, transferred to a nylon membrane (Biodyne A; Pall, Dreieich, Germany) and fixed by incubation at 80°C for 2 h. The membranes were hybridized with an EcoRI/EcoRI fragment of 3,560 bp including the complete coding sequence of the rat P2Z/P2X7 receptor. As additional P2Z/P2X7 cDNA probe, we used a BamHI/SacI fragment of 523 bp corresponding to the COOH-terminal part of the P2Z/P2X7 receptor. The full-length rat P2Z/P2X7 cDNA was kindly provided by Dr. G. Buell, Geneva, Switzerland. Control hybridizations were performed using a 700-bp fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. For hybridization, inserts were labeled by random primer extension (12).

Changes in plasma membrane permeability. ATP-induced pore formation in cultured mesangial cells was analyzed with the extracellular fluorescent tracer Lucifer yellow (Molecular Probes, Eugene, OR) as described (11). Mesangial cells grown on 8-well chamber slides were washed twice with PBS and incubated for 15 min at 37°C in 1 mg/ml Lucifer yellow, 5 mM probenecid, and PBS in absence or presence of 3 mM ATP. The cells were washed three times and analyzed by fluorescence microscopy. Severe plasma membrane disintegration of mesangial cells was examined by trypan blue uptake. Mesangial cells were collected from the culture dish using 0.05% trypsin/0.02% EDTA and analyzed by phase-contrast microscopy after addition of an equal volume of PBS/0.2% trypan blue.

Release of lactate dehydrogenase. Activity of the cytosolic enzyme lactate dehydrogenase (LDH) in the cell layer and the supernatant was performed using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling reaction (TUNEL). Cultured mesangial cells were seeded in 8-well chamber slides, grown to subconfluence, and cultured in serum-free DMEM medium for 24 h. Subsequently, mesangial cells were stimulated with 3 mM ATP for time periods as indicated. Thereafter, cell monolayers were processed for the TUNEL reaction. Numbers of TUNEL-positive nuclei per microscopic field (MF) were determined by counting at least five MFs per slide (fluorescence microscopy, magnification ×250).

RESULTS

DNA fragmentation in cultured mesangial cells was routinely determined using the TUNEL reaction. In all experiments, 1 day before addition of ATP, culture medium was changed from DMEM medium containing 10% FCS to DMEM medium without FCS (resting medium) to remove growth factors that may regulate cell death of mesangial cells. Removal of serum resulted in an increase of TUNEL-positive nuclei from 0.3 ± 0.1 to 6.7 ± 1.1 nuclei/microscopic field. Addition of 3 mM ATP induced a rapid increase in TUNEL-positive nuclei, which became detectable at 30 min. The maximal response (5.8-fold) was noted after 90 min (Fig. 1A) with no further increase of TUNEL-positive nuclei later on. In parallel experiments, the cytosolic...
enzyme LDH was found to be retained by mesangial cells at 60 and 120 min following ATP treatment, but was released into the culture medium at 3 h after addition of ATP (Fig. 1B).

Extracellular ATP at concentrations of 300 µM to 5 mM dose-dependently induced DNA fragmentation in cultured mesangial cells as measured by the TUNEL reaction (Fig. 2). Because purinergic P2Z/P2X7 receptors have been implicated in cell death of macrophages (9), we examined whether the P2Z/P2X7 agonist Bz-ATP may reproduce the effect of ATP on DNA fragmentation in mesangial cells. Bz-ATP induced DNA fragmentation in mesangial cells in concentrations one order of magnitude lower than ATP (Fig. 2). Additional purinergic receptor agonists were examined to evaluate a role of other P2 receptors in mediating cell death (Fig. 3). In addition to ATP and Bz-ATP, the metabolically stable ATP analog ATPγS had an intermediate effect on DNA fragmentation in cultured mesangial cells (Fig. 3A). ATPγS has been found to moderately activate the P2Z/P2X7 receptor (44) but is not selective for a specific P2 receptor type. Agonists for P1 receptors (adenosine) and the other P2 receptors (APCP, MeS-ATP, UTP, and UDP) showed no effects or minor effects. Furthermore, nucleotides that occur naturally, such as GTP, ITP, CTP, and TTP did not induce DNA fragmentation in cultured mesangial cells (data not shown). P2Z/P2X7 receptors of macrophages have been shown to be irreversibly inhibited after treatment for 2 h with 100 µM ox-ATP (30). When cultured mesangial cells were pretreated with ox-ATP according to this protocol, ATP- and Bz-ATP-induced DNA fragmentation were reduced by 47% and 56%, respectively. As control, DNA fragmentation caused by glio was not reduced by ox-ATP (Fig. 3B).

TUNEL-positive mesangial cell nuclei exhibited a bright fluorescence that was easily identified by fluorescence microscopy (Fig. 4A). In controls (Fig. 4B), a low basal rate of TUNEL-positive nuclei appeared, which was most likely due to growth factor deprivation (absence of serum for 24 h). In parallel experiments, mesangial cell cultures treated with 3 mM ATP for 90 min or with PBS as control were stained with the DNA-specific fluorochrome Hoechst 33258 (Fig. 5). In ATP-treated cells, we found a significant increase in condensed and fragmented cell nuclei (Fig. 5A), whereas in nontreated cells only a few positive nuclei were noted. Both methods, staining with Hoechst 33258 and the TUNEL assay, revealed a significant increase in nuclei with fragmented DNA upon treatment of mesan-

![Fig. 2. Dose-response relationship of DNA fragmentation induced by ATP and the P2Z/P2X7 receptor agonist 3'-O-(4-benzoylbenzoyl)-ATP (Bz-ATP) in cultured mesangial cells. Cells were treated with different concentrations of ATP (3 µM to 3 mM) and Bz-ATP (3–500 µM) for 90 min. Cell culture conditions and TUNEL assay were as described in the legend of Fig. 1. Control values for TUNEL-positive mesangial cell nuclei/MF in nontreated cells are shown at 0 mM of ATP and Bz-ATP, respectively. Results are means ± SD (n = 4). *P < 0.05; significant increase in TUNEL-positive nuclei of experimental values compared with untreated control.](http://journals.physiology.org/doi/10.1152/ajprenal.00355.2017)

![Fig. 3. Role of purinergic P2Z/P2X7 receptors in ATP-induced DNA fragmentation. A: induction of DNA fragmentation by various P2 receptor agonists in cultured mesangial cells. Experimental conditions were as described in legend of Fig. 1. Resting cultured mesangial cells were treated for 90 min with different P2 receptor agonists at 5 mM. ATPγS, adenosine 5’-(3-thiotriphosphate); APCPP, αβ-methyleneadenosine 5’-triphosphate; MeS-ATP, 2-methylthio-ATP; Ado, adenosine. TUNEL-positive nuclei/MF were counted by fluorescence microscopy. Results are means ± SD (n = 4). *P < 0.05; significant increase in TUNEL-positive nuclei of experimental dishes compared with nonstimulated control (Co). B: effect of the P2Z/P2X7 receptor blocker, oxidized ATP (ox-ATP), on ATP-induced DNA fragmentation. Cultured mesangial cells were pretreated with 100 µM ox-ATP for 2 h and stimulated with 5 mM ATP, 5 mM Bz-ATP, and 100 µM glio (Glio) for 90 min. Parallel dishes received PBS with or without 100 µM ox-ATP as control (Co). *P < 0.05; significant increase in TUNEL-positive nuclei of ox-ATP-treated dishes compared with corresponding controls.](http://journals.physiology.org/doi/10.1152/ajprenal.00355.2017)
gial cells with 3 mM ATP (Table 1). Fragmentation of genomic DNA upon treatment of mesangial cells with ATP was confirmed using the diphenylamine assay (Table 2). Specific DNA fragmentation increased from 11% in controls to 43% following the treatment with ATP.

Because increase of the tumor suppressor protein p53 has been shown to occur in response to DNA fragmentation and is thought to regulate cell death (15), we examined the levels of p53 in ATP-treated mesangial cells using immunoprecipitation and subsequent Western blot analysis (Fig. 6). In nontreated mesangial cells, p53 protein was barely detectable. A significant increase in p53 protein levels was observed at 30 min after addition of 3 mM ATP. The maximal stimulatory effect (4.8-fold increase) was found at 45–120 min after addition of ATP (Fig. 6). Analysis of the dose-response relationship of ATP-induced increase in p53 protein revealed a threshold dose of ~30 μM ATP and a maximal effect at 300 μM to 5 mM ATP (Fig. 7).

We then examined the effect of various P2 receptor agonists on p53 accumulation at 90 min in mesangial cells (Fig. 8) and found a significant p53 response following the addition of ATP, Bz-ATP, and ATPγS (all at 3 mM). Other P1 (adenosine) and P2 receptor agonists (APCPP, MeS-ATP, and UTP) induced minor increases in p53 protein. Because the ability of Bz-ATP to induce cell death and p53 expression indicates involvement of P2Z/P2X7 receptors, we examined whether p53 accumulation is blocked by ox-ATP, a P2Z/P2X7 antagonist. As shown in Fig. 9, pretreatment...
of cultured mesangial cells with 100 mM ox-ATP for 2 h completely abolished ATP-induced p53 expression in cultured mesangial cells.

In previous reports, P2Z/P2X7 receptors have been described primarily in macrophages and mast cells (9, 11). Therefore, we examined the expression of P2Z/P2X7 receptor mRNA in cultured mesangial cells using Northern blot analysis. Hybridization of membranes with size-fractionated total RNA from resting mesangial cells using a 3,560-bp full-length cDNA for the P2X7 receptor revealed a single band with a size of ~3.5 kb (Fig. 10). Because this size is different from previously reported mRNA transcripts in brain of 6 kb (8), we performed additional hybridization experiments using a 523-bp fragment of the P2Z/P2X7 receptor that does not display homology with other members of the P2X receptor family. This shorter cDNA fragment hybridized to the same 3.5-kb band as the full-length cDNA. Conclusively, this hybridization signal corresponded to transcripts of P2Z/P2X7 receptors expressed in cultured mesangial cells. P2Z/P2X7 receptor mRNA levels were unchanged when determined after induction of mesangial cell death by addition of 3 mM ATP for 90 min (data not shown).

Activation of P2Z/P2X7 receptors in macrophages has been shown to be associated with an increase in plasma membrane permeability for hydrophilic molecules with a molecular mass <900 Da (11, 30, 44). We studied the P2Z/P2X7 receptor-regulated change in membrane permeability by examining uptake of the fluorescent dye, Lucifer yellow (M₄, 463), in ATP-treated cultured mesangial cells (11, 21, 30, 33). As early as 15 min after addition of 3 mM ATP, 71 ± 11% of the mesangial cells were found to be permeable for Lucifer yellow, whereas untreated control cells largely excluded the dye (Table 3). Lucifer yellow-positive mesangial cells after stimulation with extracellular ATP for 15 min are shown in Fig. 11. As control, trypan blue (M₄, 960) was excluded by mesangial cells after 15 min of ATP treatment (Table 3).

Although only few mesangial cells (≤5%) detached from the tissue culture plate within 90 min of ATP treatment, ~50–60% of the cells were found in the culture medium after prolonged incubation (24 h) with 3 mM ATP. When we examined the mesangial cells that remained adherent at 24 h, 85% of these cells were permeable for trypan blue, indicating severe plasma membrane disintegration. By contrast, DNA fragmentation (TUNEL assay) was detected only in 20% of the remaining adherent mesangial cells. The results on early pore formation (Lucifer yellow assay), severe membrane disintegration (trypan blue uptake), and DNA fragmentation (TUNEL assay) in ATP-treated mesangial cells are summarized in Table 3.
DISCUSSION

We have demonstrated that stimulation of purinergic P2Z/P2X7 receptors by addition of extracellular ATP causes apoptosis and necrosis of cultured mesangial cells. The critical role of P2Z/P2X7 receptors for this effect was substantiated by several findings. First, the effect of ATP on mesangial cell death and p53 accumulation was reproduced by the selective P2Z receptor agonist Bz-ATP (Figs. 2 and 8), but not by agonists for other purinergic receptors and other naturally occurring nucleotides. Second, the inhibitor of P2Z/P2X7 receptor activation, ox-ATP, attenuated ATP-induced DNA fragmentation (Fig. 3) and completely abolished ATP-induced p53 upregulation (Fig. 9). The millimolar concentrations of ATP used in this study are in agreement with reported dose-response curves of P2Z/P2X7 receptor-mediated effects (9). These high concentrations of ATP are required because only the tetrabasic form of ATP (ATP4\(^2\)) functions as a ligand at this receptor (9). In addition, we successfully detected transcripts encoding the P2Z/P2X7 receptor in cultured mesangial cells using Northern blot analysis. Finally, Table 3.

Table 3. Early pore formation, severe plasma membrane disintegration, and DNA fragmentation in ATP-treated mesangial cells

<table>
<thead>
<tr>
<th></th>
<th>ATP (3 mM)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lucifer yellow uptake</td>
<td>71 ± 11*</td>
<td>3 ± 0.6</td>
</tr>
<tr>
<td>Trypan blue uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>3 ± 1</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>90 min</td>
<td>4 ± 2</td>
<td>3 ± 0.9</td>
</tr>
<tr>
<td>3 h</td>
<td>26 ± 5*</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>24 h</td>
<td>85 ± 13*</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>DNA fragmentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 min</td>
<td>32 ± 8*</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>24 h</td>
<td>20 ± 3*</td>
<td>6 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SD of 4 experiments and are expressed as relative number of positive cells in %. Cultured mesangial cells were treated with 3 mM ATP or PBS as control for different periods of time. Early pore formation was examined by the Lucifer yellow assay, severe plasma membrane disintegration by trypan blue uptake, and DNA fragmentation by the TUNEL assay. *P < 0.05, statistically significant increase of experimental values compared with corresponding controls.
we examined activation of the pore-forming P2Z/P2X<sub>7</sub> receptor in ATP-treated cultured mesangial cells using the fluorescent dye, Lucifer yellow. We found an early increase in plasma membrane permeability as indicated by uptake of Lucifer yellow in 71% of ATP-treated cells. It is likely that early pore formation in the plasma membrane (e.g., by strong influx of calcium, or by breakdown of the plasma membrane electric potential) activates apoptotic pathways including upregulation of p53 and DNA fragmentation. Similar to ATP-induced cell death in mesangial cells, the mechanism of excitotoxicity of glutamate in neuronal cells has been attributed to activation of ion channels leading to apoptotic and necrotic cell death (32).

When cultured mesangial cells were treated with 3 mM ATP for 90 min, apoptosis was the predominant type of cell death as judged by nuclear chromatin condensation, DNA fragmentation, upregulation of p53 (Fig. 1; Table 1; Fig. 6), and an intact plasma membrane impermeable to LDH and trypan blue (Fig. 1; Table 3). Apoptotic cells in culture usually detach from the tissue culture plate and undergo secondary plasma membrane disintegration, a phenomenon referred to as "secondary necrosis" (23). We assume that apoptotic mesangial cells contributed to the late release of LDH measured in the cell culture supernatant, because 50–60% of the mesangial cells detached from the tissue culture plate within 24 h of ATP treatment. In addition to the mesangial cells that died by apoptosis, a significant number of cells apparently underwent primary necrosis without developing apoptotic features such as DNA fragmentation. This conclusion is based on the following observation: when we examined the mesangial cells that remained adherent after 24 h of ATP treatment, 85% were trypan blue positive, indicating severe plasma membrane damage and irreversible cell death, but most of these cells did not show nuclear DNA fragmentation as measured by the TUNEL assay (Table 3). Therefore, we conclude that the complex response of cultured mesangial cells to extracellular ATP causes both apoptotic and necrotic cell death.

The pore-forming P2Z/P2X<sub>7</sub> receptor has been initially described in macrophages (11) and has been shown to be upregulated by inflammatory cytokines in monocytic cell lines (17). P2Z/P2X<sub>7</sub> receptors have been implicated to play a role in ATP-induced cell death (33), killing of microorganisms (21), and cell fusion (6). The molecular cloning of the cDNA encoding the P2Z/P2X<sub>7</sub> receptor identified this molecule as a unique member of the P2X receptor family (44). The first 395 amino acids of the P2Z/P2X<sub>7</sub> receptor are 35–40% identical to those of the other P2X receptors (P2X<sub>1</sub>–P2X<sub>6</sub>). By contrast, the COOH-terminal 200 amino acids showed no sequence homology with known proteins (44). Similar to the P2Z/P2X<sub>7</sub> receptor, the other P2X receptors (P2X<sub>1</sub>–P2X<sub>6</sub>) confer permeability for Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> but not for hydrophilic molecules with a molecular mass up to 900 Da. In a first study of tissue injury in vivo, Collo et al. (8) demonstrated an increased expression of the P2Z/P2X<sub>7</sub> receptor in microglial cells aligning an ischemic area of brain tissue. Correspondingly, Chessel et al. (5) reported the expression of the P2Z/P2X<sub>7</sub> receptor in the microglial cell line NTW8. Furthermore, this receptor showed a widespread expression in bone marrow (8). These reports imply an in vivo expression of the P2Z/P2X<sub>7</sub> receptor in phagocytic cell types such as macrophages, microglia, and mesangial cells. Thus the physiological role of this receptor may be related to specific functions of these cells. It is conceivable that P2Z/P2X<sub>7</sub> receptors play a crucial role in cell death of phagocytic cells.

To further analyze the molecular events involved in ATP-induced cell death, we examined the expression of the transcription factor p53. Although p53 was initially described as a tumor suppressor with increased frequency of mutations in malignant cells (15), the multiple functions of this important regulatory molecule as cell cycle gate keeper (20), in DNA repair (31), and in...
propagating cell death (24) have been clearly demonstrated. Upregulation of p53 has been associated with apoptosis in several cell types (26). Extracellular ATP rapidly (within 30 min) increased p53 protein accumulation with a dose-response relationship that paralleled cell death (Figs. 6 and 7). This effect was mediated by the P2Z/P2X7 receptor, because it was reproduced by the P2Z/P2X7 agonist Bz-ATP and inhibited by the P2Z/P2X7 blocker ox-ATP.

Since p53 upregulation occurs as a result of DNA damage and DNA fragmentation (15), it is likely that these events promote p53 protein accumulation in ATP-induced mesangial cell death. The precise mechanism by which p53 upregulation causes cell death is incompletely understood. p53 has been shown to induce expression of bax which is thought to be a further downstream regulator of the cell death pathway (28). Bax may ultimately cause activation of endonucleases and caspases, the final executors of cell death (35).

Previously, we and others have reported that extracellular ATP (at micromolar concentrations) stimulates proliferation of cultured mesangial cells (18, 40). The mitogenic effect of ATP has been ascribed to stimulation of heptahelical P2Y2 receptors (18). In our earlier report we noted inhibition of DNA synthesis at higher concentration of ATP (≥ 300 µM ATP) (40). We interpret the biphasic effect of ATP on mesangial cell replication to be a consequence of the stimulation of mitogenic P2Y receptors at lower concentrations (micromolar range) and activation of P2Z/P2X7 receptors at higher concentrations (millimolar range). The antimitogenic effect of ATP in the millimolar range may be due to cell loss as well as antimitogenic effects as a consequence of p53 upregulation. Activation of p53 has been shown in other cell systems to induce cell cycle arrest via activation of p21 (10). Therefore, mitogenic effects via G protein-coupled P2Y receptors and opposite effects causing cell death may determine survival and replication of mesangial cells depending on the expression of P2 receptors and the amount of nucleotides released into the extracellular space.

Because of the critical role of P2Z/P2X7 receptors for cell survival, it is mandatory to determine the expression of these receptors in the kidney under normal and disease states. Following reports that the receptor is upregulated by proinflammatory cytokines in a monocytic cell line (17), it is possible that an increased expression of this receptor by mesangial cells or other glomerular cell types may critically determine survival and cell death of glomerular cells under inflammatory conditions. It is conceivable that P2Z/P2X7 receptor-mediated cell death serves to remove partially damaged mesangial cells during severe glomerular injury when large amounts of nucleotides are released into the extracellular space. In this context, the development of more selective receptor blockers and genetically modified animals overexpressing or lacking P2Z/P2X7 receptors will help to determine the role of this intriguing receptor in diseases of the kidney.


