P2X₇ receptor-mediated apoptosis of human cervical epithelial cells

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Wang, Qifang, Liqin Wang, Ying-Hong Feng, Xin Li, Robin Zeng, and George I. Gorodeski. P2X7 receptor-mediated apoptosis of human cervical epithelial cells. Am J Physiol Cell Physiol 287:C1349–C1358, 2004. First published July 21, 2004; doi:10.1152/ajpcell.00256.2004.—Normal human ectocervical epithelial (hECE) cells undergo apoptosis in culture. Baseline apoptosis could be increased by shifting cells to serum-free medium and blocked by lowering extracellular calcium. Treatment with the ATPase apyrase attenuated baseline apoptosis, suggesting that extracellular ATP and purinergic mechanisms control the apoptosis. Treatment with ATP and the P2X7 receptor analog 2′-3′-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP) increased apoptosis significantly, in a time- and dose-related manner. The threshold of ATP effect was 0.5 μM in hECE cells and ~1 μM in CaSki cancer cells. The apoptotic effect of BzATP was additive in part to that of tumor necrosis factor (TNF-α) and it could be attenuated by lowering extracellular calcium and by treatment with the caspase-9 inhibitor Leu-Glu-His-Asp-O-methylfluoromethylketone (LEHD-FMK). Treatment with BzATP activated caspase-9, and, in contrast to TNF-α, it had only a mild effect on caspase-8. Both BzATP and TNF-α activated caspase-3, suggesting that BzATP activates the mitochondrial apoptotic pathway. Both hECE and CaSki cells secrete ATP into the extracellular fluid, and mean ATP activity in conditioned medium was ~0.5 μM, which is in the range of values that suffice to activate the P2X7 receptor. On the basis of these findings we propose a novel autocrine-paracrine mechanism of cervical cell apoptosis that operates by P2X7 receptor control of cytosolic calcium and utilizes the mitochondrial apoptotic pathway.

cervix; epithelium; ATP; 2′-3′-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate

THE FEMALE REPRODUCTIVE TRACT is lined by epithelia that regulate lubrication of the genital canal and provide necessary conditions for reproduction. Proper function of the epithelia depends on coordinated growth and differentiation of the epithelial cells. Deviations from these well-controlled functions can cause infertility and states of disease and can lead to dysplasia, neoplasia, and cancer. Cervical dysplasia affects 10–15% of women, and ~1% will progress to invasive cervical cancer, which is the second leading death-related cancer in women (2). Dysplasia and cancer usually arise from foci at the transformation zone of the cervix, where columnar cells of the endocervix undergo squamous metaplasia and transdifferentiate into the squamous ectocervical epithelium.

The ectocervical epithelium is maintained by a balance between proliferation of the basal layer of cells and death of cells in upper layers. Cells in the basal layer can either replicate or cease proliferation and exit from the mitotic cycle, stratify, and undergo terminal differentiation into cornified envelopes (superficial cells) (14). Our current state of knowledge suggests three levels of growth control of cervical epithelial cells: proliferation, controlled by mitogenic signals [e.g., estrogen and epidermal growth factor (EGF)] vs. growth inhibitory factors [e.g., transforming growth factor (TGF)-β] (21); terminal differentiation, controlled mainly by estrogen (57); and senescence of cells evading growth control and terminal differentiation due to the erosion of telomeres (32). Preliminary data from our lab (13) suggest that growth of human cervical epithelial cells is also regulated by apoptosis, but the mechanisms of apoptosis-induced growth control of the normal cells are unknown. The objective of the present study was to better understand the phenomenon and the mechanisms involved.

Apoptosis is a regulated homeostatic process, orchestrated by the host’s genome, of selective cell deletion without stimulating inflammatory response (7, 8, 61). Under certain conditions such as deficiency of necessary growth factors or hormones, apoptosis can lead to premature death of cells, loss of tissue, and aging (50). Dysregulation of apoptotic cell death has been implicated in the neoplastic transformation and in states of disease (7, 8, 44, 61). Many studies have examined apoptosis of cervical cancer cells in response to therapies, but only a few looked into regulation of apoptosis in normal human cervical cells. Ter-Harmsel et al. (55) reported higher expression of markers of proliferation and apoptosis protection in premalignant lesions compared with normal cervical tissues. Lee et al. (27) reported that tumor progression in the cervical epithelium was associated with an increase in both cell proliferation and cell apoptosis. In contrast, Mozzetti et al. (34) did not find a significant difference in the expression of apoptosis-related markers between normal and cancer cervical cells. A number of studies used cervical cells immortalized with human papillomavirus (HPV) to determine the effects of drugs on apoptosis. Rorke and Jacobberger (45) reported that TGF-β1 enhanced apoptosis in HPV type 16-immortalized human ectocervical epithelial cells. Brown et al. (4) found that HPV-16 E6 sensitized cells to atractyloside-induced apoptosis. Rorke et al. (46) reported that HPV-immortalized cervical cells are less sensitive to toxicant damage, including apoptosis induction, than normal cervical epithelial cells. More recently, Thomas et al. (56) and others have shown that in HPV-infected cells the HPV E6 protein complexes with the tumor suppressor p53 and targets it for rapid proteasome-mediated degradation, abrogating p53’s growth-arrest and apoptosis-inducing activities. Because of the mechanism by which HPV infection alters...
regulation of apoptosis, it remains difficult to extrapolate these results to the normal cervix.

A number of studies also looked into apoptosis of vaginal-cervical cells in rodents. Perfettini et al. (40) reported that infection of female mice with *Chlamydia trachomatis* enhances secretion of tumor necrosis factor (TNF-α) and augments apoptosis of endocervical cells. The authors also showed that infection of human HeLa cells with *C. trachomatis* increases secretion of TNF-α and results in apoptosis. Sato et al. (47) reported that in the middle and basal layers of the rat vaginal epithelium the apoptotic index was high at metestrus and negatively correlated with the mitotic rate in that tissue. Rao et al. (42) confirmed those results and suggested that the apoptosis is part of the terminal differentiation of the epithelium. The authors also found a few scattered cells throughout the thickness of the vaginal epithelium undergoing apoptotic death involving DNA fragmentation. Unaltered levels of Bcl-2 message on estradiol administration prompted them to question the role of Bcl-2 in preventing death of the vaginal-cervical epithelium by apoptosis.

The above studies suggest that apoptosis plays a role in the regulation of proliferation and extinction of cervical cells. However, our present state of knowledge is incomplete with regard to phenomena and mechanisms in the normal human cervix. These data could be important for the understanding of cervical cell biology and tumorigenesis. In preliminary experiments we observed (13) that normal human cervical epithelial cells undergo apoptosis spontaneously and that the effect could be blocked by incubating cells in low extracellular calcium. The main objective of the present study was to understand the regulation and mechanisms of this effect. Because the only agent that could stimulate prolonged increases in cytosolic calcium in human cervical epithelial cells is ATP (Gorodeski GI, unpublished observations), we hypothesized that ATP is the mediator of the baseline apoptosis. The experiments utilized cultures of normal human ectocervical epithelial (hECE) cells, an experimental system previously characterized (14, 16) and found to be adequate and useful for apoptosis research. The results show that ATP, acting via the P2X7 receptor mechanism, stimulates apoptosis that involves predominantly the calcium-dependent caspase-9-mediated mitochondrial pathway.

**METHODS**

**Cell cultures.** The experiments utilized two types of cells: hECE cells and CaSkI cells. Secondary-tertiary cultures of hECE cells were generated from minces of the ectocervix of premenopausal women aged 35–45 yr as described previously (16). Tissues were obtained in accordance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society. Cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM)-Ham’s F-12 (3:1) supplemented with nonessential amino acids, adenine (1.8 × 10⁻⁴ M), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), l-glutamine (2 mM), insulin (5 μg/ml), hydrocortisone (1 × 10⁻⁶ M), transferrin (5 μg/ml), triiodothyronine (2 × 10⁻² M), EGF (10 ng/ml), and 8% fetal calf serum (FCS; Sigma, St. Louis, MO) at 37°C in a 91% O₂-9% CO₂ humidified incubator. Cells were routinely tested for mycoplasma, and cells chosen for experiments were obtained from cervix tissues reported as HPV negative. CaSkI cells are a stable line of transformed cervical epithelial cells that were previously characterized (16). CaSkI cells were grown and subcultured in RPMI-1640 supplemented with 8% FCS, 0.2% NaHCO₃, nonessential amino acids, l-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and gentamicin (50 μg/ml) at 37°C in a 91% O₂-9% CO₂ humidified incubator and routinely tested for mycoplasma (16).

Preliminary experiments were conducted on cells plated on culture plates, and definitive experiments were repeated on cells plated on filters. The latter method improves culturing conditions and promotes epithelial cell polarization and differentiation (16). Anocell-10 filters (Sigma) are ceramic-base filters with a pore size of 0.02-μm width and 0.1-μm depth. Filters were coated with their upper (luminal) surface with 3–5 μg/cm² collagen type IV and incubated at 37°C overnight. The remaining collagen solution was aspirated, and the filter was dried at 37°C. Before plating, both sides of the filters were rinsed three times with Hanks’ balanced salt solution. Cells were plated on the upper surface of the filter at 3 × 10⁵ cells/cm². Plated at this high density, the cultures became confluent within 12 h after plating.

In some experiments, treatments with apoptosis-inducing drugs were carried out on cells incubated in their respective culture medium. In other experiments, cells were shifted to modified Ringer solution composed of (in mM) 120 NaCl, 1.2 CaCl₂, 1.2 MgSO₄, 5 KCl, 10 NaHCO₃, 10 HEPES, and 5 glucose with 0.1% BSA, pH 7.2. Levels of extracellular Ca²⁺ were controlled by adding EGTA. All treatments involved adding drugs to both the luminal and subluminal solutions.

**Western blot analysis.** The postnuclear supernatant of cells was solubilized in lysis buffer [50 mM Tris·HCl, pH 6.8, 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), and 5 mM EDTA, pH 8.0] containing 50 μg/ml PMSF, 10 μg/ml benzamidine, 10 μg/ml bacitracin, 10 μg/ml of leupeptin, and 2 μg/ml aprotinin. Aliquots normalized to 15 μg of protein (~45 μl; determined by Bio-Rad protein assay solution, Hercules, CA) were loaded on 10% polyacrylamide-SDS gel, and vertical electrophoresis was conducted at 50 mA for 1.5 h. Gels were transferred onto Immobilon membrane (Millipore, Bedford, MA) at 200 V for 1.5 h; membranes were blocked in 5% milk and exposed to the primary antibody at 4°C overnight. Membranes were washed three times in PBS and fluorescently stained for 1 min with an enhanced chemiluminescence (ECL) kit of peroxidase-conjugated secondary antibody from Amersham (Piscataway, NJ).

**DNA fragmentation assay.** The DNA fragmentation assay was modified from Lizard et al. (30). Cells attached on plates were harvested and combined with floaters recovered from the medium. Cells were washed in ice-cold PBS lacking Ca²⁺ and Mg²⁺ and resuspended in the same medium, and cellular DNA was extracted with a DNA extraction kit (Stratagene, La Jolla, CA). In some experiments the latter step was done by using lysis buffer composed of (in mM) 10 EDTA, 400 NaCl, 35 SDS, and 10 Tris·HCl, pH 8.2, with 1 mg/ml proteinase K. Cells were spun briefly at 180 g, and the pellet was resuspended in the lysis buffer, transferred to an Eppendorf tube, and incubated overnight at 37°C. The tubes were spun for 5 min at Eppendorf high speed, and the supernatant containing the DNA was precipitated in 2 volumes of 100% ethanol and incubated overnight at −20°C. Tubes were spun for 5 min at Eppendorf high speed, and the pellet containing the DNA was saved. For assays, the DNA-containing pellets were resuspended in 100 μl of TE buffer (composed of 10 mM Tris·HCl and 0.2 mM Na-EDTA, pH 7.5) and equal amounts of DNA (determined by spectrophotometry) were separated on 1.8% agarose gel electrophoresis for 15 h at 20 V. Gels were prepared in Tris-buffere ethanolamine buffer (9 mM Tris-borate, pH 8, 2 mM EDTA) plus 0.1 μg/ml ethidium bromide and photographed under ultraviolet light.

**DNA solubilization assay.** Twenty-four hours before the end of the experiment cells were labeled with [3H]thymidine (specific activity 100 Ci/mmol; 5 μCi per 1 × 10⁵ cells) for 16 h. The medium was removed, and cells were washed three times with fresh medium lacking [3H]thymidine and incubated in the same medium for an additional 6 h. At the end of incubation the “supernatant” was stored...
and cells were lysed in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, + 1 mM EDTA-0.2% Triton X-100) for 1 h at 4°C. The intact chromatin was separated from the fragmented DNA by 5-min centrifugation at 4°C in an Eppendorf microcentrifuge at 12,000 g. The supernatant (referred to as “lysate”) was stored, and the pellet was resuspended in 0.5 ml of 1% SDS. The radioactivity contained in the supernatant (referred to as \(-3^2\)H11032-triphosphate (A-2392), 2-(4-benzoyl-hexyl]carbamoylmethyl)adenosine 5\(^\text{methyl-}\text{H11032}-\text{H11032}\) and cells were lysed in 0.5 ml of lysis buffer (10 mM Tris-7.5, adipase, and ATPase (apyrase) were then incubated with TdT and fluorescein-dUTP for 60 min at 37°C in the dark, and the reaction was terminated by immersing the cells in 0.3 M NaCl plus 30 mM sodium citrate solution for 15 min. After two PBS washes (5 min each) cells were costained with propidium iodide and mounted in antifade solution.

**In situ detection of DNA fragmentation.** In situ detection of DNA fragmentation was done by terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling (TUNEL) with an ApoAlert DNA fragmentation assay kit from ClonTech (Palo Alto, CA); procedures were carried out at room temperature according to the manufacturer’s instructions. Cells on filters were fixed with 4% paraformaldehyde for 15 min, washed with PBS, and treated with 20 μg/ml proteinase K for 5 min. Cells were fixed again with 4% paraformaldehyde for 5 min, reincubated for 10 min with PBS, and equilibrated with TdT equilibration buffer for 10 min. Cells were then incubated with TdT and fluorescent-dUTP for 60 min at 37°C and, after 30 °C, the reaction was terminated by immersing the cells in 0.3 M NaCl plus 30 mM sodium citrate solution for 15 min. After two PBS washes (5 min each) cells were costained with propidium iodide and mounted in antifade solution.

**ATP assays.** Cells were grown on plates, and for experiments cells were maintained in a volume of 300 μl of either regular medium or modified Ringer solution. Aliquots of 50 μl were collected into a polypropylene tube 20 min after stabilization and left at 4°C for 2 h. Two hundred microliters (3.33 mg/ml proteinase K for 5 min. Cells were fixed again with 4% paraformaldehyde for 5 min, reincubated for 10 min with PBS, and equilibrated with TdT equilibration buffer for 10 min. Cells were then incubated with TdT and fluorescein-dUTP for 60 min at 37°C in the dark, and the reaction was terminated by immersing the cells in 0.3 M NaCl plus 30 mM sodium citrate solution for 15 min. After two PBS washes (5 min each) cells were costained with propidium iodide and mounted in antifade solution. The limit of detection for ATP was 0.25 nM.

**Antibodies.** Mouse monoclonal anti-human caspase-3, -8, and -9 antibodies were from Chemicon (Temecula, CA). Mouse monoclonal anti-human β-actin antibody was from Zymed Laboratory (San Francisco, CA). The antibodies were used according to the manufacturers’ instructions.

**Statistical analysis of the data.** Data are presented as means ± SD, and significance of differences among means was estimated by performing Student’s t-test. Trends were calculated with GB-STAT V5.3 (1995; Dynamic Microsystems, Silver Spring, MD) and analyzed by ANOVA.

**Chemicals and supplies.** ATP, ADP, AMP, adenosine, adenosine 5'-O-(3-thiotriophosphate) (ATP-S), β,γ-methyleneadenosine 5'-triphosphate (AMPPCP), α,β-methyleneadenosine 5'-triphosphate (AMP-CPP), adenosine 5'-β,γ-imido-triphosphate (AMP-PNP), N6-(6-amino-5-methyl-2-thiophenyl)amine (AMP-SCF), 8-azidoadenosine 5'-triphosphate (A-2392), 2'-3'-O-(4-benzoyl-benzoyl)-adenosine 5'-triphosphate (BzATP), adenosine 5'-O-(2-thiodiphosphate) (ADP-PBS), ADPase, and ATPase (apyrase) were from Sigma. 2-Methylthio-ATP (2-MeS-ATP) was obtained from Research Biochemicals (Natick, MA). Nucleotides were dissolved in water or dimethyl sulfoxide as appropriate. Leu-Glu-His-Asp-O-methyl-fluoromethylketone (LEHD-FMK), Asp-Glu-Val-Asp-O-methyl-fluoromethylketone (DEVD-FMK), Ile-Glu-Thr-Asp-O-methyl-fluoromethylketone (IETD-FMK), and N-benzoyloxycarbonyl-Val-Ala-Asp-O-methyl-fluoromethylketone (Z-VADEMK) were from Calbiochem (La Jolla, CA) and used at a concentration of 50 μM. All other culture medium additives and chemicals were obtained from Sigma, unless specified otherwise.

**RESULTS**

**Role of ATP in baseline apoptosis of cultured human cervical epithelial cells.** Apoptosis is characterized by morphological alterations of plasma- and nuclear membrane blebbing, organelle compaction, chromatin condensation, and formation of membrane-enclosed apoptotic bodies. Central to these changes is the activation of endogenous endonucleases, resulting in the production of 50- to 300-kb DNA fragments followed by oligonucleosomal cleavage products of 20–200 bp (“DNA ladders”) and an increase in hypodiploidity (48). In the present study we used three different methods to detect DNA fragmentation: DNA fragmentation assay, which detects DNA fragments in broken cell preparations; the more sensitive DNA solubilization assay, which can yield semiquantitative evaluation of apoptosis; and TUNEL, which describes morphologically the extent of DNA fragmentation in situ, including in cells in culture.

DNA fragmentation assay on homogenates of hECE or CaSki cells grown in medium enriched with serum did not detect significant laddering (not shown). However, DNA solubilization assays in those cells revealed values of 1.4% and 1%, respectively (Fig. 1), indicating a low baseline degree of apoptosis. When cells were shifted to serum-free medium these levels increased to 2.4% and 1.6%, respectively (Fig. 1; P < 0.05), and in some experiments laddering was observed (not shown). Shifting cells to low Ca\(^{2+}\) (1.2 mM → 0.6 mM) resulted in low levels of DNA solubilization (0.4–0.8%), regardless of whether cells were incubated in serum-enriched medium or in serum-free medium (Fig. 1).

One of our objectives was to determine the effect of ATP on apoptosis. Activation of ATP purinergic P2 receptors stimulates calcium influx and increases cytosolic calcium (reviewed in Refs. 6 and 41). In human cervical epithelial cells ATP activates at least three types of purinergic P2 receptors, P2Y

![Fig. 1. Effects of serum and extracellular calcium on apoptosis of human cervical epithelial cells.](http://ajpcell.physiology.org/)
and P2X$_4$ receptors (11, 12) and the P2X$_7$ receptor, and activation of the P2X$_7$ receptor stimulates prolonged and persistent increases in cytosolic calcium (Gorodeski GI, unpublished observations). In view of the finding that baseline apoptosis depends on extracellular calcium and the fact that ATP is secreted from most eukaryotic cells into the extracellular milieu (6), we hypothesized that the effects shown in Fig. 1 could be induced constitutively by ATP. To test this hypothesis we determined the effects of the ATPase apyrase on DNA solubilization. Treatment with apyrase significantly decreased DNA solubilization, regardless of whether cells were incubated in medium containing serum or in serum-free medium (Fig. 2). This result supports a role for ATP in the baseline apoptosis of cervical cells. It also suggests that ATP is the active ligand that is involved in the apoptotic effect, rather than ADP, AMP, adenosine, or adenine, and therefore rules out significant contribution of P1 (adenosine) receptors to the apoptosis.

**Effect of ATP involves activation of P2X$_7$ receptor.** Treatment of hECE cells with 50 μM ATP for 12 h resulted in DNA fragmentation in the form of laddering (Fig. 3A). Similar results were obtained in CaSki cells (not shown). The effect of ATP occurred in cells grown either in regular medium (Fig. 3) or in serum-free medium (not shown) and was similar to that obtained after treatment for 14 h with 10 ng/ml TNF-α (Fig. 3A). TNF-α was previously used in various types of cells for the induction of apoptosis, including in HeLa cells (40), and it was used here as the positive control. At 10 ng/ml, TNF-α was found to have maximal effect on DNA solubilization in human cervical epithelial cells (Table 1).

To gain a better understanding of the effect of ATP, effects of other adenine nucleotides and nucleosides, nucleoside triphosphates, and nonhydrolyzable analogs of ATP were studied. At an equimolar concentration of 50 μM administered for 12 h, only BzATP, the specific P2X$_7$ receptor analog (6, 41), induced significant DNA solubilization, whereas ADP, adenosine, ATP$_7$S, and UTP had minimal effects (Table 1 and Fig. 3A). The following agents produced a negligible degree of DNA solubilization (≤0.5%): AMP, adenosine, GTP, CTP, ITP, ATP, TTP, AMP-PCP, AMP-PNP, A-8889, A-2392, ADP$_7$S, and 2-MeS-ATP. These data suggest that the effect of ATP depends on activation of the P2X$_7$ receptor mechanism, with no significant involvement of the P2Y$_7$ or P2X$_4$ receptors. Similar to its effect on baseline apoptosis, apyrase blocked the ATP-induced apoptosis (Fig. 3B), but, as

![Fig. 2. Effects of serum and ATPase on apoptosis of human cervical epithelial cells. Four-day cultures of hECE cells on filters were maintained in regular medium or shifted to serum-free medium and treated for 12 h with ATPase [apyrase; 0.1 U/ml, added from a concentrated (1,000×) stock in PBS] or vehicle. Apoptosis was determined in terms of DNA solubilization. Shown are means ± SD of 3–4 experiments for each point. *P < 0.01.](image)

![Fig. 3. A: effects of tumor necrosis factor (TNF)-α, ATP, and ATP analogs on apoptosis of human cervical epithelial cells. Four-day cultures of hECE cells on filters were maintained in regular medium and treated for 12 h with 10 ng/ml TNF-α or with 50 μM of UTP, ATP, or 2',3'-O-(4-benzoylbenzoyl)-adenosine 5'-triphosphate (BzATP). Apoptosis was determined in terms of DNA fragmentation. m, Markers; C, control (vehicle). The experiment was repeated 3 times with similar trends. Results were the same regardless of whether UTP and ATP were added once at the beginning of the experiments or replenished every 30–60 min. B: experiment as in A, except that cultures were treated with 250 μM ATP in the absence or presence of apyrase (as in Fig. 2). The experiment was repeated 3 times with similar trends.](image)

**Table 1. Agonist-induced DNA solubilization**

<table>
<thead>
<tr>
<th>Ligand</th>
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<th>CaSki cells</th>
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<td></td>
<td>Regular medium</td>
<td>SFM</td>
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<td>SFM</td>
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<tr>
<td>ATP</td>
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<td>3.6 ± 0.6</td>
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<td>0.1</td>
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Values are means ± SD. Cells were treated for 12 h with 1 of the agonists (all at 50 μM), except for tumor necrosis factor (TNF)-α, which was administered for 14 h. hECE, human ectocervical epithelium; SFM, serum-free medium; BzATP, 2',3'-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate; ATP$_7$S, adenosine 5'-O-(3-thiotriphosphate).
expected, it had no effect on the BzATP-induced apoptosis (not shown).

The apoptotic effect of ATP was time and dose related. In CaSki cells DNA fragmentation was already observed 1–3 h after addition of ATP and reached submaximal effect 6–9 h after treatment (Fig. 4). Similar effects were obtained in hECE cells (not shown). In hECE cells DNA fragmentation began already with 0.1–1 μM ATP (Fig. 5A) and in CaSki cells with 1–10 μM ATP (Fig. 5B). Also determined were the effects of ATP and BzATP on DNA solubilization. In hECE cells (Fig. 6) and in CaSki cells (not shown) the dose-response curves of DNA solubilization vs. ATP or BzATP did not reach saturation even at millimolar concentrations of the nucleotides (Fig. 6). BzATP had a more efficacious and potent apoptosis-inducing effect than ATP (Fig. 6), similar to effects described in other types of cells (6, 41).

Interestingly, ATP and BzATP were capable of inducing apoptosis in hECE cells incubated in low (0.6 mM) Ca2+ (Fig. 7); under these conditions hECE do not stratify and remain as a monolayer of germinative cells with proliferative capacity (16, 22). The dose requirements for ATP and BzATP were greater than in normal (1.2 mM) Ca2+, and even in low Ca2+ BzATP had a more efficacious effect than ATP (Fig. 6). These data suggest that activation of the P2X7 receptor can induce apoptosis even in proliferating cervical epithelial cells.

Mechanisms of P2X7 receptor-induced apoptosis. Apoptosis can be mediated by the cell surface receptor and/or mitochondrial pathways. The former usually involves the Fas and TNF mechanisms followed by activation of caspase-8 (3, 35, 43). The mitochondrial pathway involves the release of cytochrome c (29) and activation of caspase-9 (53). The cell surface receptor and mitochondria-dependent pathways integrate at the level of the effector caspases, so that activation of caspase-9 and -8 triggers the nonreversible execution of apoptosis by caspase-6, -7, and -3 (3).

The objective of the next set of experiments was to determine the mechanism by which P2X7 receptor activation induces apoptosis in human cervical epithelial cells. As shown in Fig. 8, the effect of BzATP could be attenuated by lowering extracellular Ca2+ (Fig. 7); in contrast, the effect of TNF-α was independent of extracellular Ca2+, suggesting that TNF-α and BzATP operate via different mechanisms. To determine the degree to which BzATP and TNF-α utilize different signaling mechanisms cells were treated with both BzATP and TNF-α. Cotreatment with 10 ng/ml TNF-α had an effect on DNA solubilization partially additive to that of BzATP (Fig. 9). This was observed in the range of 0–50 μM BzATP, which are doses that produce submaximal effects for both BzATP (Fig. 6) and TNF-α (Table 1). In contrast, at 100 μM BzATP and TNF-α had no additional effect on DNA solubilization (Fig. 9).
The nonadditive effect in Fig. 9 could be the result of sharing of a common signaling pathway by both TNF-α and BzATP. Alternatively, and in view of the nonadditive effect seen predominantly at high BzATP concentrations, it is possible that the effect has reached saturation, namely, that cells have reached their maximal capacity to undergo apoptosis. The latter argument is therefore still consistent with activation of two partially independent mechanisms. Support for this speculation comes from the experimental data in Fig. 10. The caspase-9 inhibitor LEHD-FMK blocked BzATP-induced DNA solubilization to levels observed in baseline conditions (7.2% vs. 2.4%; P < 0.01). In contrast, the caspase-8 inhibitor IETD-FMK reduced DNA solubilization only to 6.2% (Fig. 10). The positive controls were DEVD-FMK (specific inhibitor of the terminal caspase-3) and Z-VAD-FMK (nonspecific pan-caspase inhibitor), which blocked the BzATP-induced DNA solubilization to levels that were lower than baseline apoptosis (0.6% and 0.8%, respectively; Fig. 10).

A more direct approach to the question of which caspases are involved in the P2X7 receptor apoptotic effect was taken by

Fig. 7. Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) effects of ATP and BzATP. Four-day cultures of hECE cells on filters were maintained in regular (serum enriched) medium and were shifted to low calcium (0.6 mM) for 12 h by adding 0.6 mM EGTA, followed by 9-h treatment with 250 μM ATP (B), 100 μM BzATP (C), or vehicle (control; A). Green structures indicate apoptotic changes/bodies. Magnification, ×20. The experiment was repeated twice with similar trends.

Fig. 8. Effects of extracellular calcium, TNF-α, and BzATP on apoptosis of human cervical epithelial cells. Four-day cultures of hECE cells on filters were shifted to serum-free medium in the absence or presence of 0.6 mM EGTA (Cao of 1.2 or 0.6 mM, respectively) and treated for 12 h with 10 ng/ml TNF-α or 100 μM BzATP, alone or in combination. Apoptosis was determined in terms of DNA solubilization. Shown are means ± SD of 3 experiments for each point. *P < 0.01 compared with 1.2 mM Ca o. **P < 0.01 and 0.02 compared with 1.2 mM Ca o and TNF-α, respectively.

Fig. 9. Effects of TNF-α and BzATP on apoptosis of human cervical epithelial cells. Four-day cultures of hECE cells on filters were shifted to serum-free medium and treated for 12 h with 10 ng/ml TNF-α plus 1 of the indicated concentrations of BzATP. Apoptosis was determined in terms of DNA solubilization. Shown are means ± SD of 3–4 experiments for each point.
studying the effects of BzATP on caspase activation. Caspases are synthesized as inactive zymogens and are converted to an active tetrameric complex composed of two heterodimeric subunits. On activation, the caspases are cleaved to subunits that can be detected by Western blots (52, 53). Treatment of hECE cells with 100 μM BzATP significantly increased the expression of the 10-kDa form of caspase-9 and had only a mild effect on the expression of caspase-8, suggesting that BzATP activates predominantly caspase-9 (Fig. 11). Treatment with 10 ng/ml TNF-α increased expression of caspase-8 43-, 18-, and 10-kDa forms, but it had little effect on the expression of caspase-9 (Fig. 11), suggesting activation predominantly of caspase-8. Both BzATP and TNF-α increased expression of the 17-kDa form of caspase-3, suggesting activation of the terminal caspase-3. Neither BzATP nor TNF-α affected expression of the constitutive protein β-actin (Fig. 11).

Collectively, the data in Figs. 8–11 support the hypothesis that the apoptotic effect of BzATP is mediated predominantly by the caspase-9 (mitochondrial) pathway. However, there is also a contribution of the caspase-8 pathway, via mechanisms different from those activated by TNF-α.

**ATP is secreted from human cervical epithelial cells.** One of the questions regarding the physiological role of P2X7 receptors in the regulation of apoptosis is that of ATP activity in the extracellular fluid. ATP is present in millimolar concentrations in the cytosol of all cell types, but extracellular levels of the nucleotide are normally maintained at lower levels because of minimal permeation of ATP across lipid bilayers and because ubiquitous ecto-ATPases and nucleotidases hydrolyze extracellular nucleotides (reviewed in Ref. 6). To determine the degree to which human cervical epithelial cells secrete ATP into the extracellular fluid, aliquots of conditioned medium from hECE and CaSki cultures were collected and analyzed by an ECL method linked to firefly luciferase-luciferin. ATP values ranged from 5 nM to 1 μM, with mean ± SD of 485 ± 217 nM. ATP levels in conditioned medium of hECE cells tended to be higher than in CaSki cells (570 ± 170 vs. 350 ± 219 nM). Lowering extracellular Ca2+ from 1.2 mM to 0.6 mM tended to lower extracellular ATP (to 405 ± 125 and 175 ± 110 nM in hECE and CaSki cells, respectively), whereas shifting cells to serum-free medium tended to increase extracellular ATP (to 650 ± 170 and 575 ± 130 nM, respectively). However, none of the differences was significant. These data indicate that cultured human cervical cells secrete ATP into the extracellular fluid, and the mean steady-state ATP activity is ~0.5 μM, which is within the range of values that suffice to activate the P2X7 receptor (Fig. 6).

**DISCUSSION**

Baseline apoptosis in normal human cervical epithelial cells depends on serum factors and calcium. Some of the serum factors are probably mitogens, e.g., EGF, which can prevent apoptosis by stimulating entry into the cell cycle. Prolonged quiescence promotes apoptosis (49), and withdrawal of serum could deplete the cells of factors that are necessary to stimulate proliferation and replication. This would allow cells to remain in quiescence (G0/G1 phase) and undergo apoptosis. Another explanation for the proapoptotic effect of serum deprivation is the omission of factors that control activity of surface ectonucleotidases. This speculation is supported by the findings that apyrase decreased and ATP, acting via the P2X7 receptor mechanism, increased apoptosis. Because extracellular levels of ATP are determined by the amount of ATP released from cells and by ATP degradation by surface ectonucleotidases, it is possible that serum deprivation decreased ecto-ATPase activity (36) and secondarily increased the availability of ATP at the cell surface. Alternatively, serum deprivation...
could have increased ectonucleotide pyrophosphatase and nucleoside diphosphokinase activity, which block ecto-ATPase activity (26).

The role of calcium in apoptosis was discussed previously (39). However, the present data are novel with regard to the role of calcium as mediator of ATP-induced apoptosis. In squamous epithelial cells, including cervical cells, augmented calcium influx stimulates terminal differentiation and envelope formation (22). Because terminal differentiation and apoptosis share signaling pathways (33, 60), a number of authors have used those terms interchangeably to describe the process in which dividing basal cells withdraw from the cell cycle and progressively differentiate as they are displaced toward the epithelial surface (see, e.g., Ref. 23). It is now realized that terminal differentiation and apoptosis are distinct phenomena. Terminal differentiation follows a structured topographical pattern whereby cells in suprabasal layers begin to lose their nucleus and other organelles to become flattened squames. These are finally shed from the surface as bags of cross-linked keratin filaments enclosed in a cornified envelope (60). Apoptosis, on the other hand, can be observed throughout the thickness of the epithelium, including in basal/parabasal layers (Refs. 38 and 60 and the present results).

In the present study we show, for the first time, that apoptosis can be induced unrelated to terminal differentiation. hECE cells cultured in low (0.6 mM) Ca\(^{2+}\) remain as a monolayer of proliferating cells, resembling the basal/parabasal layers of the ectocervical epithelium (16). Switching cells to normal (1.2 mM) Ca\(^{2+}\) induces terminal differentiation (22) and biochemical changes of apoptosis (present results). However, in cells maintained in 0.6 mM Ca\(^{2+}\), TNF-\(\alpha\) and BzATP induced apoptosis without stimulating terminal differentiation. Interestingly, low extracellular calcium had little effect on the TNF-\(\alpha\)-induced apoptosis but it attenuated the BzATP effect, suggesting that the apoptotic effect of BzATP utilizes predominantly a calcium-dependent mechanism.

In human cervical epithelial cells the apoptotic effect of ATP utilizes mainly the caspase-9-mitochondrial pathway. This pathway involves disruption of the inner mitochondrial transmembrane potential by hyperoxidation and elevated cytosolic calcium, and it leads to the formation of mitochondrial permeability transition pores (25, 28). The present data are consistent with this model, because lowered extracellular calcium blocked the effect of BzATP. Human cervical epithelial cells express the P2X\(_7\) receptor, and activation of the receptor leads to prolonged and sustained calcium influx and increased cytosolic calcium (15). It is therefore suggested that the apoptosis in human cervical epithelial cells involves calcium-induced mitochondrial dysfunction.

The mechanism by which activation of the P2X\(_7\) receptor induces uncontrolled increases in cytosolic calcium is unclear, but it probably involves increased plasma membrane permeability to Ca\(^{2+}\). The P2X\(_7\) receptor is unique in its ability to form pores in the plasma membrane in the continued presence of the ligand (58). Plasma membrane pore formation depends on the long COOH terminus of the receptor (5), and oligomerization of neighboring molecules is believed to cause progressive dilatation of the pore to a diameter of ~4 nm and an increase in the permeation path to molecules of 400- to 900-Da molecular mass (24). In its “final” size the pore is relatively permeable to Ca\(^{2+}\), but it remains selective to other cations and is impermeable to anions (41).

Activation of P2X\(_7\) receptors can also induce apoptosis by other mechanisms, including IL-1\(\beta\) (9), TNF-\(\alpha\)-TNF-related apoptosis-inducing ligand (TRAIL) (1), and the p38, JNK/SAPK (19), and NF-\(\kappa\)B (10) pathways. We found that the caspase-8 inhibitor IETD-FMK mildly attenuated the BzATP-induced apoptosis, suggesting involvement of the caspase-8 pathway. One explanation is that, in addition to the mitochondrial pathway, ATP activated another pathway such as the p38, JNK/SAPK, or NF-\(\kappa\)B cascades. The TNF-\(\alpha\)-TRAIL pathway could be one such mechanism because effects of BzATP were partially additive to those of TNF-\(\alpha\), suggesting some degree of sharing of signaling pathways. Another explanation is cross-interaction between the caspase-9 and caspase-8 pathways and amplification of caspase-3 activation (20). Regardless of the mechanism involved, the contribution of the caspase-8 pathway was significantly smaller compared with that of the caspase-9 pathway.

The present results provide insight into the physiological relevance of P2X\(_7\) receptor actions. In vivo, the only known ligand of the P2X\(_7\) receptor is ATP. Extracellular levels of ATP are determined by the amount of ATP released from cells and by ATP degradation. Intracellular levels of ATP range from 3 to 5 mM, and most cells constitutively secrete ATP into the extracellular fluid via specialized transporters (6). Extracellular ATP is usually hydrolyzed to ADP and AMP by surface ectonucleotidases, and AMP can be converted into adenosine by 5’-nucleotidases (62). Similar to other recent reports (17, 18, 31, 51), in the conditioned medium of cultured human cervical epithelial cells ATP steady-state activity was ~0.5 \(\mu\)M, which is in the range that is sufficient to activate the human P2X\(_7\) receptor (present study). This finding can explain activation of the P2X\(_7\) receptor by ATP in vivo; accordingly, cervical cells self-regulate cytosolic calcium and apoptosis by secreting ATP into the extracellular fluid. A similar concept was previously suggested for the regulation of cell volume (59) and for the regulation of renal microcirculation (37). In summary, our findings suggest a novel autocrine-paracrine mechanism of P2X\(_7\) receptor control of cervical cell apoptosis. This model could be important for the understanding of cervical cell biology and cancer development.

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