Purinergic Receptors Are Part of a Signaling System for Keratinocyte Proliferation, Differentiation, and Apoptosis in Human Fetal Epidermis

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We have investigated the expression of P2X5, P2X7, P2Y1, and P2Y2 receptor subtypes in 8- to 11-wk-old human fetal epidermis in relation to markers of proliferation (proliferating cell nuclear antigen (PCNA) and Ki-67), keratinocyte differentiation (cytokeratin K10 and involucrin), and markers of apoptosis (TdT-mediated dUTP nick end labeling (TUNEL) and anti-caspase-3). Immunohistochemistry showed that each of the four receptors was expressed in spatially distinct zones of the developing epidermis: P2Y1 receptors were found in the basal layer, P2X5 receptors were predominantly in the basal and intermediate layers, and both P2Y2 and P2X7 receptors were in the periderm. Co-localization experiments suggested different functional roles for these receptors. P2Y1 receptors were found in fetal keratinocytes positive for PCNA and Ki-67, suggesting a role in proliferation. P2X5 receptors double labeled with differentiated fetal keratinocytes that were positive for cytokeratin K10, suggesting a role in differentiation. P2X7 receptors colocalized with anti-caspase-3 antibody and were also expressed in periderm cells positive for TUNEL, suggesting a role in periderm cell apoptosis. P2Y2 receptors were found only in periderm cells and may have a role in chloride and fluid secretion into the amniotic fluid. Key words: apoptosis cell differentiation/proliferation receptors/periderm/purinergic P2.

Original Article

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A denosine 5'-triphosphate (ATP) is now recognized as an important messenger molecule for cell-cell communication, with ATP binding specifically to purinergic receptors (Ralevic and Burnstock, 1998). There is considerable evidence that purinergic signaling can have long-term, trophic effects in embryonic development, cell proliferation, differentiation, and death (Abbracchio and Burnstock, 1998; Burnstock, 2001).

Purinergic receptors are classified into two groups: P1 receptors are selective for adenosine and P2 receptors are selective for ATP and adenosine 5'-diphosphate, which act as extracellular signaling molecules (Burnstock, 1998). P2 receptors are divided into two main families: P2X (ligand-gated ion channels) and P2Y (G-protein-coupled receptors), based on molecular structure, transduction mechanisms, and pharmacologic properties (Abbracchio and Burnstock, 1994). Eight subtypes of P2Y receptors have been described, and seven subtypes of P2X receptors are recognized (Burnstock, 2003).

ATP is likely to be an important local messenger in the epidermis. Both P2X5 and P2X7 receptors are expressed on adult rat cutaneous keratinocytes and functional roles in the regulation of proliferation, differentiation, and cell death have been proposed (Gröschel-Stewart et al., 1999). P2Y2 receptors, found in the basal layer of normal adult epidermis, are claimed to be involved in keratinocyte proliferation (Dixon et al., 1999). Studies on adult human epidermis and primary keratinocyte cultures have suggested that P2Y1 and P2Y2 receptors are involved in keratinocyte proliferation, that P2X5 receptors are likely to be involved in keratinocyte differentiation, and that P2X7 receptors are likely to be part of the machinery of end stage terminal differentiation/apoptosis of keratinocytes (Greig et al., 2003).

Human fetal skin is unique in structure and function and is in a continuum of change until birth. The transition from fetal to mature epidermis of the neonate and adult involves formation of new epidermal layers, invagination and remodeling of epidermal appendages, and finally loss of fetal-specific cell types and onset of adult-type differentiation (see Holbrook and Hoff, 1984, for a review). The primitive epidermis is established at 7 to 8 d when ectoderm and endoderm are defined in the inner cell mass of the implanted blastocyst. At this stage the epidermis is a single-layered "indifferent ectoderm." A second epidermal layer forms at the end of the fourth week. The outermost of the two layers is the periderm. The periderm is the transient, protective covering of the epidermis that is sloughed into the amniotic fluid as soon as differentiation of the underlying epidermal layers is complete at 20 to 24 wk. Beneath the periderm, basal keratinocytes comprise the single layer of the "epidermis proper." At the end of the ninth week, the embryonic-fetal transition, basal cells divide to give rise to daughter cells that move vertically to form the first intermediate cell layer that characterizes the fetal epidermis. Two or three more layers of intermediate cells are added to the epidermis in the second trimester (12–24 wk). Once a granular layer is established in the sixth month, the intermediate cells become known as spinous cells; thus each layer of the epidermis assumes...
the adult nomenclature as the tissue assumes the adult characteristics. The first stratum corneum develops at the end of the second trimester (24 wk), consisting of only a few cell layers, which increase in thickness during the third trimester and, at birth, it is approximately equivalent to that of the adult.

This study demonstrates the distribution of P2X and P2Y receptors in human fetal epidermis. We propose that these receptors are part of the normal mechanisms controlling fetal epidermal development in relation to keratinocyte proliferation, differentiation, and apoptosis.

MATERIALS AND METHODS

Tissues Twenty-five samples of normal human fetal skin were examined from nine fetuses at 8 to 11 wk estimated gestational age, which was determined by fetal measurements after pregnancy termination. Samples were obtained from fully informed, consenting patients undergoing elective terminations of pregnancy. Ethics Committee approval was obtained to harvest human fetal samples. Tissue was frozen in isopentane precooled in liquid nitrogen. Blocks were sectioned at 10 μm on a cryostat (Reichert Jung CM1800), collected on gelatin-coated slides, and air-dried at room temperature. The slides were stored at −20°C.

Antibodies The immunogens used for production of polyclonal P2X5 and P2X7 antibodies were synthetic peptides corresponding to 15 receptor-type-specific amino acids in the intracellular C-termini of the cloned rat and human P2X receptors, as described previously (Gröschel-Stewart et al., 1999; Oglesby et al., 1999). P2X5 and P2X7 antibodies (provided by Roche Bioscience, Palo Alto, CA) were kept frozen at a stock concentration of 1 mg per mL (P2Y1, P2Y2). Sections were mounted in Citi£uor (Citi£uor Ltd, Leicester, UK). P2X or P2Y receptor antibodies were applied at a dilution of 1:200 for 1 h, and then sections were washed and incubated at room temperature for 2 h with one of the following antibodies: rabbit anti-human Ki-67 antigen (Dako) 1:50 or rabbit anti-human active caspase-3 (Abcam). Sections were then washed and incubated with the directly labeled secondary antibody Oregon-green-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory), diluted 1:200 for 45 min. Sections were then washed and mounted in Citifluor (Citifluor Ltd). P2X or P2Y receptor immunostaining appeared red and Ki-67 and caspase-3 stained green.

Double-labeling of P2X or P2Y receptor antibodies with either Ki-67 antigen or antihuman caspase-3 Sections were fixed and incubated with P2X or P2Y antibodies overnight, as described above. After being washed, sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratory) diluted to 1:500 in 1% normal horse serum in PBS for 1 h, followed by ExtrAvidin peroxidase conjugate (Sigma Chemical Co.) diluted to 1/500 in PBS for 1 h, tyramide amplification for 8 min (tyramide amplification kit, NEN Life Science Products, Boston, MA) and then streptavidin–Texas red (Amersham International plc.), diluted 1:200 in PBS–merthiolate for 10 min. Sections were washed three times in PBS after each of the above steps. Sections were preincubated for 20 min in 10% normal goat serum and then incubated at room temperature for 2 h with one of the following antibodies: rabbit anti-human Ki-67 antigen (Dako) 1:50 or rabbit anti-human active caspase-3 (Abcam). Sections were then washed and incubated with the directly labeled secondary antibody Oregon-green-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory), diluted 1:200 for 45 min. Sections were then washed and mounted in Citifluor (Citifluor Ltd). P2X or P2Y receptor immunostaining appeared red and Ki-67 and caspase-3 stained green.

Double labeling of P2X7 receptor antibodies with TdT-mediated dUTP nick end labeling (TUNEL) TUNEL identifies cells undergoing apoptosis by labeling nuclear DNA fragments that have been cleaved during apoptosis (Gavrieli et al., 1992). TUNEL labeling was performed using a kit (Boehringer Mannheim, Germany). After overnight incubation with P2X7 receptor antibody diluted to 1:200 as above, sections were washed in PBS and then incubated with the TUNEL reaction mixture for 1 h at 37°C. As a negative control, sections were incubated with the TUNEL solution only. After additional washes in PBS, sections were incubated for 1 h with biotinylated donkey anti-rabbit antibody at a concentration of 1:500. Sections were washed in PBS and then incubated with 1 h with streptavidin–Texas red (Amersham International plc.) at a concentration of 1:200. Sections were washed in PBS and mounted in Citifluor (Citifluor Ltd). P2X7 receptor immunostaining appeared red and TUNEL labeling was green.

Controls Control experiments were carried out with primary antibodies omitted from the staining procedure or the primary antibodies preabsorbed with the corresponding peptides.

Photography The results were photographed using a Zeiss Axioplan, high-definition light microscope (Oberkochen, Germany) mounted with a Leica DC 200 digital camera (Heerbrugg, Switzerland).

RESULTS

P2X5, P2X7, P2Y1, and P2Y2 receptor expression in human fetal epidermis P2X5 and P2X7 receptor immunoreactivity was observed in the epidermal keratinocytes of all human fetal skin samples (Fig 1a,b). P2X5 receptor immunoreactivity was found mainly in the basal layer and to a lesser extent in the intermediate cell layer (Fig 1c). P2X5 receptors were found in the cell membranes and sometimes in the cell cytoplasm, but not in the nucleus. The P2X7 receptor staining on keratinocytes in the stratum basale appeared to be polarized, being concentrated on the basal aspects of cells associated with the basement membrane (Fig 1c). In the epidermis, P2X5 receptor immunoreactivity was associated with periderm cells only (Fig 1d). P2X7 receptor immunostaining also labeled fetal blood cells. P2Y1 receptors were found in the basal layer of the epidermis (Fig 1e) and P2Y2 receptors were expressed only in the periderm (Fig 1f).

Double labeling of P2Y1 and P2Y2 receptors with markers for cellular proliferation Fetal skin was double-stained for Ki-67 and P2Y1 receptors (Fig 1g) and PCNA and P2Y2 receptors (Fig 1h). The proliferation markers identified a proliferating subpopulation of basal keratinocytes. Cells positive for these two markers were also positive for P2Y1, but not for P2Y2 receptors, which were only found in periderm cells.
Figure 1. Expression of P2X5, P2X7, P2Y1, and P2Y2 receptors in human fetal epidermis and double labeling with markers for cellular proliferation, keratinocyte differentiation, and markers for apoptosis. (a) Cross-section of hand of 9-wk-old fetus showing P2X5 receptor immunostaining. Bar, 375 μm. (b) Cross-section of hand of 9-wk-old fetus showing P2X7 receptor immunostaining, with a predominance on the dorsal surface (arrow). Bar, 375 μm. (c) High-power view of (a): P2X5 receptor staining was mainly found in the basal layer (B) of the epidermis and to a lesser extent in the intermediate layer (I). There was little staining in the periderm (P). Bar, 375 μm. (d) High-power view of (b): P2X7 receptor immunoreactivity was associated with cells within the periderm (P). Bar, 375 μm. (e) P2Y1 receptors were found in the basal layer (B) of the epidermis. Bar, 25 μm. (f) P2Y2 receptors were found in the periderm (P). Bar, 25 μm. (g) Double labeling of fetal epidermis with P2Y1 receptors (red) and Ki-67 (green) to show that P2Y1 receptors are found in proliferating cells in the basal layer (B). Bar, 25 μm. (h) Double labeling of P2Y2 receptors (red) and PCNA (green). PCNA identified a proliferating subpopulation of only basal keratinocytes (B), but P2Y2 receptors were only found in periderm cells (P). Bar, 25 μm. (i) Double labeling of P2X5 receptors (red) with cytokeratin K10 (green). Cytokeratin K10 was found in cells in the intermediate cell layer (I) and not in basal keratinocytes (B). The stratum basale (B) stained only for P2X5 receptors, indicating that no differentiation was taking place in these cells. Bar, 25 μm. (j) Double labeling of P2X7 receptors (red) with involucrin (green). Involucrin was expressed only in the periderm cells (P) and did not colocalize with P2X7 receptor staining in the basal (B) and intermediate (I) layers. Bar, 25 μm. (k) Double labeling (yellow) of P2X7 receptors (red) with TUNEL (green). Only intermittent, rounded periderm cells (P) had nuclei that stained strongly positive for TUNEL although P2X7 receptors were expressed in most of the cells in the periderm layer. Bar, 25 μm. (l) Double labeling (yellow) of P2X7 receptors (red) with anti-caspase-3 (green) showed colocalization within the periderm (P). Bar, 25 μm.

**Double labeling of P2X5 receptors with markers for keratinocyte differentiation** Double labeling of P2X5 receptors with cytokeratin K10 (Fig 1j) showed that P2X5 receptors were expressed in differentiating keratinocytes within the fetal epidermis. Cytokeratin K10, an early marker of keratinocyte differentiation, was found in cells in the intermediate cell layer and not in basal keratinocytes. The basal layer stained only for P2X5 receptors, and not for markers of differentiation, indicating that no differentiation was taking place in these cells. Involucrin, a late marker of keratinocyte differentiation in adult skin, was expressed only in the periderm cells and did not colocalize with P2X5 receptor staining (Fig 1j).

**Double labeling of P2X7 receptors with markers for apoptosis in fetal epidermis show colocalization** Individual rounded periderm cells had nuclei that stained strongly positive for TUNEL (Fig 1k). This corresponded to a transitional stage when the periderm is beginning the formation of a series of complex surface blebs. Double staining of the P2X7 receptor and TUNEL showed that only intermittent cells were positive for TUNEL, although P2X7 receptors were expressed in most of the cells in the periderm layer. The negative control for TUNEL showed no reaction. Double labeling of P2X7 receptors with anti-caspase-3 showed colocalization within the periderm (Fig 1l).

**Controls** Both the omission of the primary antibody and the preabsorption with corresponding peptides were performed as controls. The immunoreaction was abolished after preabsorption of the P2X5, P2X7, P2Y1, or P2Y2 receptor antibodies with the corresponding peptides, confirming the specificity of the immunoreaction.

**DISCUSSION**

This study has shown the first direct evidence for the expression of P2X5, P2X7, P2Y1, and P2Y2 receptors in 8- to 11-wk-old
human fetal epidermis, using immunohistochemistry. Four periods of human epidermal development have been identified (Dale et al., 1985). The first is the embryonic period (before 9 wk estimated gestational age) and the others are within the fetal period: stratification (9–14 wk), follicular keratinization (14–24 wk), and interfollicular keratinization (beginning approximately 24 wk). We have studied epidermal tissue at the end of the embryonic period and during the period of stratification. It would be of interest to have studied fetal tissue from different stages of gestation but fresh-frozen tissue was not available for other time points.

Purinergic receptors bind ATP, which is found in all cells. Intracellular ATP can be released passively from dying, necrotic cells undergoing cytosis, or it can be actively released from living cells by vesicular release or via ATP-binding cassette proteins (Bodin and Burnstock, 2001). In our study, we have seen that purinceptors are distributed on cell membranes and in the cytoplasm. Internazionalization of purinceptors has been observed in a variety of circumstances including synthesis of receptors in the cytoplasm and transport to the cell membrane (Loesch and Burnstock, 2001) and following prolonged exposure to ATP (Li et al., 2000).

P2Y1 receptors showed a strong immunopositive signal in the basal layer of the fetal epidermis, which is known to be where keratinocyte proliferation occurs (Bickenholt and Holbrook, 1987). Double labeling of P2Y1 receptors and keratinocyte proliferation markers Ki-67 and PCNA was performed. This confirmed the presence of P2Y1 receptors in proliferating cells, indicating that this receptor is involved in keratinocyte proliferation. The distribution of P2Y1 receptors was the same as in adult epidermis (Greig et al., 2003).

P2X5 receptors were expressed in the basal layer of human fetal epidermis and were also expressed in the intermediate cell layer, where cytokeratin K10, an early marker of keratinocyte differentiation, was found. It is known that keratinocytes that initially occupy the intermediate layer of fetal epidermis from 9 to 20 wk gestation undergo only an incomplete type of differentiation (Holbrook, 1991) that is not characteristic of the mature epidermis. Although not morphologically different, the intermediate cells at this stage have initiated biochemical differentiation, characterized by the synthesis of epidermal differentiation-specific markers, keratins 1 and 10 (Dale et al., 1985) as seen in our study. The expression of these keratins is thought to herald the commitment of the keratinocyte to a program of epidermal keratinocyte differentiation (Haake and Cooklis, 1997). Double labeling of P2X5 receptors with cytokeratin K10 showed that P2X5 receptors were expressed in differentiating keratinocytes within the fetal epidermis, so it seems likely that P2X5 receptors are involved in fetal epidermal keratinocyte differentiation. The basal layer stained only for P2X5 receptors and not cytokeratin K10, indicating that basal cells were not differentiated, so P2X5 receptors may also have an additional role in basal keratinocytes. There is evidence from other tissues regarding the role of P2X5 receptors. In fetal rat skeletal muscle, P2X5 receptors are sequentially expressed during development (Ryten et al., 2001). P2X5 receptors have been implicated in the regulation of osteoblastic differentiation and proliferation (Hoeberitz et al., 2000) and in triggering the differentiation of skeletal muscle satellite cells (Ryten et al., 2002).

Involucrin, a late marker of keratinocyte differentiation in adult skin and a cornified cell envelope precursor protein, was expressed only in periderm cells and did not colocalize with P2X5 receptor staining. The lack of dual labeling might simply be due to the incomplete nature of epidermal differentiation at this stage in development, because involucrin is a late marker of differentiation. Involucrin has previously been found to be expressed only on periderm cells in fetal epidermis at the same stage of development (Akiyama et al., 1999) and is thought to be representative of a process of keratinization within the periderm, separate from the keratinization of the underlying "proper" epidermal layers that occurs later in development. In adult epidermis, we found that involucrin colocalized with P2X5 receptors only in the upper layers of the stratum spinosum (Greig et al., 2003).

Cells in the periderm were intensely stained for TUNEL and caspase-3. Double staining of the P2X7 receptor and TUNEL showed that only intermittent cells were positive for TUNEL, although P2X7 receptors were expressed in most of the cells in the periderm layer. Double labeling of P2X7 receptors with anti-caspase-3 showed colocalization within the periderm. The P2X7 receptor is unlike other P2X receptors because it is a bifunctional molecule that can be triggered to act as a channel permeable to small cations or on prolonged stimulation can form a cytoplasmic pore permeable to large hydrophilic molecules of up to 900 Da (Surprent et al., 1996). The opening of this pore results in the increase in intracellular cytosolic free calcium ions and the induction of cell death and there is increasing evidence that this process is dependent on the caspase signaling cascade (Ferrari et al., 1999). Caspase-3 is expressed in adult terminally differentiating keratinocytes (Wei et al., 1999). During apoptosis, the nucleus condenses and DNA is fragmented by endonucleases, which can be detected by TUNEL. TUNEL-positive keratinocytes have been found in the upper regions of the granular layer of the adult epidermis, before cornification (Polakowska et al., 1994; Tamada et al., 1994; Gandarillas et al., 1999). Nuclei of individual periderm cells have been previously reported to stain positively for TUNEL from about 14 wk estimated gestational age (Polakowska et al., 1994). In our study, the periderm was shown to exhibit characteristics consistent with apoptosis before its loss from the epidermis, which occurs at midgestation (20–24 wk). The periderm undergoes a series of morphologic changes that have been described ultrastructurally including: blebbing of the cell surface and pinching off of cytoplasmic fragments, accumulation of cytoplasmic filaments, formation of a submembranous cell envelope, and a pyknotic nucleus (Holbrook and Oldland, 1975). These morphologic changes in the periderm are similar to those described for other cell types undergoing apoptosis (Kerr et al., 1972) and the time of their onset correlates with the appearance of markers of apoptosis (Polakowska et al., 1994). Following the bleb stage, periderm cells also exhibit a number of regressive changes involving partial organelle degeneration and accumulation of filaments in the cytoplasm. It is thought that apoptosis is the mechanism by which periderm cells are deleted from the epidermis after serving their function (Polakowska et al., 1994). In this study, it is likely that periderm cells at 8 to 11 wk estimated gestational age, which double label for P2X5 receptors and markers of apoptosis, are showing a preliminary stage in the journey toward apoptosis, because they do not have the characteristic histologic appearance of apoptosis. The expression of P2X7 receptors may be important in the regulation of apoptosis intrinsic to correct development.

Interestingly, P2Y2 receptors were also found only in periderm cells. In adult skin, P2Y2 receptors were found in the basal layer of the epidermis, where proliferation markers Ki-67 and PCNA were also found (Greig et al., 2003). The P2Y2 receptor agonist, uridine 5′-triphosphate (UTP), caused a significant increase in human keratinocyte cell number in vitro (Greig et al., 2003). Previous work has localized P2Y2 receptor mRNA in adult human epidermal basal cells via in situ hybridization (Dixon et al., 1999). UTP, the potent P2Y2 receptor agonist, has also been shown to cause proliferation in keratinocytes (Dixon et al., 1999; Greig et al., 2003). Nevertheless, in fetal epidermis, P2Y2 receptor-positive periderm cells were not positive for proliferation markers PCNA and Ki-67. Therefore, P2Y2 receptors may have an alternative role in periderm cells. For example, P2Y2 receptors on periderm cells could be involved in chloride and fluid secretion into the amniotic fluid. P2Y2 receptors are involved in ion and fluid secretion in lung and conjunctival epithelial cells (Yerxa, 2001). Studies have suggested that nonkeratinized fetal epidermis may have an osmoregulatory function and also that the fetal epidermis contributes to the formation of amniotic fluid in the first trimester and
becomes the primary source in the second trimester (Holbrook, 1991). Periderm cells have microvilli and surface blebs that increase the surface area of the plasma membrane exposed to the amniotic fluid, there are large numbers of membrane-bound vesicles adjacent to the plasma membrane, and tight junctions join adjacent periderm cells. This suggests that the periderm cell layer may have potential for fluid and ion transport (Holbrook, 1991).

In summary, P2 purinergic receptors are likely to be involved in fetal keratinocyte proliferation via P2Y1 receptors found on basal cells and fetal keratinocyte differentiation via activation of P2X7 receptors. P2X7 receptors are likely to be involved in apoptosis of periderm cells, but the role of P2Y2 receptors in periderm cells is currently unknown; it is suggested that they may play a role in chloride and fluid secretion into the amniotic fluid.

The support of Roche Bioscience (Palo Alto, CA), who provided P2X5 and P2X7 receptor antibodies, is gratefully acknowledged. A.V.H.G. was the recipient of a Research Fellowship from The Wellcome Trust and The Simpson Surgical Research Fellowship from The Royal College of Surgeons of England.

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