Purinergic receptors are part of a signalling system for proliferation and differentiation in distinct cell lineages in human anagen hair follicles

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Abstract We investigated the expression of P2X5, P2X7, P2Y1 and P2Y2 receptor subtypes in adult human anagen hair follicles and in relation to markers of proliferation [proliferating cell nuclear antigen (PCNA) and Ki-67], keratinocyte differentiation (involucrin) and apoptosis (anticaspase-3). Using immunohistochemistry, we showed that P2X5, P2Y1 and P2Y2 receptors were expressed in spatially distinct zones of the anagen hair follicle: P2Y1 receptors in the outer root sheath and bulb, P2X5 receptors in the inner and outer root sheaths and medulla and P2Y2 receptors in living cells at the edge of the cortex/medulla. P2X7 receptors were not expressed. Colocalisation experiments suggested different functional roles for these receptors: P2Y1 receptors were associated with bulb and outer root sheath keratinocyte proliferation, P2X5 receptors were associated with differentiation of cells of the medulla and inner root sheaths and P2Y2 receptors were associated with early differentiated cells in the cortex/medulla that contribute to the formation of the hair shaft. The therapeutic potential of purinergic agonists and antagonists for controlling hair growth is discussed.

Keywords Receptors · Purinergic · Hair follicle · Proliferation · Differentiation

Abbreviations

AA amino acids
ATP adenosine 5'-triphosphate
ADP adenosine 5'-diphosphate
DAB diaminobenzidine
NGS normal goat serum
NHS normal horse serum
IRS inner root sheath
ORS outer root sheath
PBS phosphate buffered saline
PCNA proliferating cell nuclear antigen

Introduction

The cells of the lower portion of the hair follicle bulb are undifferentiated matrix cells. These are rapidly dividing cells that give rise to eight different cell lineages [1]. From within outwards, these include the medulla, cortex and hair cuticle cell lineages that make up the hair shaft; the inner root sheath cuticle, Huxley’s and Henle’s layers that make up the inner root sheath (IRS); and the companion layer and the outer root sheath (ORS) [1]. The hair cycle is divided into periods of hair growth (anagen), which are followed by a regression phase (catagen), when the lower part of the hair follicle undergoes programmed cell death [2], and a resting phase (telogen), before onset of a new growth phase.

The growing anagen hair follicle is an attractive system for studying proliferation and differentiation. There is increasing evidence that purinergic signalling can have long-term,
trophic effects on these processes [3, 4]. Adenosine 5'-triphosphate (ATP) is now recognised as an important messenger molecule for cell–cell communication, with ATP binding specifically to purinergic receptors [5, 6]. Purinergic receptors are classified into two groups: P1 receptors are selective for adenosine, and P2 receptors are selective for ATP and adenosine 5'-diphosphate (ADP), which act as extracellular signalling molecules [6]. P2 receptors are divided into two main families: P2X receptors, which are ligand-gated ion channels, and P2Y receptors, which are G protein-coupled, based on molecular structure, transduction mechanisms and pharmacological properties [7]. Seven subtypes of P2X receptors [8] and eight subtypes of P2Y receptors are recognised [9]. There is growing evidence that ATP may act as an important local messenger in the epidermis. Purinergic receptors are expressed on rat cutaneous keratinocytes, and functional roles in the regulation of proliferation, differentiation and cell death have been proposed [10]. In particular, P2X5 receptors are expressed on cells undergoing proliferation and differentiation, whereas P2X7 receptors are associated with keratinised dead cells. P2Y2 receptors, found in the basal layer of normal epidermis, are claimed to be involved in keratinocyte proliferation [11]. P2Y1 receptors are thought to be mitogenic in endothelial cells [4]. Previous work on adult human interfollicular epidermis and primary keratinocyte cultures has suggested that P2Y1 and P2Y2 receptors are involved in keratinocyte proliferation and that P2X5 receptors are likely to be involved in keratinocyte differentiation, whereas P2X7 receptors are likely to be part of the machinery of end-stage terminal differentiation of keratinocytes [12].

This study demonstrates for the first time the distribution of P2X and P2Y receptors in human anagen hair follicles. We propose that these receptors are part of the normal homeostatic mechanisms controlling hair keratinocyte proliferation and differentiation.

Materials and methods

Tissues

Eight samples of normal hair-bearing human skin were examined immunohistochemically in this study. Ethical Committee approval was obtained to harvest human skin. Samples of postoperatively redundant skin from otoplasty, preauricular skin tags and from the leg were obtained. Tissue was frozen in isopentane precooled in liquid nitrogen to cryoprotect the tissue and prevent damage to the tissue architecture from ice crystals. Blocks were sectioned at 10 µm on a cryostat (Reichert Jung CM1800), collected on gelatine-coated slides and air-dried at room temperature. The slides were stored at −20°C.

Antibodies

The immunogens used to produce polyclonal P2X5 and P2X7 antibodies were synthetic peptides corresponding to 15 receptor-type-specific amino acids (AA) in the intracellular C termini of the cloned rat and human P2X receptors, as previously described [10, 13]. P2X5 and P2X7 antibodies (provided by Roche Bioscience, Palo Alto, CA, USA) were kept frozen at a stock concentration of 1 mg/ml. Polyclonal anti-P2Y1 and P2Y2 antibodies were obtained from Alomone Labs (Jerusalem, Israel) and corresponded to the third extracellular loop of the P2Y1 (AA 242–258) and P2Y2 receptor (AA 227–244). Antibodies were kept frozen at a stock concentration of 0.6 mg/ml (P2Y1, P2Y2). Proliferating cell nuclear antigen (PCNA) is a marker for proliferation in normal adult human keratinocytes [14]. Involutrin is a marker for keratinocyte differentiation [15]. PCNA (monoclonal antiproliferating cell nuclear antigen, clone PC10, raised in the mouse. Ki-67 antigen is a marker for keratinocyte proliferation [16, 17]. Active caspase-3 is part of the apoptotic machinery of the cell and is expressed in terminally differentiating keratinocytes [18]. Ki-67 (DAKO, Heverlee, Denmark) and active caspase-3 (Abcam, Cambridge, UK) antibodies were both raised in the rabbit.

Immunohistochemistry

For immunostaining of cryostat sections, the avidin-biotin technique was used according to a revised protocol [19, 20]. Air-dried sections were fixed for 2 min in 4% formaldehyde in 0.1M phosphate buffer containing 0.2% of a saturated solution of picric acid (pH 7.4). Fixation was carried out at this point, as earlier fixation of the entire tissue can result in alteration of the antibody binding sites, interfering with staining. Endogenous peroxidase was blocked for 10 min with 50% methanol containing 0.4% hydrogen peroxide. Non-specific binding sites were blocked by a 20-min preincubation in 10% normal horse serum (NHS) in 0.1 M phosphate buffer containing 0.05% merthiolate (Sigma), followed by incubation with the primary antibodies diluted to 1:100 or 1:200 in antibody diluent [10% NHS in PBS + 2.5% sodium chloride (NaCl)] at 4°C overnight. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit immunoglobulin G (IgG) (Jackson Immunoresearch Lab, West Grove, PA, USA) diluted to 1:500 in 1% NHS in PBS for 30 min, followed by ExtrAvidin peroxidase conjugate (Sigma) diluted to 1:1000 in PBS for 30 min at room temperature. After a wash step, a nickel-diaminobenzidine (DAB) enhancement technique was used to visualise the reaction product. Sections were washed three
times with PBS after each of the above steps except for after preincubation with 10% NHS. After the last wash, sections were dehydrated twice in isopropanol and mounted with EUKITT (BDH Laboratory Supplies, Poole, UK). Control experiments were carried out with primary antibodies omitted from the staining procedure or the primary antibodies preabsorbed with the corresponding peptides.

**Double labelling of P2X or P2Y receptor antibodies with either PCNA or involucrin**

Sections were fixed and incubated with P2X or P2Y antibodies overnight, as described above. After a wash step, biotinylated donkey anti-rabbit IgG antibody, diluted 1:500 in 1% NHS in PBS, was then applied for 1 h, followed by Streptavidin Texas red (Amersham Plc., Little Chalfont, UK), diluted 1:200 in PBS-merthiolate for 1 h at room temperature. Sections were preincubated for 30 min with 10% normal goat serum (NGS) diluted in 0.1 M phosphate buffer containing 0.05% merthiolate (Sigma). They were then incubated for 2 h at room temperature with one of the following antibodies: PCNA antibody (Sigma) diluted 1:1000; mouse monoclonal anti-involucrin (Sigma) diluted 1:50. After a wash step, the directly labelled secondary antibody goat anti-mouse FITC (Nordic Immunological Laboratories, Tilburg, The Netherlands) was applied at a dilution of 1:200 for 1 h, and sections were washed and mounted in Citifluor (Citifluor Ltd., Leicester, UK). P2X or P2Y receptor immunostaining appeared red and PCNA and involucrin stained green.

**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**

Only hair follicles in the anagen (growth) phase were studied, as hair follicles in catagen (regression) or telogen (resting) phases of the hair cycle were not seen in the sections analysed.

P2X5, P2X7, P2Y1 and P2Y2 receptors are expressed in human anagen hair follicles

P2X5 receptor immunoreactivity was observed in the hair keratinocytes of all human skin samples. P2X5 receptor staining was restricted to the medulla and both the IRS and ORS (Fig. 1a,b), which were continuous with the surface epidermis. P2X7 receptor immunostaining was present in the stratum corneum of the overlying epidermis, but there was no staining in the anagen hair follicle (Fig. 1c,d). P2Y1 receptors were found in the ORS and bulb of anagen hair follicles but not in the IRS (Fig. 2a,b). P2Y2 receptors were found in cells in the cortex, with possibly a few positive cells at the edge of the medulla, but no staining in the central medulla (Fig. 2c,d). Double labelling of P2Y1 receptors with Ki-67 antigen showed that P2Y1 receptors were found in proliferating cells in the ORS and bulb of anagen hair follicles (Fig. 3a,b). Double labelling of P2Y2 receptors with PCNA showed that P2Y2 receptors were expressed in the cortex and possibly at the edge of the medulla (Fig. 3c) and PCNA was expressed in the ORS and bulb (Fig. 3c,d). P2Y2 receptors were only expressed in viable cells and not in the keratinised hair shaft or the central medulla (Fig. 3d). Involucrin was expressed both in the cortex and IRS but not in the ORS (Fig. 3e,f). P2X5 receptors were expressed in the medulla, IRS and ORS. There was colocalisation of involucrin with P2X5 receptor staining in the IRS and in a few cell layers at the edge of the medulla (Fig. 3e,f). Double labelling of P2X7 receptors with active caspase-3 did not show any immunoreaction within the anagen hair follicle.

Control immunostaining experiments

Both omission of the primary antibody and preabsorption with corresponding peptides were performed as controls. The immunoreaction was abolished after preabsorption of the P2X5 (Fig. 1e), P2X7 (Fig. 1f), P2Y1 (Fig. 2e) or P2Y2 (Fig. 2f)
antibody (Fig. 2f) with the corresponding peptides, confirming the specificity of the immunoreaction.

Discussion

In this study, we have obtained using immunohistochemistry the first direct evidence for the expression of P2Y1, P2Y2 and P2X5 receptors in human anagen hair follicles. Double labelling of P2Y1 receptors with proliferation markers Ki-67 and PCNA showed that P2Y1 receptors were found in proliferating cells in the ORS and bulb in anagen hair follicles. The ORS is established during the early stages of anagen by the downwards migration of the regenerating epithelium and then maintains itself (in contrast to the IRS and hair shaft), independent of the bulbar matrix, by basal cell growth [21]. Thickness and cellularity of the ORS varies with the level of the follicle: it is single-layered near the bulb, higher up it is composed of multilayered cuboidal cells, and from the level of the sebaceous gland upwards it becomes multilayered and is structurally similar to the epidermis [22]. Previous work on normal interfollicular epidermis has shown that P2Y1 receptors show a strong immunopositive signal in the basal layer, and double labelling of P2Y1 receptors with keratinocyte proliferation markers Ki-67 and PCNA in the
interfollicular epidermis confirmed the presence of P2Y1 receptors in proliferating cells [12]. The P2Y1 receptor-selective agonist 2-methylthio ADP caused an increase in the number of human keratinocytes in vitro. It is therefore consistent that P2Y1 receptors would have a proliferative role in the anagen hair follicle ORS.

In anagen hair follicles, P2Y2 receptors were confined to a spatially distinct group of cells in the cortex and at the edge of the medulla, where there was no double labelling with proliferation markers. Commitment to the formation of the three hair shaft cell lineages (medulla, cortex, cuticle) takes place in a subcompartment of the hair matrix known as the precortex [1]. P2Y2 receptors seemed to be expressed on early differentiated cells in the hair shaft, because they were found on living cells of the cortex but not in the differentiated hair cuticle. Previous work on normal interfollicular epidermis has shown that P2Y2 receptors are expressed in all cells of the basal layer and in a few cells within the stratum spinosum [12]. Stem cells in undamaged epidermis divide infrequently, and it is the nonstem daughters of stem cells, i.e. transit amplifying cells, which are actively dividing [1]. Double labelling of P2Y2 receptors with keratinocyte proliferation markers Ki-67 and PCNA in the interfollicular epidermis confirmed the presence of P2Y2 receptors in proliferating cells [12]. The P2Y2 receptor agonist uridine 5’-triphosphate also caused significant proliferation in keratinocytes [11, 12] and HaCaT keratinocytes [23]. P2Y2 receptors stained all the different cell populations within the basal layer of the epidermis (i.e. stem cells, transit amplifying cells and postmitotic cells). P2Y2 receptors may then cause proliferation in transit-amplifying cells in the epidermis. Although

![Fig. 2 Expression of P2Y1 and P2Y2 receptors in human anagen hair follicles.](image)
Fig. 3  Double labelling of P2Y₁ and P2Y₂ receptors with markers for cellular proliferation, and double labelling of P2X₅ receptors with markers for keratinocyte differentiation in anagen hair follicles. a Double labelling of P2Y₁ receptors (red) with Ki-67, a nuclear marker for proliferating cells (green), to show that P2Y₁ receptors are found in proliferating basal cells in the outer root sheath (ORS) and bulb region of the hair follicle in longitudinal section. Scale bar=50 μm. b Transverse section: double labelling of P2Y₁ receptors (red) with Ki-67, a nuclear marker (green), to show that P2Y₁ receptors are found in proliferating cells in the outer root sheath (ORS) of the hair follicle. Scale bar=50 μm. c Longitudinal section of anagen hair follicle through the dermal papilla (DP); double labelling of P2Y₂ receptors (red) with proliferating cell nuclear antigen (PCNA), a marker for proliferating cells (green), showed that P2Y₂ receptors were found in the cortex (CTX) and at the edge of the medulla (M) but not in the central medulla. P2Y₂ receptors were not found in the matrix (Ma), where cells were positive for PCNA. Scale bar=75 μm. d Longitudinal section of anagen hair follicle: P2Y₂ receptors were absent from the keratinised cuticle (Cu) of the hair shaft. PCNA was also found in cells of the outer root sheath (ORS). Scale bar=75 μm. e Double labelling of P2X₅ receptors (red-brown) with involucrin, a marker for differentiating cells (green). Involucrin was expressed both in the inner root sheath (IRS), cortex (CTX) and in the outermost edge of the medulla (M). P2X₅ receptors were expressed in the inner (IRS) and outer root sheaths (ORS) and in the medulla (M) and matrix cells (Ma). There was yellow colocalisation with P2X₅ receptors in the inner root sheath and in cells at the outermost edge of the medulla (arrow). The cortex only stained positive for involucrin, not P2X₅ receptors. Scale bar=50 μm. f Transverse section: double labelling of P2X₅ receptors (red) with involucrin (green). Involucrin was expressed in the inner root sheath (IRS) and cortex (CTX) and colocalised (yellow) with P2X₅ receptor staining in the inner root sheath (IRS). Scale bar=50 μm.
it is convenient to think of stem and transit-amplifying cells as discrete populations from fully differentiated keratinocytes, there might be gradients of cell behaviour, ranging from a cell that has maximum self-renewal and zero differentiation capacity to one that has completed terminal differentiation and cannot divide again. Transit-amplifying cells would lie in the middle of each gradient [1]. In anagen hair follicles, P2Y2 receptors are expressed on early differentiated hair keratinocytes that contribute to the formation of the hair shaft. Cells positive for P2Y2 receptors in hair follicles could still have some proliferative capacity given the right stimulus.

In anagen hair follicles, P2X5 receptors were found on cells in the IRS, ORS and medulla. P2X5 receptors are expressed in the basal layer of the interfollicular epidermis but are expressed more strongly in the stratum spinosum and variably into the stratum granulosum [12]. The expression of P2X5 receptors in the IRS was similar to that seen in the epidermis, with basal cells labelling less strongly than suprabasal cells. It has been proposed that P2X5 receptors are involved in human epidermal keratinocyte differentiation [10, 12]. There is evidence from other tissues regarding the role of P2X5 receptors. In foetal rat skeletal muscle, P2X5 receptors are sequentially expressed during development [24] and associated with differentiating cells [25]. P2X5 receptors have also been implicated in the regulation of osteoblastic differentiation and proliferation [26]. Involutin is a marker of keratinocyte differentiation [27], which is found in the epidermis in the upper stratum spinosum and in the stratum granulosum. In the hair follicle, involucrin is expressed in the hair cortex, medulla, IRS and the innermost cells of the lower ORS [28]. Involutin colocalised with P2X5 receptor staining in the IRS and in a few cell layers at the edge of the medulla. This suggests a role for P2X5 receptors in the differentiation of cells of the medulla and IRS.

There was no staining with antibodies to P2X7 receptors or active caspase-3 in the anagen hair follicle. Further work on the expression of P2X7 receptors in different stages of the hair cycle would be of interest. Periods of hair growth (anagen) are followed by a regression phase (catagen), when the hair follicle undergoes programmed cell death [2], and a resting phase (telogen), before onset of a new growth phase. In this study, only hair follicles in the anagen phase were studied, as hair follicles in catagen or telogen phases of the hair cycle were not found in the sections analysed. At any one time, 84% of scalp hairs are in anagen, 2% in catagen and 14% in telogen [29]. The P2X7 receptor is unlike other P2X receptors because it is a bifunctional molecule that, in addition to forming a channel permeable to small cations, can be triggered to form a cytolytic pore permeable to large hydrophilic molecules up to 900 daltons [30]. The opening of this pore results in the increase in intracellular cytosolic free calcium ions and the induction of cell death [31, 32]. P2X7 receptors are also found on dendritic cells, macrophages and microglial cells, where extracellular ATP can trigger apoptosis via these receptors. There is increasing evidence that this process is dependent on the caspase signalling cascade [33, 34]. It would be of interest to see whether P2X7 receptors are involved in the process of programmed cell death that occurs during the catagen phase of the hair cycle.

In summary, P2 purinergic receptors are likely to be involved in ORS cell proliferation via P2Y1 receptors found in the bulb and ORS; IRS and medulla differentiation via activation of P2X5 receptors; and early differentiation of cells of the cortex and medulla of the hair shaft via activation of P2Y2 receptors. Expression of P2 purinergic receptors in the anagen hair follicle may have therapeutic implications for treating hair loss. The expression of P2 receptors in different cell lineages of the anagen hair follicle could provide the prospect of engineering hair follicles in those with hair loss or excess growth by using purinergic receptor agonists and antagonists. Lack of growth reflects follicular dynamics and represents the central mechanisms of most common causes of alopecia. In androgenetic alopecia, the hair-shaft diameter is reduced, and there is a progressive decrease in anagen hair percentage, as well as a significant reduction in proliferating cells [35]. The anagen phase is also shorter [36]. This results in the formation of progressively thinner and shorter hair. In some men, there is also prolongation of the interval between shedding of the club hair in telogen and the emergence of a replacement hair in the anagen phase, giving rise to an increase in the number of “empty” follicles [37]. Functional work on hair-follicle cycling, cell proliferation and differentiation using purinergic receptor agonists and antagonists may lead to new approaches in the treatment of hair loss.

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