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$P2X_5$ and $P2X_7$ receptors in human warts and CIN 612 organotypic raft cultures of human papillomavirus infected keratinocytes

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Abstract

Purinergic receptors, which bind adenosine 5′-triphosphate (ATP), are expressed on human cutaneous keratinocytes and in squamous cell carcinomas. Studies on normal human epidermis and primary keratinocyte cultures have suggested that $P2X_5$ receptors are likely to be involved in keratinocyte differentiation and $P2X_7$ receptors are likely to be part of the machinery of end stage terminal differentiation/apoptosis of keratinocytes. $P2X_7$ receptor agonists can significantly reduce primary keratinocyte cell numbers in culture. Human papillomaviruses are increasingly recognised as important human carcinogens in the development of non-melanoma skin cancers. In our study, immunohistochemical analysis for $P2X_5$ and $P2X_7$ receptors was performed on paraffin sections of normal human skin, warts, raft cultures of normal human keratinocytes and raft cultures of CIN 612 cells, a model of keratinocytes infected with human papillomavirus type 31. In warts there was up-regulation of the expression of $P2X_5$ receptors. A similar pattern was seen in the CIN 612 raft cultures. Both $P2X_5$ and $P2X_7$ receptors were found in the nuclei of koilocytes, abnormal keratinocytes characteristic of human papillomavirus infection. $P2X_5$ and $P2X_7$ receptors may provide a new focus for therapeutic research into treatments for warts because these receptors can induce cell differentiation and cell death.

Abbreviations: ADP – adenosine 5′-diphosphate; ATP – adenosine 5′-triphosphate; BCC – basal cell carcinoma; DAB – diaminobenzidine; GFP – green fluorescent protein; HPV – human papillomavirus; NHEK – normal human epidermal keratinocytes; SCC – squamous cell carcinoma

Introduction

Non-melanoma skin cancer is the most frequently occurring malignancy worldwide in the Caucasian population [1]. Ultraviolet radiation is the major environmental factor in the pathogenesis of basal cell carcinomas (BCC) and squamous cell carcinomas (SCC), which tend to occur in sun-exposed sites [2]. The ratio of BCCs to SCCs is 5:1 in immunocompetent populations, whereas in immunosuppressed patients the ratio is reversed, with the risk of developing a SCC up to 250 times greater and the risk of developing a BCC 10 times greater than that in the general population [3]. The difference in incidence of these tumours in immunocompromised patients is thought to be due to the involvement of papillomaviruses. The association between warts and skin cancer was first noted in renal transplant recipients [4]. Human papillomaviruses (HPVs) are small double-stranded DNA viruses that are widespread in the human population. They are strictly epitheliotropic and infect only cutaneous and mucosal skin sites. Over 120 different HPV types have been identified, of which 80 have been characterised in full [3].

There is increasing evidence that purinergic signalling can have long-term, trophic effects in cell growth, proliferation, differentiation and death [5, 6]. Purinergic receptors are classified into two groups: P1 receptors are selective for adenosine and P2 receptors are selective for adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP), which act as extracellular signalling molecules [7]. P2 receptors are sub-divided into P2X and P2Y receptor families [8, 9]. P2X receptors are ligand-gated ion channels, and are activated by extracellular ATP to elicit a flow of cations (Na^+, K^+ and Ca^{2+}) across the plasma membrane. Seven subtypes of P2X receptors are recognised [10]. In contrast, P2Y receptors are G protein-coupled and eight subtypes of P2Y receptors have been described [11]. P2X receptors are largely viewed as mediators of short term, fast intercellular communication.

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Recent studies suggest that P2X receptors could also mediate trophic effects. $P2X_5$ receptors have been implicated in the regulation of osteoblastic differentiation and proliferation [12], and triggering the differentiation of skeletal muscle satellite cells [13]. $P2X_7$ receptors have been shown to mediate ATP-induced apoptosis [14, 15]. ATP is likely to be an important local messenger in the epidermis. Both $P2X_5$ and $P2X_7$ receptors are expressed on adult rat cutaneous keratinocytes and functional roles in the regulation of cell turnover have been proposed [16]. Studies on adult human epidermis and primary keratinocyte cultures [17] have suggested that $P2X_5$ receptors are likely to be involved in early keratinocyte differentiation and $P2X_7$ receptors are likely to be part of the machinery of end stage terminal differentiation/apoptosis of keratinocytes. $P2X_5$ and $P2X_7$ receptors have altered expression in both BCCs and in SCCs and functional experiments using a cutaneous squamous cell carcinoma cell line, A431, have shown that purinergic receptors could in future provide novel therapeutic targets in non-melanoma skin cancers [18]. $P2X_5$ receptors are also involved in the differentiation of human fetal epidermis [19] and in rat wound healing [20].

This study compares the distribution of $P2X_5$ and $P2X_7$ receptors in normal human skin, with that in human warts. Vegetative reproduction of HPV particles can only take place in highly differentiated keratinocytes. This has made it difficult to study the effects of HPV *in vitro*. We studied the distribution of $P2X_5$ and $P2X_7$ receptors in HPV infected keratinocytes grown as an organotypic raft culture, where the cells are permitted to differentiate and stratify. These receptors may be a useful target for further research into both HPV and the carcinogenesis of cutaneous SCCs using organotypic raft cultures of HPV infected keratinocytes as a research tool.

Materials and methods

Tissues

Paraffin sections of samples of normal skin, human warts, normal human epidermal keratinocyte (NHEK) raft cultures and CIN 612 (HPV 31 infected cell line) raft cultures were stained for $P2X_5$ and $P2X_7$ receptors. Ethics Committee Approval was obtained to harvest human skin samples. Paraffin blocks of warts, NHEK raft cultures and CIN 612 raft cultures were prepared by Roche Discovery, Welwyn. Six samples of each were used in the study, with at least four sections of each sample. $P2X_5$ and $P2X_7$ receptors were examined because they are involved in early keratinocyte differentiation and terminal keratinocyte differentiation/apoptosis respectively.

Raft cultures of differentiated HPV-infected keratinocytes

The method for raft culture of keratinocytes has already been described [21]. Briefly, primary human foreskin keratinocytes (NHEKs) were obtained from Clonetics (San Diego, USA), the CIN 612 cell line was established from a CIN I biopsy and contained HPV31b DNA. Epithelial cells were seeded onto collagen matrices containing J2 3T3 fibroblast feeders. When the epithelial cells had grown to confluence, collagen matrices were lifted onto stainless steel grids and the cells were fed by diffusion from under the matrix. The cells were allowed to stratify and differentiate at the air–liquid interface over a 16-day period. Raft cultures were then harvested, fixed in 4% paraformaldehyde and embedded in paraffin.

Antibodies

The immunogens used for production of polyclonal $P2X_5$ and $P2X_7$ 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 previously described [16, 22]. $P2X_5$ and $P2X_7$ antibodies were kept frozen at a stock concentration of 1 mg/ml and used at a dilution of 1:200.

Immunohistochemical method for paraffin sections

The method below was an adaptation of the routine method used for immunohistochemistry in paraffin sections at RAFT and was developed by Elizabeth Clayton, Histology Department, RAFT. The method is described in detail in [18]. Briefly, Microwave antigen retrieval was used for the visualization of both $P2X_5$ and $P2X_7$ receptors in 4 μm paraffin sections. $P2X_5$ receptors were demonstrated via tyramide amplification and a diaminobenzidine (DAB) final substrate system, so that receptors were stained brown. $P2X_7$ receptors were demonstrated using a routine Streptavidin Alkaline Phosphatase method and a Vector Red final substrate system (Vector Laboratories, Peterborough, UK), so that receptors were stained pink. Nuclei were counterstained blue with Harris′s haematoxylin. Control experiments were carried out with the primary antibody omitted from the staining procedure. From previous work in frozen sections, there was no staining in both the no primary controls and upon pre-absorption of the primary antibody with the corresponding peptide [17].

Photography

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

Results

$P2X_5$ and $P2X_7$ receptors in paraffin sections of normal human skin

$P2X_5$ immunoreactivity was present mainly in the viable cell layers of normal human skin (Figure 1a), where the

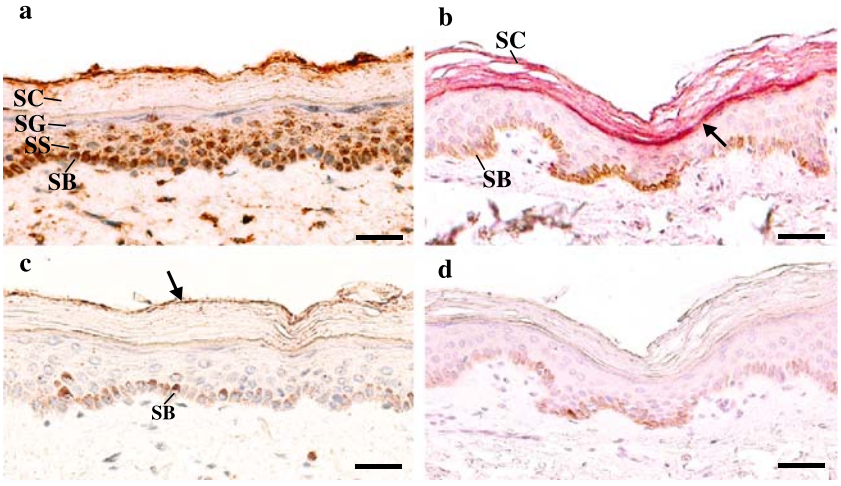

Figure 1. Expression of $P2X_5$ and $P2X_7$ receptors in paraffin sections of normal human skin. Nuclei were counterstained blue with haematoxylin. **a** In normal skin, $P2X_5$ immunoreactivity (*brown*) was found in basal keratinocytes (B) (although this was not easy to distinguish from melanin), and in the stratum spinosum (SS), and in very few cells in the stratum granulosum (SG). $P2X_5$ receptor staining was absent from the stratum corneum (SC), apart from at the outer edge. $P2X_5$ receptor staining was confined largely to the cell membranes in the basal layer, and found in the cytoplasm, and occasionally in the nucleus in both basal and suprabasal keratinocytes. *Scale bar.* 25 μm. **b** $P2X_7$ immunoreactivity (*pink*) was present in the epidermis of all normal skin samples, and was associated with cells and cell fragments (*arrow*) in the stratum corneum (SC). Note the brown melanin in the basal layer (B). *Scale bar*, 25 μm. **c** There was residual staining of the outermost edge of the stratum corneum (*arrow*) with the $P2X_5$ receptor antibody no primary control, and therefore this was non-specific staining. Note the brown melanin in the basal layer (B). *Scale bar*, 25 μm. **d** There was no staining in the no primary control for the $P2X_7$ receptor antibody *Scale bar.* 25 μm.

staining was confined largely to the cell membranes and the cytoplasm in epidermal keratinocytes, with occasional nuclear staining. $P2X_7$ immunoreactivity was present in the epidermis of all normal skin samples, and was associated with cells and cell fragments in the stratum corneum (Figure 1b). There was some staining of the outermost edge of the stratum corneum with the $P2X_5$ receptor antibody, which was only slightly reduced in the no primary antibody control (Figure 1c), and therefore non-specific staining. There was no staining in the no primary control for the $P2X_7$ receptor antibody (Figure 1d).

$P2X_5$ and $P2X_7$ receptors in paraffin sections of human warts

In warts, there was marked hyperkeratosis and parakeratosis within the stratum corneum. $P2X_5$ receptors were present in nucleated keratinocytes in areas of parakeratosis, but not within the hyperkeratotic areas of the stratum corneum (Figure 2a). In contrast, $P2X_7$ receptors were found in hyperkeratotic areas of the stratum corneum but not in parakeratotic areas of the wart (Figure 2b). $P2X_5$ immunoreactivity was present in the majority of wart keratinocytes (Figure 2c), but few cells in the basal layer were positive, with most of the positively stained cells in the suprabasal layers. $P2X_7$ receptors were found in the nuclei of suprabasal cells (Figure 2d). There was a prominent granular layer in the wart with koilocytes. Koilocytes are the characteristic cytological feature of HPV infection. Koilocytes are keratinocytes with pyknotic, deeply blue nuclei surrounded by a halo and clear cytoplasm with a paucity of keratohyaline granules. They usually indicate the presence of human papilloma virus. Both $P2X_5$ and $P2X_7$ receptors were found in the nuclei of koilocytes (Figure 2c,d). There was a band of heavy staining of the outermost edge of the stratum corneum with the $P2X_5$ receptor antibody (Figure 2a), which was still present in the no primary antibody control (Figure 2e), and therefore was non-specific staining. There was no staining in the no primary control for the $P2X_7$ receptor antibody.

$P2X_5$ and $P2X_7$ receptors in paraffin sections of raft cultures of normal human keratinocytes and of CIN 612 (HPV 31) cells

$P2X_5$ immunoreactivity was present throughout all the layers of the raft cultures of normal human foreskin keratinocytes (Figure 3a), where the staining was confined largely to the cell membranes and the cytoplasm. $P2X_7$ immunoreactivity was present in the raft cultures of normal human foreskin keratinocytes, staining weakly within the uppermost layer (rudimentary stratum corneum) (Figure 3b). The $P2X_7$ receptor staining was not as strikingly positive as with the paraffin section of normal skin (Figure 1b).

$P2X_5$ immunoreactivity was present in the CIN 612 (HPV 31) raft keratinocytes (Figure 3c), where the staining was seen within all layers of the raft. $P2X_7$ immunoreactivity was present in the CIN 612 raft (Figure 3d) and was associated with the cell cytoplasm in the HPV infected cells. At higher power, the uppermost layers are highly disorganised, with nucleated cells at the surface of the raft (Figure 3e,f). There was positive staining in the cytoplasm of mitotic cells within the raft for both $P2X_5$ (Figure 3e) and $P2X_7$ (Figure 3f) receptors.

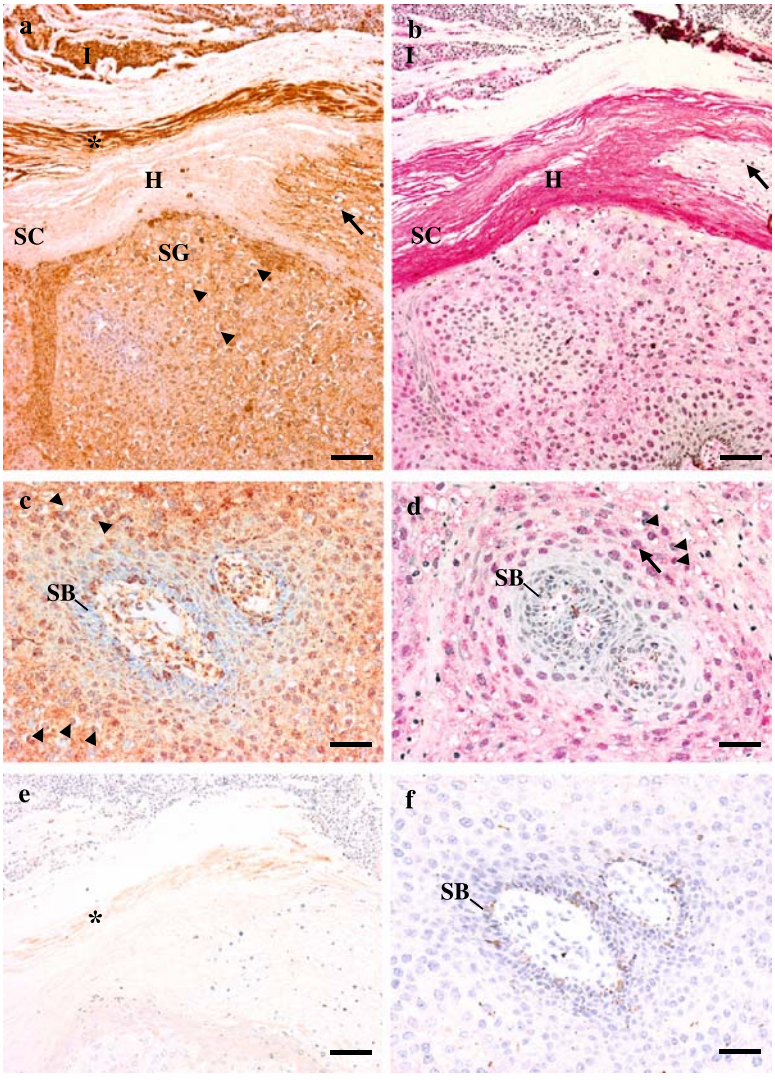

Figure 2. Expression of $P2X_5$ and $P2X_7$ receptors in paraffin sections of human warts. Nuclei were counterstained blue with haematoxylin. **a** Low power view of $P2X_5$ immunoreactivity (*brown*) in the wart. $P2X_5$ receptors were present within the keratinocytes of the wart. There was marked hyperkeratosis (H), which was negative for $P2X_5$ receptors, although areas of parakeratosis were positive (*arrow*). At the outer edge of the stratum corneum there was a band of heavy staining (*asterisk*). $P2X_5$ receptors were also found in the inflammatory cell infiltrate (I) above the stratum corneum (SC). There was a prominent granular layer (SG), within which cells (koilocytes) showed typical cytoplasmic vacuolation (*arrowheads*). *Scale bar.* 100 μm. **b** Low power view of $P2X_7$ immunoreactivity (*pink*) in the wart. $P2X_7$ receptors were strongly present within the hyperkeratotic (H) areas of the stratum corneum (SC), but not in areas of parakeratosis (*arrow*). $P2X_7$ receptors were weakly present in the wart keratinocytes, and mainly found in the nucleus. $P2X_7$ receptors were also weakly found in the inflammatory cell infiltrate (I) above the stratum corneum. *Scale bar*, 100 μm. **c** High power view of $P2X_5$ immunoreactivity (*brown*) in wart keratinocytes. There were a few positive cells in the basal layer (B), but most of the positively stained cells were in the suprabasal layers. Koilocytes showed $P2X_5$ receptor staining in the nucleus (*arrowheads*). *Scale bar*, 50 μm. **d** $P2X_7$ immunoreactivity (*pink*) was present in the suprabasal layers of the wart, in either large, flat nuclei with an obvious nuclear membrane (*arrow*), or in koilocytes, where the receptor was prominent in shrunken, pynotic nuclei, (*arrowheads*). $P2X_7$ receptors were not found in the basal layer (B) of the wart. *Scale bar*, 50 μm. **e** There was residual staining of the outermost edge of the stratum corneum (*asterisk*) with the $P2X_5$ receptor antibody no primary control, and therefore this was non-specific staining. *Scale bar*, 100 μm. **f** There was no staining in keratinocytes of the wart with the $P2X_5$ receptor antibody no primary control. There was some melanin in the basal layer (B) of the wart. *Scale bar*, 50 μm.

Discussion

This paper adds extra understanding of the role of $P2X_5$ and $P2X_7$ receptors in keratinocytes differentiating under abnormal circumstances, for example during human papilloma virus infection. Human papilloma viruses are increasingly recognised as an important human carcinogen and have been implicated in non-melanoma skin cancers [23, 24]. The association between skin warts and skin cancer was first noted in renal transplant recipients [4] who have a marked increase in susceptibility to both viral warts and non-melanoma skin cancer. Clinical and histological features of transplant SCCs indirectly support the progression of viral warts through increasingly dysplastic squamous lesions to invasive SCCs [25]. $P2X_5$ and $P2X_7$ receptors have altered expression in both BCCs and in SCCs [18]. We examined the expression of these receptors in human papilloma virus infected keratinocytes with the long-term aim of establishing whether they may also have a role as a potential therapeutic target.

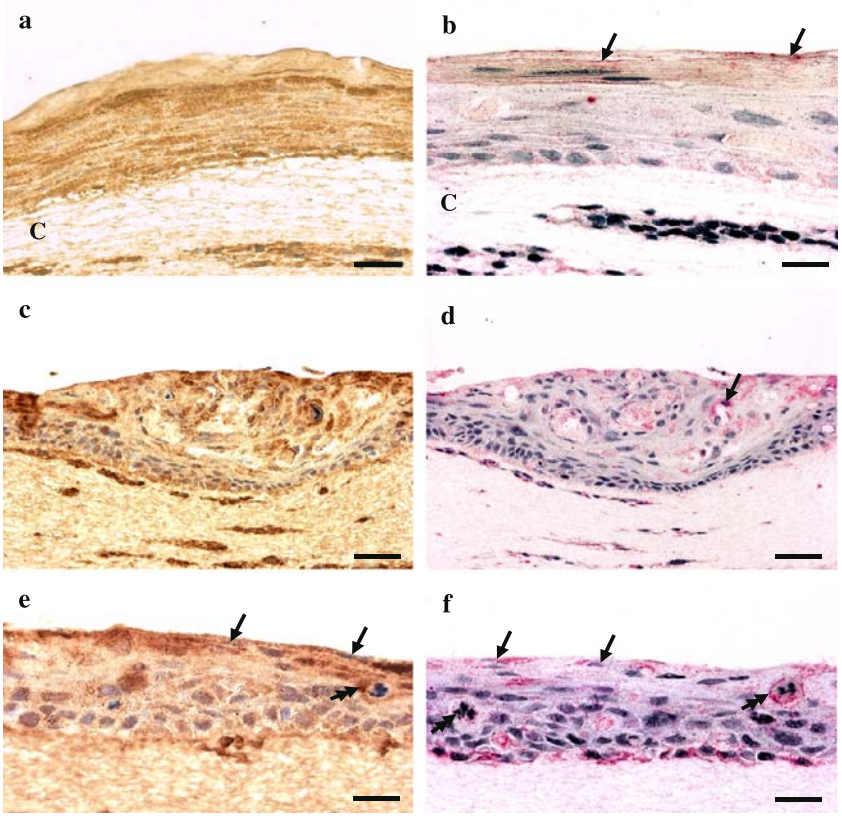

Figure 3. Expression of $P2X_5$ and $P2X_7$ receptors in paraffin sections of raft cultures of normal human keratinocytes and of CIN 612 (HPV 31) cells. Nuclei were counterstained blue with haematoxylin. **a** $P2X_5$ immunoreactivity (*brown*) was present throughout all layers of the raft cultures of normal human foreskin keratinocytes, where the staining was confined largely to the cell membranes and the cytoplasm. The raft culture was supported on a collagen matrix (C). *Scale bar.* 25 μm. **b** $P2X_7$ immunoreactivity (*pink*) was present in the raft cultures of normal human foreskin keratinocytes, staining weakly within the uppermost layer (*arrows*). *Scale bar.* 25 μm. **c** $P2X_5$ immunoreactivity (*brown*) was present in the CIN 612 (HPV 31) raft keratinocytes, staining all layers of the raft. *Scale bar.* 50 μm. **d** $P2X_7$ immunoreactivity (*pink*) was present in the CIN 612 raft and was associated with the cell cytoplasm and nucleus (*arrow*). *Scale bar.* 50 μm. **e**, **f** High power views of CIN 612 (HPV 31) raft cultures: the uppermost layers are highly disorganised, with nucleated cells at the surface of the raft (*arrows*). There was also positive staining in the cytoplasm of mitotic cells (double arrows) within the raft for both **e** $P2X_5$ receptors (*brown*) *Scale bar* 25 μm. and **f** $P2X_7$ receptors (*pink*). *Scale bar.* 25 μm.

In this paper changes in $P2X_5$ and $P2X_7$ receptor expression were examined within human papilloma virus infected differentiated keratinocytes in both warts and in a model system. The organotypic raft model was chosen because it would allow an *in vitro* model of both the normal epidermis and of a wart that could later be compared and manipulated with drugs. This model allows keratinocytes to differentiate and stratify at an air fluid interface. Since human papilloma virus replication tends to take place in differentiated keratinocytes, this model has the advantage of allowing us to study differentiated cells, which would be much harder to do with monolayer keratinocyte culture systems. Previous work has proposed that $P2X_5$ receptors are involved in early differentiation of keratinocytes and that $P2X_7$ receptors are involved in the terminal differentiation of keratinocytes [17]. The draw back of the organotypic raft culture model is that the keratinocytes only differentiate to form a rudimentary stratum corneum, making it harder to study the expression of $P2X_7$ receptors.

In this study, we obtained the first direct evidence for the expression of $P2X_5$ and $P2X_7$ receptors in human warts, using immunohistochemistry. Studies on normal human epidermis and functional studies on primary keratinocyte cultures [17] have suggested that $P2X_5$ receptors are likely to be involved in keratinocyte differentiation and $P2X_7$ receptors are likely to be part of the machinery of end stage terminal differentiation of keratinocytes. 2′- and 3′-0-(4-Benzoyl–benzoyl) ATP, a potent $P2X_7$ receptor agonist, causes a significant decrease in cell number via a direct effect on $P2X_7$ receptors [17], which are also involved in mediating apoptosis [26, 27].

In normal skin, $P2X_5$ receptors were found in the basal layer, stratum spinosum and weakly in the stratum granulosum, with occasional nuclear staining. There was little or no $P2X_5$ receptor staining in the stratum corneum, apart from some staining artefact. In the raft cultures of normal human foreskin keratinocytes, $P2X_5$ receptors were found throughout all layers, from the basal layer to the most differentiated layer. Warts arise because human papillomaviruses infect the basal keratinocyte of the epidermis, presumably through disruptions of the skin or mucosal surface [28]. The virus remains latent in basal cells as a circular episome. As keratinocytes differentiate and migrate to the surface, the virus is triggered to undergo replication and maturation. Hybridisation studies *in situ* of

HPV lesions have shown that viral DNA synthesis occurs in the skin in the superficial stratum spinosum and full virus assembly with capsid production occurs in the stratum granulosum. In warts there is a prominent granular cell layer, within which there are vacuolated cells called koilocytes, characteristic of HPV infection. The process of virus replication alters the character of the epidermis, resulting in cutaneous or mucosal excrescences known as warts. In warts, $P2X_5$ receptor staining was increased compared to that in normal skin. There were few $P2X_5$ receptor positive cells in the basal layer, most of the positively stained cells being in the suprabasal layers.

HPV infections are classified into cutaneous, cutaneous involved in epidermodysplasia verruciformis, cutaneous and mucosal, and mucosal of low and high risk [29]. HPVs are linked with cervical cancer and SCC of the anus [30], which are associated with high risk genital HPV types 16, 18, 31, 33 and the low risk genital HPV types 6 and 11 [31]. Examination of immunostaining of $P2X_5$ and $P2X_7$ receptors on paraffin sections of cervical epithelium have shown that $P2X_5$ receptors are expressed in the suprabasal, differentiated layers of the epithelium, but not in the basal layer. $P2X_7$ receptor immunostaining was weakly present in the terminally differentiated cells of this non-keratinised epithelium. So these receptors are expressed in cervical cells. In the CIN 612 (HPV 31) raft cultures, $P2X_5$ receptors were found in all cell layers and the level of staining was more intense than in the normal keratinocyte raft cultures.

Hyperproliferation is a feature of warts. Immunohistochemical labelling of frozen sections of other hyperproliferative lesions e.g. psoriasis, with $P2X_5$ receptor antibodies has shown increased expression of the receptor in hyperproliferative areas of the epidermis. In psoriasis, rapid proliferation of keratinocytes leads to the production of immature keratin at the surface that has not completed its terminal differentiation process. This shows as silvery scaly psoriatic plaques on the skin surface. The receptor is again more prominent in suprabasal layers of the epidermis in differentiating and differentiated keratinocytes. This would suggest that this receptor is part of the differentiation process rather than part of a proliferative process. Interestingly a high prevalence of HPV DNA has also been found in psoriasis [32, 33].

$P2X_5$ receptors have also been implicated in the regulation of osteoblastic differentiation and proliferation [12], and triggering the differentiation of skeletal muscle satellite cells [13]. In fetal rat skeletal muscle, $P2X_5$ receptors are sequentially expressed during development [34]. $P2X_5$ receptors are also involved in the differentiation of the human fetal epidermis [19] and play a role in wound healing in the rat epidermis [20].

In normal human skin, $P2X_7$ receptor immunoreactivity was solely associated with cells and cell fragments within the stratum corneum [17]. In the raft cultures $P2X_7$ receptor staining was very weak compared to normal skin. This may be due to the phenomenon of incomplete differentiation that occurs in raft cultures, thought to be associated with the presence of retinoids in the medium [35]. In warts, $P2X_7$ immunoreactivity was associated with hyperkeratotic areas of the stratum corneum, as well as in nuclei of koilocytes in the suprabasal layers. The nuclei that were positive for $P2X_7$ receptors were not normal: nuclei were shrunken, with much more intense, pink $P2X_7$ receptor staining. In the CIN 612 raft cultures, $P2X_7$ immunoreactivity was positive in both the cell cytoplasm and in the nucleus of HPV infected cells. The $P2X_7$ receptor is a bifunctional molecule that can be triggered to act as a channel, permeable to small cations, or on prolonged stimulation form a cytolytic pore permeable to large hydrophilic molecules up to 900 Da [36]. The opening of this pore results in the increase in intracellular cytosolic free calcium ions and the induction of cell death [26, 27]. It is possible that the presence of $P2X_7$ receptors in the nucleus of HPV infected cells indicates a severe disruption of the cellular machinery. It might be possible to use $P2X_7$ receptor agonists to trigger apoptosis in these virally infected cells. $P2X_7$ receptors are also found on dendritic cells, macrophages and microglial cells, where extracellular ATP can trigger apoptosis via these receptors and there is increasing evidence that this process is dependent on the caspase signalling cascade [14, 37].

In summary, $P2X_5$ and $P2X_7$ receptors may provide a useful focus for more research into new treatment modalities for warts and SCCs because these receptors can induce cell differentiation as well as cell death. Vegetative reproduction of HPV particles can only take place in highly differentiated keratinocytes. Raft cultures of both normal human keratinocytes and HPV infected cells could prove to be a useful tool for further study of these receptors *in vitro*.

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