Expression of Purinergic Receptors in Non-melanoma Skin Cancers and Their Functional Roles in A431 Cells

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We investigated the use of purinergic receptors as a new treatment modality for nonmelanoma skin cancers. Purinergic receptors, which bind adenosine 5′-triphosphate, are expressed on human cutaneous keratinocytes. Previous work in rat and human epidermis suggested functional roles for purinergic receptors in the regulation of proliferation, differentiation, and apoptosis. Immunohistochemical analysis of frozen sections in human basal cell carcinomas and squamous cell carcinomas for $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors was performed, accompanied by detailed analysis of archive material of tumor subtypes in paraffin sections. Functional studies were performed using a human cutaneous squamous cell carcinoma cell line (A431), where purinergic receptor subtype agonists were applied to cells and changes in cell number were quantified via a colorimetric assay. Immunostaining in paraffin sections was essentially the same as that in frozen sections, although more detail of the subcellular composition was visible. $P2X_5$ and $P2Y_2$ receptors were heavily expressed in basal cell carcinomas and squamous cell carcinomas. $P2X_7$ receptors were expressed in the necrotic center of nodular basal cell carcinomas and in apoptotic cells in superficial multifocal and infiltrative basal cell carcinomas, and squamous cell carcinomas. $P2Y_1$ receptors were only expressed in the stroma surrounding tumors. $P2Y_4$ receptors were found in basal cell carcinomas but not in squamous cell carcinomas. $P2X_5$ receptors appear to be associated with differentiation. The $P2X_7$ receptor agonist benzoylbenzoyl-adenosine 5′-triphosphate and high concentrations of adenosine 5′-triphosphate (1000–5000 μM) caused a significant reduction in A431 cell number ($p < 0.001$), whereas the $P2Y_2$ receptor agonist uridine 5′-triphosphate caused a significant amount of proliferation ($p < 0.001$). We have demonstrated that non-melanoma skin cancers express functional purinergic receptors and that $P2X_7$ receptor agonists significantly reduce cell numbers *in vitro*. *Key words: adenosine triphosphate/apoptosis/basal cell/carcinoma/carcinoma/purinergic P2/receptors/squamous cell. J Invest Dermatol 121:315–327, 2003*

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) of the skin are the commonest malignancies in White people, accounting for more than 95% of non-melanoma skin cancers (NMSC) (Miller and Weinstock, 1994). The incidence of NMSC has risen significantly over the last 10 y (Holme *et al*, 2000). An estimated 2.75 million cases of NMSC are diagnosed per year worldwide (Strom and Yamamura, 1997), with approximately one million cases per year diagnosed in the USA (Miller and Weinstock, 1994). Multiple treatment modalities are available for NMSC, and surgical management is the most common approach. In patients with multiple lesions or in cases of tumors on difficult locations (e.g., eyelids or lips), surgery can cause significant disfigurement. Topical drug treatment is indicated for superficial cutaneous neoplasms, which because of their multiplicity and involvement of large surfaces are difficult to treat with other methods. The purpose of this study was to investigate whether purinergic receptors could represent a suitable target for drug treatment of NMSC.

There is increasing evidence that purinergic signaling can have long-term, trophic effects in embryonic development, cell growth, differentiation, and proliferation (Abbracchio and Burnstock, 1998; Burnstock, 2001, 2002). 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 signaling molecules (Burnstock, 1978). P2 receptors are divided into two main families: P2X and P2Y, based on molecular structure, transduction mechanisms and pharmacologic properties (Abbracchio and Burnstock, 1994). P2Y receptors are G-protein-coupled and the principal signal transduction pathway

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Abbreviations: αβMEATP, α, β-methylene ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-(3-thiotriphosphate); ADP, adenosine 5′-diphosphate; BCC, basal cell carcinoma; BzATP, 2′- 3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate; 2MeSADP, 2-methylthioadenosine 5′-diphosphate; PCNA, proliferating cell nuclear antigen; SCC, squamous cell carcinoma; TUNEL, TdT-mediated dUTP nick end labeling; UTP, uridine 5′-triphosphate

involves phospholipase C, which leads to the formation of inositol 1,4,5-triphosphate (IP_3) and mobilization of intracellular calcium. IP_3 regulates cell growth and DNA replication (Berridge, 1987). Seven subtypes of P2Y receptors have been described so far (King *et al*, 2000; Communi *et al*, 2001). In contrast, 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 recognized (Khakh *et al*, 2001a); all $P2X_{1-7}$ subunits have been cloned from mammalian species and are capable of assembling into homomultimeric or heteromultimeric receptors (Torres *et al*, 1999).

There is growing evidence that ATP may act as an important local messenger in the skin, particularly 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 (Gröschel-Stewart *et al*, 1999). In particular, $P2X_5$ receptors are expressed on cells undergoing proliferation and differentiation, whereas $P2X_7$ receptors are associated with keratinized dead cells. $P2Y_2$ receptors, found in the basal layer of normal epidermis, are claimed to be involved in keratinocyte proliferation (Dixon *et al*, 1999). $P2Y_1$ receptors are thought to be mitogenic in endothelial cells and $P2Y_2$ and $P2Y_4$ receptors stimulate vascular smooth muscle cell proliferation (Burnstock, 2002).

There is evidence that there are functional purinergic receptors in human malignant cell lines, including: MCF-7 breast cancer cells (Wagstaff *et al*, 2000), endometrial cancer cell lines (Katzur *et al*, 1999), prostate carcinoma cells (Janssens and Boeynaems, 2001), colorectal carcinoma cells (Höpfner *et al*, 1998), ovarian cancer cells (Schultze-Mogasu *et al*, 2000), and HL-60 leukemia cells (Conigrave *et al*, 2000).

This study demonstrates the spatially distinct distribution patterns of different P2X and P2Y receptors in human BCC and SCC as well as evidence for the actions of purinergic agonists on cell proliferation and death in a SCC cell line. This suggests a pathophysiologic role for these receptors and raises the possibility that purinergic agonists are putative therapeutic agents.

MATERIALS AND METHODS

Tissues Frozen sections of 10 BCC (five male, five female, mean age 71) and four SCC (two male, two female, mean age 73) were examined in this study. Ethics Committee Approval was obtained to harvest human NMSC samples from consenting patients. The difficulty of obtaining fresh human tissue for frozen sections led to the development of a special method to allow staining of $P2X_5$ and $P2X_7$ receptors in paraffin sections. Paraffin blocks of a BCC and SCC belonging to a collection at Mount Vernon Hospital were examined.

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 $P2X_5$ 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 described previously (Gröschel-Stewart *et al*, 1999, Oglesby *et al*, 1999). The polyclonal antibodies were raised by multiple monthly injections of New Zealand rabbits with the peptides (performed by Research Genetics, Huntsville, Alabama). $P2X_5$ and $P2X_7$ antibodies (provided by Roche Bioscience, Palo Alto, California) were kept frozen at a stock concentration of 1 mg per mL. Polyclonal anti-$P2Y_1$, $P2Y_2$, and $P2Y_4$ receptor antibodies were obtained from Alomone Laboratories (Jerusalem, Israel), and corresponded to the third extracellular loop of the $P2Y_1$ (amino acids 242–258), $P2Y_2$ (amino acids 227–244), and $P2Y_4$ receptor (amino acids 337–350) and were also raised in rabbits. Antibodies were kept frozen at a stock concentration of 0.6 mg per mL ($P2Y_1$, $P2Y_2$) and 0.3 mg per mL ($P2Y_4$). PCNA–(monoclonal anti-proliferating cell nuclear antigen, clone PC10, raised in mouse ascites fluid; Sigma, Poole, UK) is a marker for proliferation (Miyagawa *et al*, 1989). Active caspase-3 is part of the apoptotic machinery of the cell and is expressed in terminally differentiating keratinocytes (Weil *et al*, 1999). Active caspase-3 (Abcam, Cambridge, UK) antibody was raised in rabbit.

Immunohistochemical methods for frozen sections For immunostaining of cryostat sections, the avidin–biotin technique was used according to a revised protocol (Llewellyn-Smith *et al*, 1993). Air-dried sections were fixed for 2 min in 4% formaldehyde in 0.1 M phosphate buffer, containing 0.2% of a saturated solution of picric acid (pH 7.4). Endogenous peroxidase was blocked for 10 min with 50% methanol containing 0.4% hydrogen peroxide. Nonspecific binding sites were blocked by a 20 min preincubation in 10% normal horse serum 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% normal horse serum in PBS + 2.5% sodium chloride (NaCl) at 4°C overnight. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania) diluted to 1:500 in 1% normal horse serum 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 enhancement technique was used to visualize the reaction product. Sections were washed three times with PBS after each of the above steps except after preincubation with 10% normal horse serum. After the last wash, sections were dehydrated twice in isopropanol and mounted with EUKITT (BDH Laboratory Supplies, Poole, UK).

Controls for immunohistochemical method in frozen sections Control experiments were carried out with primary antibodies omitted from the staining procedure or the primary antibodies preabsorbed with the corresponding peptides.

Double labeling of $P2X_7$ receptor antibody with either terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) or anti-human caspase 3 TUNEL was performed using a kit (Boehringer Mannheim, Germany). TUNEL identifies cells undergoing apoptosis by labeling nuclear DNA fragments that have been cleaved during apoptosis (Gavrieli *et al*, 1992). Sections were fixed and incubated with the $P2X_7$ receptor antibody overnight as described above. Then either the TUNEL or the anti-caspase 3 protocol were followed.

For double labeling with TUNEL, sections were washed in PBS and then incubated with the TUNEL reaction mixture for 1 h at 37°C; then for 1 h with biotinylated donkey anti-rabbit antibody at a concentration of 1:500; then for 1 h with streptavidin Texas red (Amersham International Plc, Amersham, UK) at a concentration of 1:200. Sections were washed three times with PBS after each of the above steps and at the end of the staining procedure mounted in Citifluor (Citifluor Ltd, Leicester, UK).

For double labeling with anti-caspase-3, sections were washed in PBS and then 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) diluted to 1:1500 in PBS for 1 h, tyramide amplification for 8 min (Tyramide Amplification Kit, NEN Life Science Products, Boston, Massachusetts) and then streptavidin Texas red, 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 rabbit anti-human active caspase-3 (Abcam). Sections were washed and incubated with the directly labeled secondary antibody, Oregon-green-labeled goat-anti-rabbit IgG (Jackson ImmunoResearch Laboratory), diluted 1:100 for 45 min. Sections were washed and mounted in Citifluor.

Double labeling of $P2Y_2$ receptor antibody with PCNA Sections were fixed and incubated with $P2Y_2$ antibody diluted 1:100 overnight as described above. After a wash step, biotinylated donkey anti-rabbit IgG antibody, diluted 1:500 in 1% normal horse serum in PBS, was then applied for 1 h followed by streptavidin Texas red, diluted 1:200 in PBS-merthiolate for 1 h at room temperature. Sections were preincubated for 30 min with 10% normal goat serum diluted in 0.1 M phosphate buffer, containing 0.05% merthiolate (Sigma), and then incubated for 2 h at room temperature with PCNA antibody (monoclonal anti-proliferating cell nuclear antigen, clone PC10, raised in mouse ascites fluid; Sigma) diluted 1:1000. After a wash step, the directly labeled secondary antibody goat anti-mouse fluorescein isothiocyanate (Nordic Immunological Laboratories, Tilburg, the Netherlands) was applied at a dilution of 1:200 for 1 h and then sections were washed and mounted in Citifluor.

Immunohistochemical method for paraffin sections The method below was an adaptation of the routine method used for immunohistochemistry in paraffin sections at The RAFT Institute, Mount Vernon Hospital, Northwood (Middlesex, UK) and was developed by Elizabeth Clayton, Histology Department, RAFT. Microwave antigen retrieval was used for the visualization of both $P2X_5$ and $P2X_7$ receptors in paraffin sections. Paraffin blocks were sectioned at 4 μm on a Reichert-Jung Microtome, and sections were taken on Snowcoat Extra slides (Surgipath, St Neots, Cambs, UK). The slides were then dried in an oven for 2 h at 60°C. $P2X_7$ receptors were demonstrated using a routine streptavidin alkaline phosphatase method and a Vector Red final substrate system (Vector Laboratories, Peterborough, UK). $P2X_5$ receptors were demonstrated via tyramide amplification and a nickeldiamino-benzidine final substrate system.

All histologic solvents, buffered formal saline, toluene, xylene and alcohol were supplied by Genta Medical (York, UK). Sections were dewaxed and rehydrated using xylene and graded concentrations of industrial methylated spirits, then washed in running tap water for 5 min Sections were microwaved (Daewoo 800 W domestic microwave) at full power in a plastic rack for 10 min in a covered but not closed container filled with 400 mL 1 mM ethylenediamine tetraacetic acid at pH 8.0 and then immediately washed in cold tap water for 2 to 3 min.

Sections were ringed with a wax pen and placed in a humidification chamber for the application of immunoreagents. After incubation with each immunoreagent, all slides were rinsed off with a wash bottle filled with Tris-buffered saline (TBS) containing Tween 20 (two drops Tween per 500 mL of TBS) (TBS + T), placed in a slide rack and put in a fresh bath of TBS + T on a magnetic stirrer for 15 min. This step was crucial in order to reduce background staining.

Method for $P2X_5$ receptor antibody immunostaining in paraffin sections Endogenous peroxidases were blocked using a 3% hydrogen peroxide solution in methanol containing 0.3% sodium azide for 30 min. Sections were washed in running tap water for 2 to 3 min and then placed in a bath of 50 mM TBS (50 mM Tris + 150 mM NaCl pH 7.6). Sections were incubated with one drop of Avidin D blocking solution for 15 min (Avidin Biotin Blocking Kit SP200, Vector Laboratories). This was then flicked off and rinsed with TBS + T. One drop of the Biotin blocking solution was then applied for 15 min (Avidin Biotin Blocking Kit SP200, Vector Laboratories). Sections were washed with TBS + T and 100 μL 1:5 normal swine serum diluted in DAKO ChemMate antibody diluent (DAKO S2022, DAKO, Ely, UK) was then applied to each section for 30 min. Excess swine serum was flicked off the slides and the primary $P2X_5$ receptor antibody (diluted 1:600 in DAKO ChemMate diluent) was applied for 15 min.

The DAKO CSA kit was used for all subsequent layers as per kit instructions (DAKO Catalyzed Serum Amplification system K1500 with biotinylated anti-rabbit secondary antibody (DAKO K1498); DAKO). After primary antibody incubation and washing, sections were incubated with 100 μL biotinylated anti-rabbit antibody (prediluted from the kit) for 15 min (solution 4). After washing with TBS + T, 100 μL of avidin–biotin complex solution (prepared 30 min prior to use (solutions 5–7)) was applied for 15 min. After washing with TBS + T, 100 μL of tyramide amplification solution was applied for 15 min (solution 8). After a wash step with TBS + T, 100 μL of streptavidin-peroxidase reagent was applied for 15 min (solution 9). Following washing with TBS + T, the nickel-diaminobenzidine substrate chromogen was applied for 5 min. $P2X_5$ receptors appeared brown and the intensity of the chromogen was checked under the microscope before washing slides in running tap water.

Method for $P2X_7$ receptor antibody immunostaining in paraffin sections The streptavidin alkaline phosphatase method was used to demonstrate $P2X_7$ receptors in paraffin sections. This method provided staining with a striking contrast for positive and negative reactions, was fluorescent when looking for low level expression and caused no confusion with endogenous pigments.

Endogenous alkaline phosphatase was blocked by a 20 min incubation in a bath of 20% acetic acid solution. Sections were washed and incubated first with avidin D blocking solution (Vector Laboratories), then biotin blocking solution (Vector Laboratories), and then 1:5 normal swine serum as above. One hundred microliters of $P2X_7$ receptor antibody, diluted 1:100 was applied for 1 h at room temperature or overnight at 4°C.

After a wash step with TBS + T, 100 μL of biotinylated anti-rabbit antibody (DAKO E0353), diluted 1:200 in DAKO ChemMate diluent (DAKO), was applied for 30 min. After washing in TBS + T, 100 μL of streptavidin alkaline phosphatase (Vector SA5100) diluted 1:200 in DAKO ChemMate diluent, was applied for 30 min After washing in TBS + T, Vector Red substrate (Vector Alkaline phosphatase substrate, SK5100) made up in 200 mM Tris–HCl (pH 8.2) was applied for 10 min. $P2X_7$ receptor staining appeared bright pink, the intensity of the chromogen was checked under the microscope prior to washing the slides in running tap water for 2 to 3 min.

Counterstaining Nuclei were counterstained blue with Harris hematoxylin for 10 to 30 s and all sections were subsequently dehydrated, cleared, and mounted.

Controls for immunohistochemical method in paraffin sections Negative controls were used: either diluent only or 1:5 normal swine serum were substituted for the $P2X_5$ or $P2X_7$ receptor antibody at an equivalent dilution. Numerous test sections showed no reaction with either the diluent alone or with the added normal swine serum; therefore, subsequently, the ChemMate diluent was used alone as a negative control.

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

A431 cell line This human epidermal SCC cell line was purchased from the European Collection of Cell Cultures (ECACC no. 85090402). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. Cultures were grown on plastic in Dulbecco minimal Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and L-glutamine. Cells were disaggregated at 70% confluency with trypsin/ethylenediamine tetraacetic acid and seeded at 1×10^4 cells per well in a 96 well plate. This seeding density gave the best growth curve over a 72 h period.

Proliferation assay Twenty-four hours after seeding into 96 well plates, medium was gently aspirated from culture wells and P2 receptor subtype agonists were applied to the A431 cells, diluted in medium. These were obtained from Sigma and included: ATP; uridine 5′-triphosphate (UTP), $P2Y_2$ receptor agonist (von Kügelgen and Wetter, 2000); adenosine 5′-O-(3-thiotriphosphate) (ATPγS), $P2X_5$ receptor agonist (Khakh *et al*, 2000); 2′- 3′-O-(4-benzoyl-benzoyl) adenosine 5′-triphosphate (BzATP), $P2X_7$ receptor agonist (Khakh *et al*, 2001a); and 2-methylthioadenosine 5′-diphosphate (2MeSADP), $P2Y_1$ receptor agonist (von Kügelgen and Wetter, 2000). The pH of the drug solutions prepared in the culture medium was 7.0.

Changes in cell number were quantified via a colorimetric assay using crystal violet (Gillies *et al*, 1986; Kueng *et al*, 1989) and read using a spectrophotometric plate reader (Model 550, Microplate Manager 4.0 Bio-Rad Laboratories, Inc. Hercules, CA USA) at 0, 24, 48, and 72 h after addition of drugs. For the colorimetric assay, a solution of 0.5 g crystal violet, 0.85 g NaCl, 5 mL 10% formal saline, 50 mL absolute ethanol, 45 mL distilled H_2O was used. Medium was gently aspirated from wells of a 96 well plate and 100 μL of the colorimetric assay mixture was added to each well and incubated at room temperature for 10 min. This mixture allowed simultaneous fixation of cells and penetration of the crystal violet dye into the cells. After washing three times in PBS, 33% acetic acid was used to elute color from cells and optical density was read at 595 nm using the spectrophotometric plate reader. The changes in cell number identified by the crystal violet assay were validated at least once for each drug set by doing actual cell counts with a hemocytometer. To confirm that the optical density of the wells correlated with cell numbers, a control assay was performed for each experiment, where known numbers of cells were seeded in ascending seeding densities and the plate read as soon as cells had attached. Cell number *versus* optical density was plotted. The R^2 value of the trend line was always greater than 0.98 (data not shown).

Statistical analysis Each proliferation assay experiment was repeated an average of eight times, each with triplicate samples. Data analysis was performed using Microsoft Excel 97 and GraphPad Prism 3.0 software. One-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test were carried out between groups.

RESULTS

$P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors were expressed in frozen sections of BCC $P2X_5$ receptors were heavily expressed in tumor cells of BCC, but slightly less heavily than in the epidermis (**Fig 1*a***). At high power, $P2X_5$ receptors were expressed within the cell cytoplasm of tumor cells and the level of staining intensity in tumor cells was comparable with that seen

in basal cells within the epidermis. In the central area of the tumor, staining was absent (**Fig 1*a***). $P2X_7$ receptors were expressed in the necrotic center of nodular BCC (**Fig 1*b***). Although nonspecific staining in necrotic areas does occur, this staining disappeared on preabsorption of the $P2X_7$ primary antibody, suggesting that it was specific. At higher magnification, the staining was seen to be associated with mast cells, macrophages, and swollen fibroblasts in the stroma surrounding the tumor. In normal skin, $P2X_7$ receptors are found in the stratum corneum. It was noticeable that in the more normal areas of skin that $P2X_7$ receptors were present, but in the area immediately over the BCC, the expression of $P2X_7$ receptors disappeared (**Fig 1*c***).

$P2Y_1$ receptors were expressed in the stroma surrounding the tumor, but not within the tumor cells (**Fig 1*d***). $P2Y_2$ receptors (**Fig 1*e***) and $P2Y_4$ receptors **(Fig 1*f*)** were expressed within the tumor but not in the surrounding stromal matrix. Both the omission of the primary antibody and preabsorption with corresponding peptides were performed as controls in sections of BCC. There was no immunoreaction when the primary antibody was omitted. The immunoreaction was abolished after preabsorption of the $P2X_5$ (**Fig 1*g***), $P2X_7$ (**Fig 1*h***), $P2Y_1$ (**Fig 1*i***), $P2Y_2$ (**Fig 1*j***), and $P2Y_4$ receptor antibodies (**Fig 1*k***) with the corresponding peptides, confirming the specificity of the immunoreaction.

$P2X_5$, $P2X_7$, $P2Y_1$, and $P2Y_2$ receptors were expressed in frozen sections of SCC $P2X_5$ receptors were more heavily expressed in the tumor cells than in the cells of the surrounding epidermis and $P2X_5$ receptors were located specifically on the cell

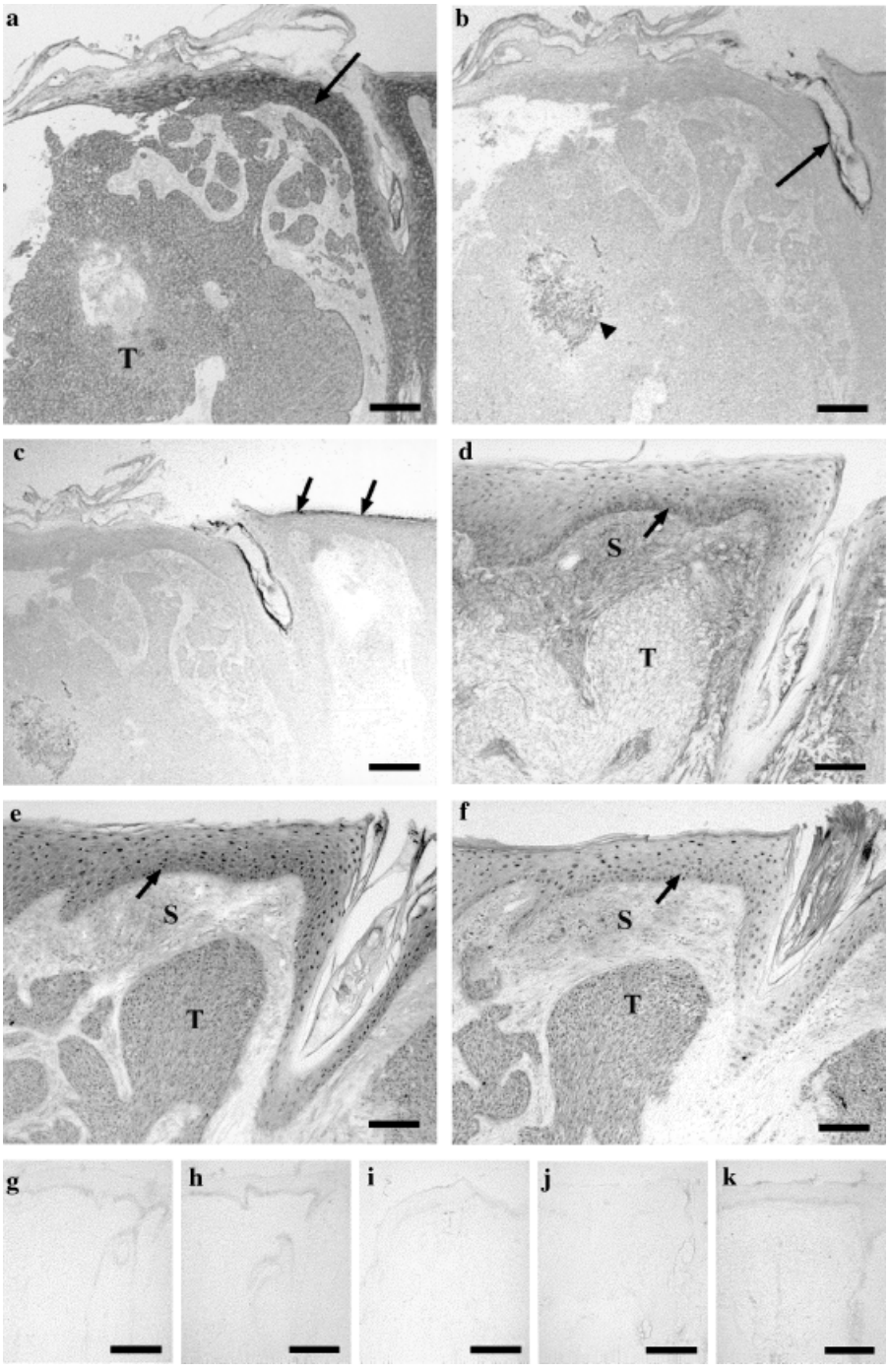

Figure 1. $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptor staining in BCC. (*a*) $P2X_5$ receptors were heavily expressed in tumor (T), but slightly less heavily than in the epidermis (*arrow*). In the central area of the tumor, staining was absent. *Scale bar* = 200 μm. (*b*) $P2X_7$ receptors were expressed in the necrotic center of the BCC (*arrowhead*), as well as in the stratum corneum (*arrow*). *Scale bar* = 200 μm. (*c*) $P2X_7$ receptors were found in the stratum corneum in the more normal areas of skin (*arrows*), but in the area immediately over the BCC, the expression of $P2X_7$ receptors disappeared. *Scale bar* = 175 μm. (*d*) $P2Y_1$ receptors were expressed in the basal layer of the epidermis (*arrow*) overlying the tumor, and in the stroma (S) surrounding the tumor (T), but not within the tumor itself. *Scale bar* = 100 μm. (*e*) $P2Y_2$ receptors were expressed in the basal layer of the epidermis (*arrow*), and within tumor (T) and not in the surrounding stromal matrix (S). There was some nuclear staining within the epidermis *Scale bar* = 100 μm. (*f*) $P2Y_4$ receptors were faintly expressed in the basal layer of the epidermis (*arrow*), and more strongly within tumor (T) and not in the surrounding stromal matrix (S). There was some nuclear staining within the epidermis. *Scale bar* = 100 μm. The immunoreaction was abolished after preabsorption of the (*g*) $P2X_5$, (*h*) $P2X_7$, (*i*) $P2Y_1$, (*j*) $P2Y_2$, and (*k*) $P2Y_4$ receptor antibodies with the corresponding peptides, confirming the specificity of the immunoreaction. There was, however, some melanin in the basal layer of the epidermis, which could be distinguished from immunostaining by its color. *Scale bars*: (*g–k*) 250 μm.

membrane of the SCC cells (**Fig 2*a***), rather than within the cell cytoplasm as in BCC tumor cells. $P2X_7$ receptors were strongly expressed in the stratum corneum of the epidermis but only weakly expressed in the nucleus of occasional tumor cells (**Fig 2*b***). $P2Y_1$ receptors were strongly expressed within the stromal matrix surrounding the tumor nests within the dermis, but not expressed within the tumor (**Fig 2*c***). $P2Y_2$ receptors were strongly expressed within the tumor, but not expressed within the surrounding stromal matrix (**Fig 2*d***). The $P2Y_2$ receptor staining appears to be both in the cell membrane and weakly in the nucleus of the tumor cells. The SCC cells appear nonuniform in size and shape, with vacuolation of the cytoplasm in some cells, which may explain the nonuniformity of $P2Y_2$ staining in some tumor cells. $P2Y_4$ receptors were not expressed in the SCC'S examined.

Both the omission of the primary antibody and preabsorption with corresponding peptides were performed as controls in sections of SCC. There was no immunoreaction when the primary antibody was omitted. The immunoreaction was abolished after preabsorption of the $P2X_5$ (**Fig 2*e***), $P2X_7$ (**Fig 2*f***), $P2Y_1$ (**Fig 2*g***), and $P2Y_2$ receptor antibodies (**Fig 2*h***) with the corresponding peptides, confirming the specificity of the immunoreaction. The distribution of P2X and P2Y receptors in both BCC and SCC is summarized in **Table I**. P2 receptor staining in normal skin is described in detail in Greig *et al* (2003).

Double labeling of $P2X_7$ receptors with TUNEL or anti-human caspase-3 showed colocalization, and double labeling of $P2Y_2$ receptors with PCNA showed overlap in NMSC Double labeling of $P2X_7$ receptors and TUNEL in the center of a nodular BCC showed colocalization on cells undergoing apoptosis, with $P2X_7$ receptors also expressed in cellular fragments (**Fig 3*a***). Double labeling with $P2X_7$ receptors and anti-human caspase-3, showed colocalization in the nuclei of cells within the tumor nests of a SCC (**Fig 3*b***).

Double labeling with $P2Y_2$ receptors (red) and PCNA (green) (**Fig 3*c***) showed that there was much proliferative activity within SCC tumor nests and that there was overlap between the cells expressing PCNA and $P2Y_2$ receptors.

$P2X_5$ and $P2X_7$ receptors were expressed in paraffin sections of BCC and SCC The immunostaining of paraffin sections showed essentially the same distribution as that in frozen sections, although more detail of the cellular and subcellular composition was visible. The development of this method also allowed us to look at archive material of paraffin sections of different tumor subtypes. $P2X_5$ and $P2X_7$ receptors were examined because they are involved in early keratinocyte differentiation and terminal keratinocyte differentiation/apoptosis, respectively. Paraffin sections of BCC were grouped as follows: 11 nodular (including three solid subtypes), nine superficial multifocal, and 24 infiltrative (morpheic). Of the 24 infiltrative BCC, seven represented a subgroup of "horrifying BCC" (Horlock *et al*, 1998), which were clinically locally aggressive and deeply invasive. The distribution of $P2X_5$ and $P2X_7$ receptors within subgroups of BCC is summarized in **Table II**.

In nodular BCC, $P2X_5$ receptor staining (brown) was present both in the tumor (in the cell cytoplasm), but also in cells of the surrounding stromal matrix (**Fig 4*a***). These positive stromal cells were likely to be endothelial cells, seen at higher power in association with capillaries. $P2X_7$ receptors (pink) were

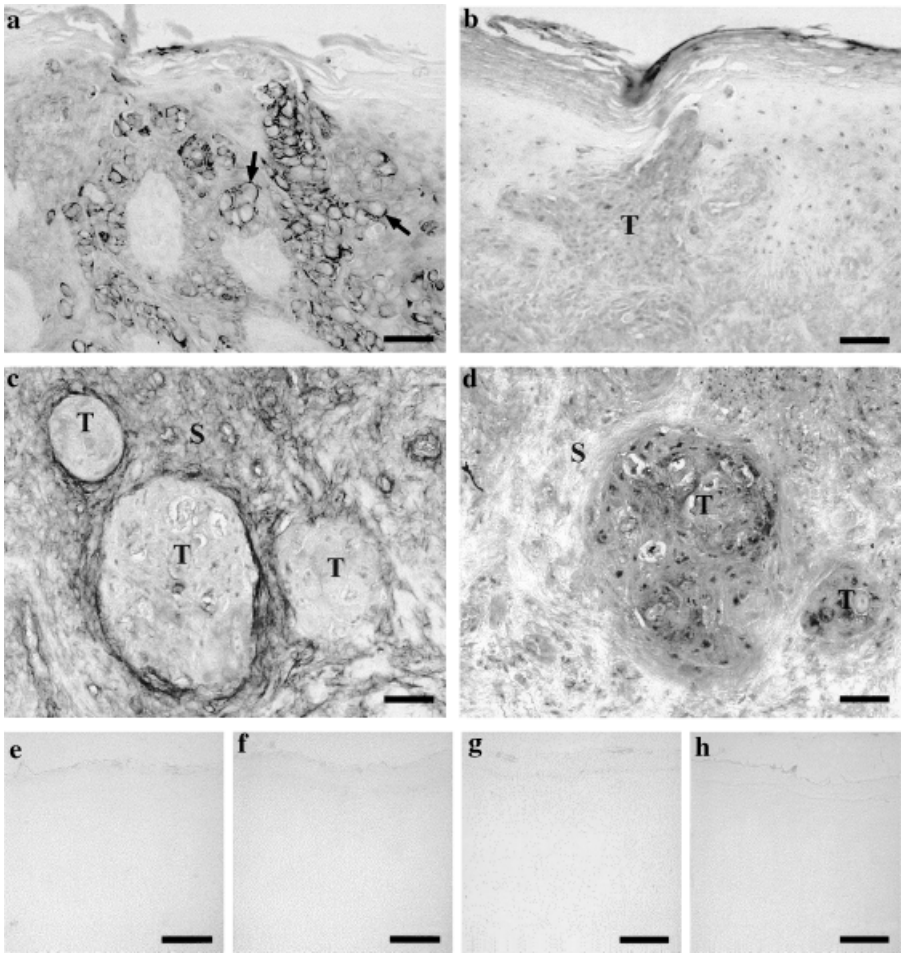

Figure 2. $P2X_5$, $P2X_7$, $P2Y_1$, and $P2Y_2$ receptor staining in SCC. (*a*) $P2X_5$ receptors were more heavily expressed on tumor cell membranes (*arrows*) than on the cells of the surrounding epidermis. *Scale bar* = 30 μm. (*b*) $P2X_7$ receptors were strongly expressed in the stratum corneum of the epidermis and only weakly expressed within the tumor (T). *Scale bar* = 30 μm. (*c*) $P2Y_1$ receptors were strongly expressed within the stromal matrix (S) surrounding the nests of tumor (T) within the dermis, but not expressed within the tumor itself. *Scale bar* = 50 μm. (*d*) $P2Y_2$ receptors were strongly expressed within the tumor (T) within the dermis, but not expressed within the surrounding stromal matrix (S). *Scale bar* = 50 μm. The immunoreaction was abolished after preabsorption of the (*e*) $P2X_5$, (*f*) $P2X_7$, (*g*) $P2Y_1$, and (*h*) $P2Y_2$ receptor antibodies with the corresponding peptides, confirming the specificity of the immunoreaction. *Scale bars*: (*g–j*) 200 μm.

Table I. Distribution of P2X and P2Y receptor subtypes in normal skin, BCC and SCC

Receptor	Normal skin	BCC	SCC
$P2X_5$	Weaker in basal layer, strong staining in stratum spinosum and granular layer keratinocytes	Tumor cell cytoplasm, endothelial cells	Tumor cell membranes, endothelial cells
$P2X_7$	Stratum corneum	Necrotic areas within tumor, mast cells, macrophages and fibroblasts	Nucleus of occasional tumor cells
$P2Y_1$	Basal epidermal keratinocytes	Stroma	Stroma
$P2Y_2$	Basal epidermal keratinocytes	Tumor cell cytoplasm	Tumor cell cytoplasm
$P2Y_4$	Weak in epidermis	Tumor cell cytoplasm	No staining

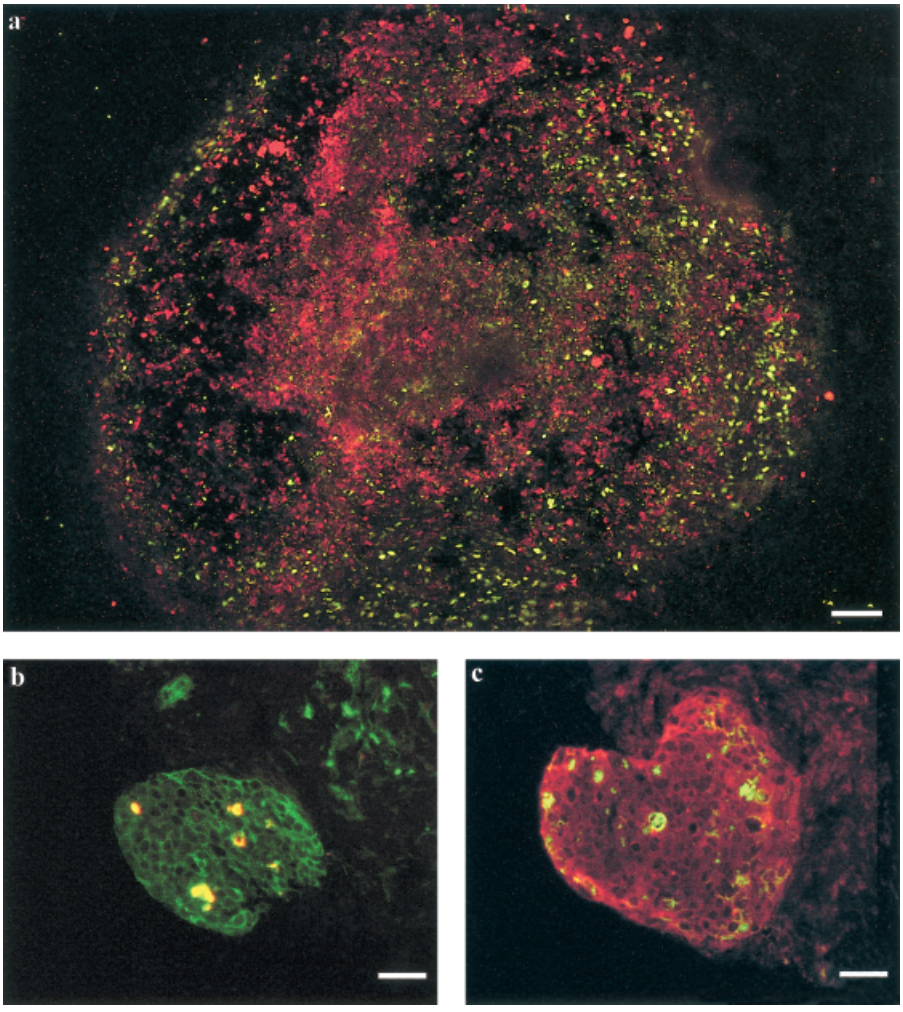

Figure 3. Double labeling of $P2X_7$ receptors with TUNEL and anti-human caspase-3 and $P2Y_2$ receptors with PCNA. (*a*) Double labeling of $P2X_7$ receptors (red) and TUNEL (green) in the center of a nodular BCC showed colocalization of cells undergoing apoptosis, with $P2X_7$ receptors also expressed in cellular fragments. *Scale bar* = 40 μm. (*b*) Double labeling with $P2X_7$ receptors (red) and anti-human caspase 3 (green), showed colocalization in the nuclei (yellow) of SCC cells within a tumor nest. *Scale bar* = 50 μm. (*c*) Double labeling with $P2Y_2$ receptors (red) and PCNA (green nuclear staining) showed that there was overlap between SCC cells expressing PCNA and $P2Y_2$ receptors. *Scale bar* = 50 μm.

Table II. Analysis of $P2X_5$ and $P2X_7$ receptor distribution in paraffin sections of BCC subgroups

BCC subtype	$P2X_5^+$	$P2X_7^+$	$P2X_5^+/P2X_7^+$	$P2X_5^-/P2X_7^-$	$P2X_5^+/P2X_7^-$	$P2X_5^-/P2X_7^+$
Nodular (11)	7	4	4	4	3	0
Superficial multifocal (9)	5	3	3	4	2	0
Infiltrative (17)	14	10	9	2	5	1
Horrifying (7)	4	1	1	3	3	0

expressed in the necrotic center of nodular BCC (**Fig 4*b***) the same as in frozen sections. In superficial multifocal BCC, $P2X_5$ receptors were expressed in a patchy fashion throughout tumor nests mostly in the tumor cell cytoplasm (**Fig 4*c***). $P2X_7$ receptors were weakly expressed in the nucleus of occasional tumor cells (**Fig 4*d***). In infiltrative BCC, the $P2X_5$ receptor staining was different in cells at the edge of the tumor nest, from cells in the center (**Fig 4*e***). Cells at the edge had positive staining in the plasma membrane, and this staining appeared to be polarized to the surface of the cells that were in contact with

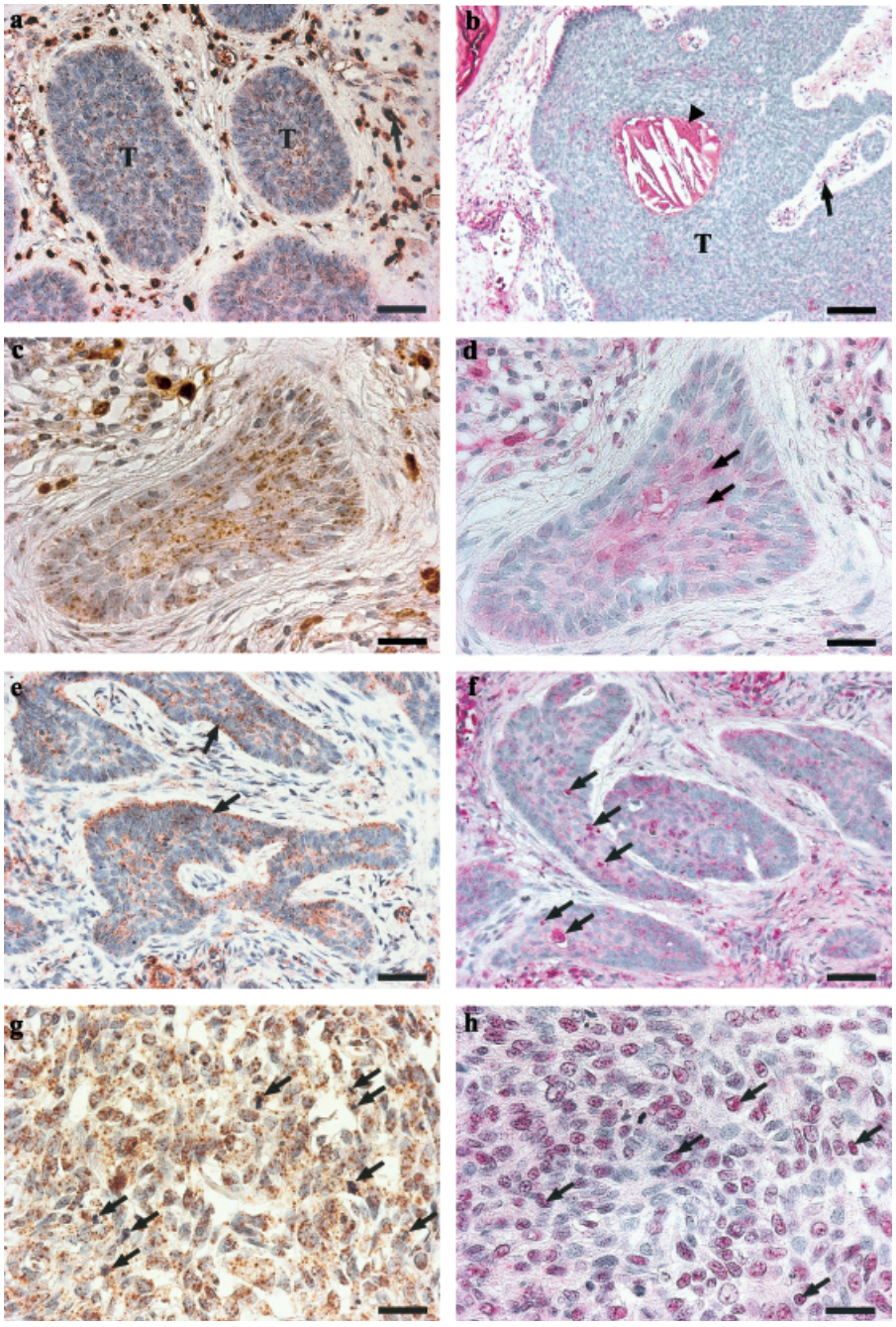

Figure 4. $P2X_5$ and $P2X_7$ receptors are expressed in paraffin sections of different subtypes of BCC; sections counterstained with hematoxylin to stain nuclei blue. (*a,b*) *Nodular BCC.* (*a*) $P2X_5$ receptors (brown) were expressed within the tumor (T) and cells of the stromal matrix also show strong labeling (*arrow*). The staining was uniform in nature throughout each tumor nest and mainly in the cell cytoplasm. *Scale bar* = 50 μm. (*b*) $P2X_7$ receptors (pink) were expressed in the necrotic center of nodular BCC (*arrowhead*) as in the frozen sections. They were also expressed on inflammatory cells (*arrow*) surrounding the tumor (T). *Scale bar* = 50 μm. (*c,d*) *Superficial multifocal BCC.* (*c*) $P2X_5$ receptors (brown) were expressed in a patchy fashion throughout tumor nests mostly in the tumor cell cytoplasm. *Scale bar* = 25 μm. (*d*) $P2X_7$ receptors (pink) were weakly expressed in the nucleus of occasional tumor cells (*arrows*). *Scale bar* = 25 μm. (*e,f*) *Infiltrative BCC* (*e*) $P2X_5$ receptors (brown) were expressed in both the basal (*arrows*) and suprabasal layers of the tumor. *Scale bar* = 50 μm. (*f*) $P2X_7$ receptor immunoreactivity (pink) was seen to be nuclear or perinuclear (*arrows*), often in cells towards the center of a tumor nest. The stained nuclei were condensed and shrunken away from surrounding tissue. *Scale bar* = 50 μm. (*g,h*) "*Horrifying BCC*", a subgroup of the infiltrative type of BCC, which is locally aggressive, and deeply invasive. (*g*) $P2X_5$ receptors (brown) were seen in the cytoplasm of tumor cells in association with numerous cells undergoing mitosis (*arrows*). *Scale bar* = 25 μm. (*h*) $P2X_7$ receptors (pink) were expressed in the nucleus of numerous cells (*arrows*) but unlike infiltrative BCC (*f*) the nuclei were not condensed. *Scale bar* = 25 μm.

the basement membrane surrounding the tumor nest. In contrast, cells in the center of the tumor nest had positive staining for $P2X_5$ receptors in the cell cytoplasm, as seen in nodular (**Fig 4*a***) and superficial multifocal BCC (**Fig 4*c***). $P2X_7$ receptor immunoreactivity in infiltrative BCC was mainly in shrunken, condensed, possibly apoptotic nuclei near the center of tumor nests (**Fig 4*f***). In "horrifying BCC", a locally aggressive and deeply invasive subgroup of infiltrative BCC, $P2X_5$ receptors were seen in the cytoplasm of tumor cells in association with numerous cells undergoing mitosis (**Fig 4*g***). In "horrifying BCC", $P2X_7$ receptors were expressed in the nucleus of numerous cells but unlike in infiltrative BCC the nuclei were not condensed or apoptotic (**Fig 4*h***).

SCC were subclassified as follows: five Bowen's disease (carcinomas *in situ*), four differentiated SCC, and five invasive SCC. Two basisquamous (metaplastic) carcinomas, showing characteristics of both BCC and SCC, were also analyzed immunohistochemically. The distribution of $P2X_5$ and $P2X_7$ receptors within subgroups of SCC is summarized in **Table III**.

All examples of Bowen's disease (SCC *in situ*) examined were positive for $P2X_5$ receptors (**Table III**). The staining was mainly in the cytoplasm and was much more intense than in the surrounding normal epidermis (**Fig 5*a***). There were also positive cells staining for $P2X_5$ receptors within the dermis (**Fig 5*a***), which were likely to be endothelial cells in association with capillaries. $P2X_7$ receptors were expressed in Civatte bodies (apoptotic keratinocytes) within the tumor (**Fig 5*b***). All four well-differentiated SCC were positive for both $P2X_5$ and $P2X_7$ receptors (**Table III**). In well-differentiated SCC, $P2X_5$ receptors were heavily expressed throughout the tumor, in the tumor cell cytoplasm (**Fig 5*c***). $P2X_5$ receptors were heavily expressed at the edges of keratin pearls, but were less intensely expressed in the more differentiated central areas of keratin pearls. In contrast, $P2X_7$ receptors were only expressed in the center of keratin

Table III. Analysis of $P2X_5$ and $P2X_7$ receptor distribution in paraffin sections of SCC subgroups

SCC subtype	$P2X_5^+$	$P2X_7^+$	$P2X_5^+/P2X_7^+$	$P2X_5^-/P2X_7^-$	$P2X_5^+/P2X_7^-$	$P2X_5^-/P2X_7^+$
Bowen's disease (carcinoma *in situ*) (5)	5	3	3	0	2	0
Differentiated (4)	4	4	4	0	0	0
Invasive (5)	5	3	3	0	2	0
Basisquamous (metaplastic) (2)	2	2	2	0	0	0

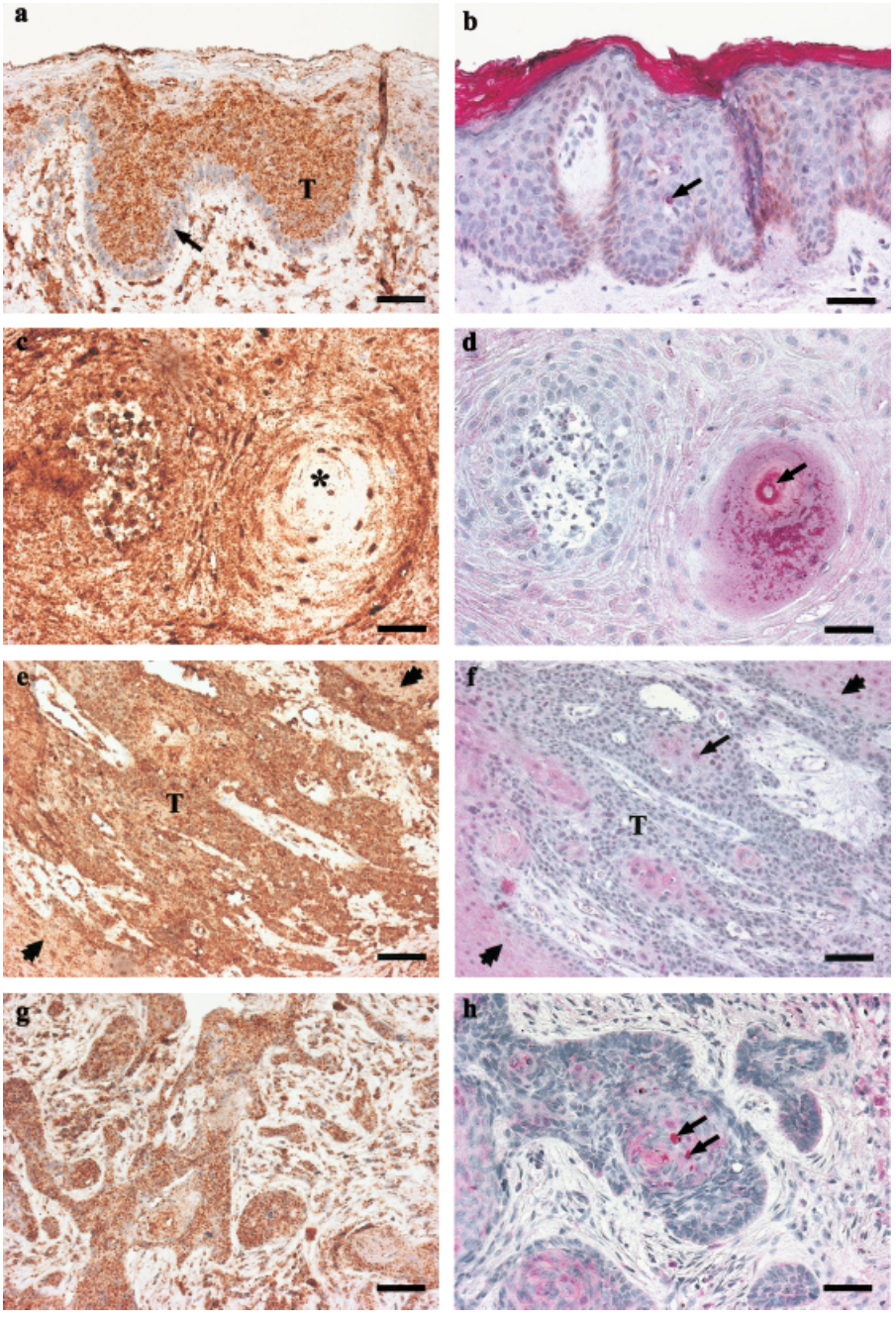

Figure 5. $P2X_5$ and $P2X_7$ receptors are expressed in paraffin sections of different subtypes of SCC; sections counterstained with hematoxylin to stain nuclei blue. (*a,b*) *Bowen's disease* (SCC *in situ*). (*a*) $P2X_5$ receptors (brown) were expressed within the tumor (T) and also in cells of the stromal matrix. There was almost no staining within the basal layer of the epidermis (*arrow*). *Scale bar* = 50 μm. (*b*) $P2X_7$ receptors (pink) were expressed in Civatte bodies (apoptotic keratinocytes—*arrow*) within the area of the tumor. *Scale bar* = 50 μm. (*c,d*) *Well-differentiated SCC.* (*c*) $P2X_5$ receptors (brown) were heavily expressed throughout the tumor, especially in areas where keratin pearls (asterisk) were forming. The receptors were also found in the tumor cell cytoplasm, but showed lower expression in the more differentiated areas of the keratin pearls (asterisk). *Scale bar* = 50 μm. (*d*) $P2X_7$ receptors (pink) were only expressed in the center of keratin pearls in the more differentiated cells and in areas where necrosis had occurred (*arrow*). *Scale bar* = 50 μm. (*e,f*) *Invasive SCC.* (*e*) $P2X_5$ receptors (brown) were more heavily expressed in the invasive tumor (T) compared with the more normal areas of epidermis nearby (*double arrows*). *Scale bar* = 100 μm. (*f*) $P2X_7$ receptor immunoreactivity was seen in the nucleus (*arrow*), often in cells towards the center of a tumor nest. *Scale bar* = 100 μm. (*g,h*) *Basisquamous (metaplastic) carcinoma.* (*g*) $P2X_5$ receptors (brown) were seen in all tumor cells. *Scale bar* = 50 μm. (*h*) $P2X_7$ receptors (pink) were expressed in nuclei (*arrows*) that were condensed and possibly apoptotic. *Scale bar* = 50 μm.

pearls in more differentiated cells and in areas where necrosis had occurred (**Fig 5*d***). In invasive SCC, $P2X_5$ receptors were more heavily expressed in the invasive tumor compared with normal areas of epidermis (**Fig 5*e***). In invasive SCC, $P2X_7$ receptor immunoreactivity was seen within shrunken, pyknotic, apoptotic-looking nuclei, often in cells towards the center of a tumor nest (**Fig 5*f***). In basisquamous (metaplastic) carcinomas, $P2X_5$ receptor labeling was found both in the tumor cell

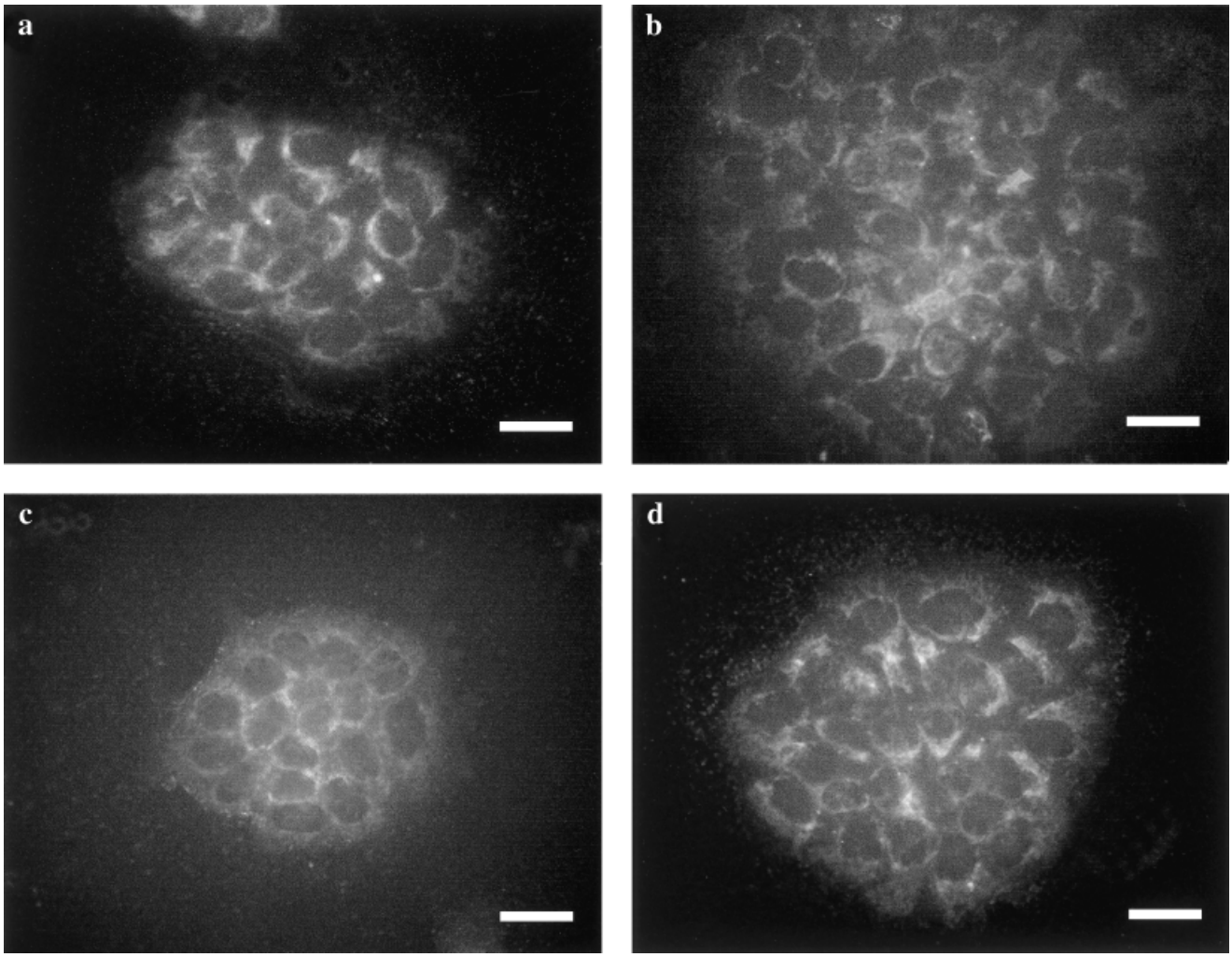

Figure 6. $P2X_5$, $P2X_7$, $P2Y_1$, and $P2Y_2$ receptor staining in SCC, A431 cells grown in culture. (*a*) $P2X_5$, (*b*) $P2X_7$, (*c*) $P2Y_1$, and (*d*) $P2Y_2$ receptor immuno-labeling on the membranes of A431 cells. *Scale bar* = 20 μm.

membrane and within the cytoplasm (**Fig 5*g***). $P2X_7$ receptors were expressed in condensed nuclei, which were possibly apoptotic (**Fig 5*h***). Both basisquamous carcinomas examined were positive for $P2X_5$ and $P2X_7$ receptors (**Table III**).

$P2X_5$, $P2X_7$, $P2Y_1$, and $P2Y_2$ receptors were expressed in A431 cells grown in culture $P2X_5$ (**Fig 6*a***), $P2X_7$ (**Fig 6*b***), $P2Y_1$ (**Fig 6*c***), and $P2Y_2$ receptors (**Fig 6*d***) were expressed on A431 cells on the cell membranes. Both the omission of the primary antibody and preabsorption with corresponding peptides were performed as controls. There was no immunoreaction when the primary antibody was omitted. Furthermore, the immunoreaction was abolished after preabsorption of the $P2X_5$, $P2X_7$, $P2Y_1$, and $P2Y_2$ receptor antibodies with the corresponding peptides, confirming the specificity of the immunoreaction.

Actions of P2 receptors agonists on cell numbers of cultured cutaneous SCC, A431 cells Low concentrations of ATP (10 μM) caused an increase in cell number, whereas high concentrations of ATP (500–5000 μM) caused a significant decrease ($p < 0.001$) in cell number at 48 h after application (**Fig 7**), with cells in the 5000 μM ATP solution appearing rounded up and dead (**Fig 7*e***). Low concentrations of ATPγS (1–10 μM) and high concentrations of UTP (1000 μM) caused an increase in cell number, with UTP causing a significant effect ($p < 0.001$). High concentrations of ATP (500 μM) and ATPγS (100–500 μM) caused a significant decrease ($p < 0.001$) in cell number, at 48 h after drug application to A431 cells (**Fig 8*a***). The $P2X_7$ receptor agonist, BzATP (500 μM) (**Fig 8*b***) caused a significant reduction in cell number ($p < 0.001$) and the $P2Y_1$ receptor agonist, 2MeSADP (1–500 μM) (**Fig 8*c***) caused a reduction in cell number, which was not statistically significant.

DISCUSSION

In this study, we have obtained the first direct evidence for the expression of $P2X_5$, $P2X_7$, $P2Y_1$, and $P2Y_2$ receptors in human BCC and SCC, using immunohistochemistry and functional studies *in vitro* with the human SCC, A431 cell line. *In vitro* experiments with A431 cells were used to give an indication of the effects of P2 receptor agonists on SCC cells *in vivo*, but further experiments are needed to assess the effects in tumors. Low concentrations of ATP increased A431 cell number. Previous work with A431 cells has shown that micromolar range concentrations of ATP are mitogenic for A431 cells (Huang *et al*, 1989) and increase intracellular calcium levels (Gonzalez *et al*, 1988). ATP has also been shown to activate cell proliferation in human ovarian tumor cells (Popper and Batra, 1993) and in MCF-7 breast cancer cells (Dixon *et al*, 1997). From our functional data in A431 cells, UTP, the $P2Y_2$ receptor agonist caused a significant increase

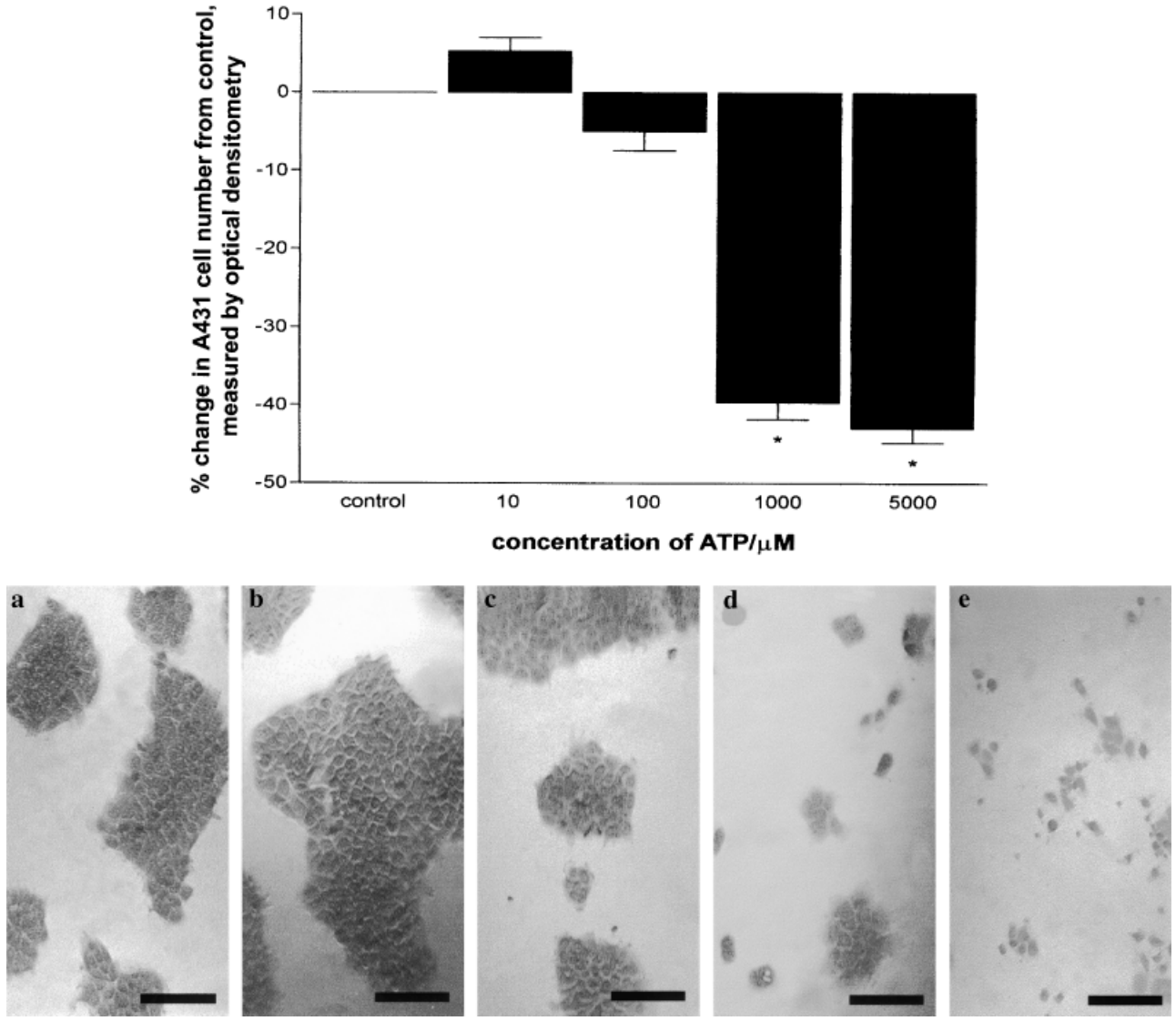

Figure 7. Change in cell number of SCC, A431 cells at 48 h after ATP application. At 48 h after application, low doses of ATP (10 μM) caused an increase in cell number, whereas high doses of ATP (1000–5000 μM) ($p < 0.001$) caused a significant decrease. Results represent the mean of 26 experiments. *$p < 0.001$ compared with control. Error bars represent mean ± SEM. These effects were shown also by the colony size and cell morphology. A431 cells were stained with crystal violet and phase contrast micrographs were taken. (*a*) Control—cells grown in A431 medium only; (*b*) 10 μM ATP caused colonies of A431 cells to appear visibly larger than in control wells; (*c*) 100 μM ATP caused no change in the colony size; (*d*) 1000 μM ATP and (*e*) 5000 μM ATP both caused a marked decrease in colony size, with cells rounding up and dying in 5000 μM ATP. *Scale bar* = 150 μm.

($p < 0.001$) in proliferation. $P2Y_2$ receptors were heavily expressed in both BCC and SCC and there was overlap between cells expressing PCNA and $P2Y_2$ receptors. The proportion of PCNA-positive cells in SCC tumor nests was higher than in normal epidermis. Where this increased expression of PCNA was found, so the expression of $P2Y_2$ receptors was proportionally increased compared with normal epidermis. Taken together, the PCNA findings further confirm our findings in the functional experiments that $P2Y_2$ receptors are likely to be involved in proliferation. Previous work has localized $P2Y_2$ receptor mRNA in human epidermal basal cells via *in situ* hybridization (Dixon *et al*, 1999). UTP has also been shown to cause proliferation of normal keratinocytes (Dixon *et al*, 1999) and HaCaT cells (Lee *et al*, 2001). Functional $P2Y_2$ receptors have also been characterized on human ovarian cancer cells (Schultze-Mogasu *et al*, 2000), colorectal carcinoma cells (Höpfner *et al*, 1998), prostate carcinoma cells (Janssens and Boeynaems, 2001), endometrial cancer cell lines (Katzur *et al*, 1999), and MCF-7 breast cancer cells (Wagstaff *et al*, 2000).

In contrast, high concentrations of ATP and the $P2X_7$ receptor agonist, BzATP caused a significant decrease in A431 cell number ($p < 0.001$). The concentrations of ATP used were anticipated to be within the physiologic range because ATP physiologically released from nerves (Sperlágh and Vizi, 1996), endothelial cells (Bodin and Burnstock, 1998), and urothelial cells (Ferguson, 1999) can reach very high levels extracellularly ($> 10^{-3}$ mM) before being broken down by ectoenzymes. $P2X_7$ receptors were heavily expressed in the necrotic center of nodular BCC and colocalized with TUNEL labeling. They were also expressed in apoptotic cells in both infiltrative BCC and in SCC and $P2X_7$ receptors colocalized with active caspase-3. The $P2X_7$ receptor is unlike other P2X receptors because it is a bifunctional molecule that can be triggered by low concentrations of ATP to act as a channel permeable to small cations, or at high concentrations (> 100 μM), forms a cytolytic pore permeable to large hydrophilic molecules up to 900 Da (Surprenant *et al*, 1996). The opening of this pore results in the increase in intracellular cytosolic free calcium ions, leading to cell death. The reduction in A431 cell

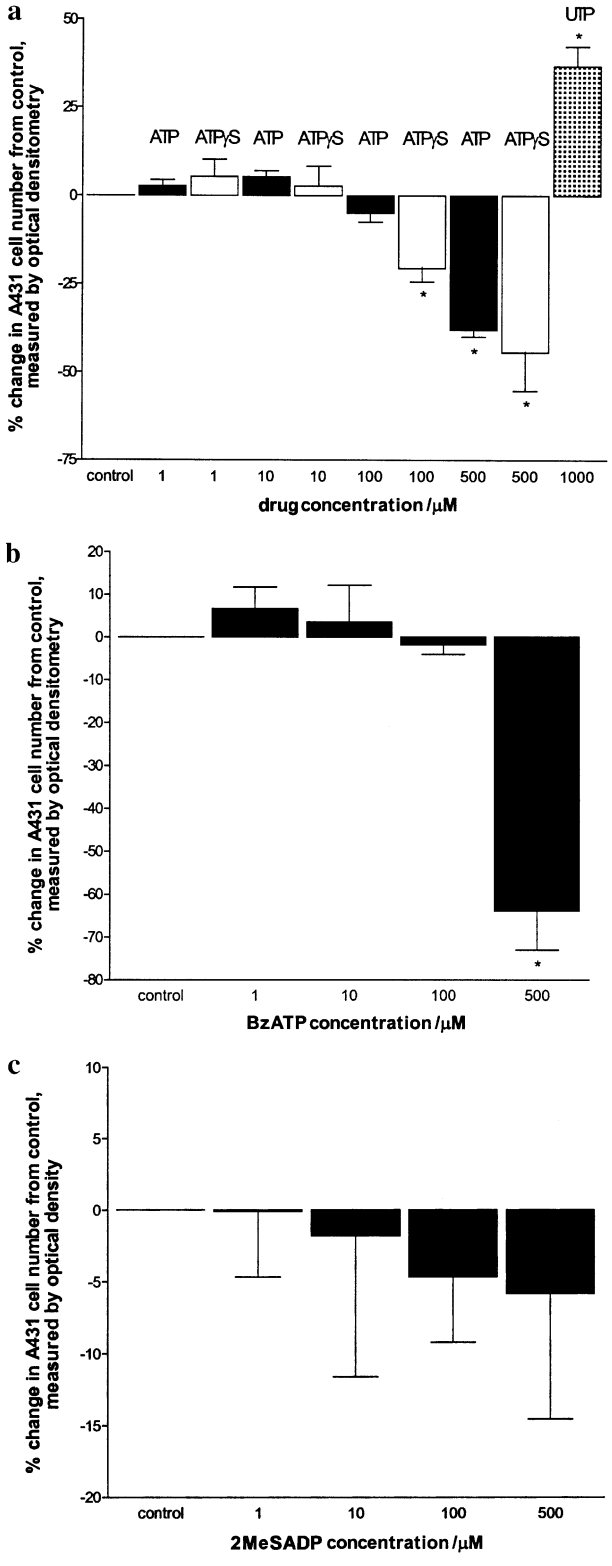

Figure 8. Comparison of effect of P2 agonists at 48 h after drug application to A431 cells. (*a*) At 48 h after application, ATP (1–10 μM), ATPγS (1–10 μM), and UTP (1000 μM) ($p < 0.001$) caused an increase in cell number of primary human keratinocyte cultures, whereas ATPγS (100–500 μM) ($p < 0.001$) and ATP (500 μM) ($p < 0.001$) caused a significant decrease in cell number. Results represent the mean of eight experiments. *$p < 0.001$ compared with control. (*b*) BzATP ($P2X_7$ receptor subtype agonist) (500 μM) caused a significant reduction in cell number (*$p < 0.001$). Results represent the mean of eight experiments. *$p < 0.001$ compared with control. (*c*) 2MeSADP ($P2Y_1$ receptor subtype agonist) (1–500 μM) caused a reduction in cell number, which was not significant. Results represent the mean of eight experiments. Error bars represent mean ± SEM.

number with increasing concentrations of ATP or BzATP could have resulted from either inhibition of proliferation or stimulation of cell death; however, BzATP is established as a 10 to 30 times more potent agonist at $P2X_7$ receptors than ATP, leading to apoptosis (North, 2002). There is no published evidence that $P2X_7$ receptors mediate inhibition of cell proliferation.

The $P2X_7$ receptor is involved in the release of interleukin-1β (Ferrari *et al*, 1997) and the induction of cell death (Zheng *et al*, 1991; Ferrari *et al*, 1996). $P2X_7$ receptors have been localized in rat and human epidermis (Gröschel-Stewart *et al*, 1999; Greig *et al*, 2003) and are also found on dendritic cells, macrophages, and microglial cells, where high concentrations of extracellular ATP can trigger apoptosis via these receptors and there is increasing evidence that this process is dependent on the caspase signaling cascade (Coutinho-Silva *et al*, 1999; Ferrari *et al*, 1999). Extracellular ATP induces apoptosis and inhibits growth of colorectal carcinoma cells in culture (Höpfner *et al*, 1999). ATP inhibits growth in breast cancer cells (Spungin and Friedberg, 1993), androgen-independent prostate carcinoma cells (Fang *et al*, 1992), and Ehrlich tumor cells (Lasso de la Vega *et al*, 1994).

BCC are slow-growing tumors with paradoxically high proliferative activity. The slow growth has been previously explained by a concurrent high apoptotic activity (Miller, 1991a, b). The interpretation of BCC as a neoplasm with high apoptotic activity has been supported by electron microscopy (Miller, 1991a, b) and an end-labeling study (Mooney *et al*, 1995). From our analysis of different subgroups of BCC in paraffin section, it appears that in moderately aggressive tumors, there is a high level of expression of $P2X_7$ receptors, but with increasing aggressiveness of tumor, this is reduced. The 24 infiltrative BCC studied can be divided into two groups: 17 infiltrative BCC and seven "horrifying BCC". In 17 infiltrative BCC, 59% expressed $P2X_7$ receptors but only 14% of "horrifying BCC" expressed $P2X_7$ receptors and within this tumor, the receptor was expressed on most of the cells within it, with positive cells not appearing to be apoptotic, in contrast to the other group of infiltrative BCC analyzed. Reduced apoptosis may lead to unstable tissue kinetics, favoring an increase in total cell numbers, which in tumors correspond to the stage of cellular expansion. A reduction in apoptosis may be responsible for the preservation of genetically aberrant cells, favoring neoplastic progression (Staibano *et al*, 1999). This may partly explain the aggressive nature of these "horrifying BCC". Analysis of paraffin sections of SCC showed that $P2X_7$ receptors were expressed in all the differentiated tumors studied, but in only three of five invasive SCC. This would suggest, despite the small sample size that more aggressive tumors also have reduced expression of $P2X_7$ receptors.

High concentrations of ATPγS, a potent $P2X_5$ receptor agonist, caused a significant decrease in cell number, at 48 h after drug application to A431 cells. This may be because cells are withdrawing from the cell cycle and differentiating. There is evidence from other tissues regarding the role of $P2X_5$ receptors. In normal rat and human epidermis (Greig *et al*, 2003) $P2X_5$ receptors are found predominantly in differentiating keratinocytes (Gröschel-Stewart *et al*, 1999). In fetal rat skeletal muscle, $P2X_5$ receptors are sequentially expressed during development (Ryten *et al*, 2001). $P2X_5$ receptors have been implicated in the regulation of osteoblastic differentiation and proliferation (Hoebertz *et al*, 2000) and in triggering the differentiation of skeletal muscle satellite cells (Ryten *et al*, 2002). In paraffin sections of SCC, $P2X_5$ receptors were highly expressed in all subgroups of tumors, with increased expression compared with the surrounding "normal" epidermis. $P2X_5$ receptors were not found in the basal layer in Bowen's disease (carcinoma *in situ*), but only in the suprabasal, differentiated cell layers. $P2X_5$ receptor staining in SCC was on the cell membrane, whereas in BCC, the receptors were expressed in the cell cytoplasm. In basisquamous (metaplastic) carcinomas, $P2X_5$ receptor labeling was found both in the tumor cell membrane and within the cytoplasm, reflecting the dual differentiation of this tumor subtype. The cytoplasmic distribution and

level of staining intensity of $P2X_5$ receptors in BCC tumor cells was comparable with that seen in basal cells within the normal epidermis, which may reflect the basal cell origin of these tumors. In this study, $P2X_5$ receptors were present either or both in the cell membrane and in the cytoplasm. Studies using $P2X_2$ receptors tagged with green fluorescent protein in hippocampal neurons have confirmed that P2X receptors can have both a cytoplasmic and cell membrane distribution (Khakh *et al*, 2001b). $P2X_1$ receptors tagged with green fluorescent protein (Dutton *et al*, 2000; Li *et al*, 2000) and native $P2X_1$ receptors (Ennion and Evans, 2001) have also been shown to undergo internalization during application of ATP or αβ-meATP to the cell. It has been proposed that $P2X_1$ receptor internalization is part of the cellular recycling of receptors and that it is part of the process of receptor activation, inactivation, internalization, and recovery (Ennion and Evans, 2001). It is likely that $P2X_5$ receptors also take part in these processes and this is why they have been localized both on the cell membrane and in the cell cytoplasm.

In paraffin sections of BCC, $P2X_5$ receptors were expressed on the majority of tumors; however, with increasing aggressiveness of tumor, the expression was reduced. Considering the two groups of infiltrative BCC: of 17 infiltrative BCC, 82% expressed $P2X_5$ receptors but only 57% of "horrifying BCC" expressed $P2X_5$ receptors. In $P2X_5$ receptor positive "horrifying BCC", the receptor was expressed on most of the cells within it, in association with numerous cells undergoing mitosis, unlike the other group of infiltrative BCC analyzed, where $P2X_5$ receptor expression was more polarized towards the basement membrane and mitoses were few.

$P2Y_1$ receptors were expressed in the stroma surrounding both BCC and SCC, but not within the tumor cells. 2MeSADP, the $P2Y_1$ receptor subtype agonist, caused a reduction in A431 cell number, although this was not statistically significant. The actions of 2MeSADP might therefore be nonspecific. It is known that for BCC to grow, they depend on interaction with their stromal matrix, and this is why it is generally thought that BCC are difficult to establish in culture. It seems that $P2Y_1$ receptors do not have a direct proliferative role in A431 cells.

In summary, $P2X_7$ receptor immunostaining may be a useful histologic tool to assess apoptosis in tumors, with the loss of expression of the marker suggesting increased tumor aggressiveness. P2 purinergic receptor agonists alter A431 cell numbers via several mechanisms: UTP and low concentrations of ATP cause an increase in cell number via $P2Y_2$ receptors; ATPγS causes a decrease in cell number, probably because cells are lost from the cell cycle by being induced to differentiate via activation of $P2X_5$ receptors; BzATP and high concentrations of ATP cause a significant decrease in cell number via a direct effect on $P2X_7$ receptors, which are known to be involved in mediating apoptosis. We have demonstrated that NMSC contain purinergic receptors and that these receptors are functional in A431 cells *in vitro*. This opens the pathway for new treatment modalities in these important skin cancers.

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