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Purinergic receptor expression in the regenerating epidermis in a rat model of normal and delayed wound healing

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Abstract: This study investigated changes in the protein expression of purinergic receptors in the regenerating rat epidermis during normal wound healing, in denervated wounds, and in denervated wounds treated with nerve growth factor (NGF), where wound healing rates are normalized. Excisional wounds were placed within denervated, pedicled, oblique, groin skin flaps, and in the contralateral abdomen to act as a control site. Six rats had NGF-treated wounds and six had untreated wounds. Tissue was harvested at day four after wounding. The re-epithelializing wound edges were analyzed immunohistochemically for $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors, and immunostaining of keratinocytes was quantified using optical densitometry.

In normal rat epidermis, $P2Y_1$ and $P2Y_2$ receptors were found in the basal layer where keratinocytes proliferate; $P2X_5$ receptors were associated with proliferating and differentiating epidermal keratinocytes in basal and suprabasal layers; $P2X_7$ receptors were associated with terminally differentiated keratinocytes in the stratum corneum. In the regenerating epidermis of denervated wounds, $P2Y_1$ receptor protein expression was significantly increased in keratinocytes ($P < 0.001$) but $P2Y_2$ receptor protein expression was significantly decreased ($P < 0.001$). Conversely, NGF treatment of denervated wounds, reduced expression of $P2Y_1$ receptors ($P < 0.001$) in keratinocytes but enhanced expression of the $P2Y_2$ receptors ($P < 0.01$) compared with untreated denervated wounds. In innervated wounds, NGF treatment enhanced $P2X_5$ ($P < 0.001$) and $P2Y_1$ receptor protein ($P < 0.001$) expression in keratinocytes. $P2X_7$ receptors were absent in all experimental wound healing preparations. $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptor protein expression in the regenerating epidermis was altered both during wound healing and also by NGF treatment. Possible roles for purinergic signalling and its relation to NGF in wound healing are discussed.

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Introduction

This study was designed to investigate changes in the expression of purinergic receptors in epidermal wound healing. Functional roles have been proposed for purinergic receptors in keratinocyte differentiation, proliferation and apoptosis (1,2). All these processes occur in wound healing. We studied the expression of purinergic receptors in normal and denervated wounds, as well as in denervated wounds treated with nerve growth factor (NGF).

Purinergic receptors are divided into two groups based on different extracellular signalling molecules. P1 receptors are selective for adenosine and P2 receptors are selective for adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) (3). P2 receptors are subdivided into P2X

Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; UTP, uridine 5′-triphosphate; NGF, nerve growth factor; NHS, normal horse serum; PBS, phosphate buffered saline; NaCl, sodium chloride; DAB, nickel-diaminobenzidine.

(ligand-gated ion channels) and P2Y (G protein-coupled) receptors (4). Seven subtypes of P2X receptors (5) and seven subtypes of P2Y receptors have been described (6,7). $P2X_5$ receptors are found in proliferating and differentiating keratinocytes in rat epidermis (1), but are thought to be more involved in keratinocyte differentiation (2). $P2X_5$ receptors are also found in other stratified squamous epithelia than from the skin, e.g. cornea, tongue and vagina (1). They are also found in urogenital tract epithelia (8), duodenal villus goblet cells (9), as well as in brain, heart, spinal cord and adrenal medulla (10). In fetal rat skeletal muscle, $P2X_5$ receptors are sequentially expressed during development (11). $P2X_5$ receptors have been implicated in the regulation of osteoblastic differentiation and proliferation (12) and in triggering the differentiation of skeletal muscle satellite cells (13).

$P2X_7$ receptors are strongly linked to apoptosis (14,15). The $P2X_7$ receptor can be triggered to form a cytolytic pore permeable to large hydrophilic molecules up to 900 Da (14). The opening of this pore results in the increase in intracellular cytosolic-free calcium ions and the induction of cell death (16,17). It has been proposed that $P2X_7$ receptors are involved in the apoptotic process of terminal differentiation of keratinocytes because $P2X_7$ receptors are associated with dying keratinocytes in the stratum corneum, and BzATP, a potent $P2X_7$ receptor agonist, can induce a significant decrease in keratinocyte number in culture (1,2).

$P2Y_1$ receptors are thought to be mitogenic in endothelial cells (18), are found in the basal layer of human epidermis and can be stimulated to increase keratinocyte number (2). $P2Y_2$ receptor mRNA has been localized in human epidermal basal cells via *in situ* hybridization. UTP, a $P2Y_2$ receptor subtype agonist, has also been shown to cause proliferation of keratinocytes (19) and HaCaT cells (20). $P2Y_2$ receptors cause proliferation in rat glomerular mesangial cells (21) and have mitogenic effects on vascular smooth muscle cells (22).

Several studies have proposed that extracellular ATP and ADP may have a role in wound healing (23). ATP released by damaged cells (24) and platelets (25,26) may be involved in wound healing, tissue repair and regeneration. ATP, acting on purinergic receptors, is involved in cell proliferation in astrocytes and epithelial cells (27) and may be an important physiological regulator of epidermal growth and differentiation, acting via inositol triphosphate and intracellular calcium levels (28).

We chose to use a rat model of normal and delayed wound healing. This model was based on the rat pedicled oblique groin flap to produce a denervated wound (29). Wound healing has been shown to be delayed in denervated wounds (30). The mechanism for this is unknown, and this study was designed to investigate whether $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors may have a role. It has been shown recently that exogenously applied nerve growth factor (NGF) has a normalizing effect on the rate of healing of denervated wounds in a rat model, reducing the time for wound closure (31), and this study aimed to investigate whether the expression pattern of the above purinergic receptors was also normalized by NGF in denervated wounds.

Materials and Methods

Study design

Maintenance and killing of the animals used in this study followed principles of good laboratory animal care and experimentation in compliance with UK national law and regulations. From previous work (32), it was established that $n = 6$ was the minimum size per group to avoid interanimal and intersample variations influencing the outcome of the results. This group size ensured that the statistical significance was at the 5% level, with an 80% statistical power. Eighteen male adult Lewis rats weighing 200–300 g were included in this study. The rats were divided into three groups of six animals. In group 1, six rats had a denervated flap, which was compared with skin taken from a control site on the contralateral abdomen in the same dermatome. In group 2, six rats had a denervated flap with a wound placed within the flap and a wound placed in the control site on the contralateral abdomen. In group 3, six rats had a denervated flap with a wound treated with NGF placed within the flap and a control wound placed in the contralateral abdomen, also treated with NGF.

Surgical technique

Rats were anaesthetized with halothane (0.5–2%) in oxygen. A 40×20-mm oblique groin flap was raised on its neurovascular pedicle, containing the superficial epigastric vessels. Sympathetic denervation was achieved by stripping all loose areolar tissue from the pedicle. Compression of the neurovascular pedicle with a clamp for 5 min completed the denervation of the flap. The flap was sutured in place orthotopically using interrupted 5/0 silk sutures. A 10×10-mm full thickness excisional wound was sited centrally within the flap, with the same-sized control wound sited on the contralateral side of the abdomen in the same dermatome.

Nerve growth factor: treated flaps

The oblique pedicled groin flap was raised and denervated, with an excisional wound made as described. Nerve growth factor at 40 μg/ml in 0.1% bovine serum albumin and PBS was topically applied as a 50-μl aliquot to both denervated wounds and normally innervated control wounds. The rats were anaesthetized for a further 60 min to allow the NGF to be absorbed locally. The rats were inspected daily and evaluated for general health and flap viability. No dressing was applied to the wounds and the flaps were harvested at 4 days postsurgery. Previous work has shown that 4 days after denervation, the wound was still open and the flap remained fully denervated (31).

Preparation of primary antibodies

The immunogens used for production of polyclonal P2X receptor antibodies were synthetic peptides corresponding to 15 receptor-type-specific amino acids (AA) in the intracellular C-termini of the cloned rat P2X receptors. The peptides were covalently linked to keyhole limpet haemocyanin. The peptide sequences are as follows: $P2X_5$, AA 437–451, RENAIVNVKQSQILH; and $P2X_7$, AA 555–569, TWRFVSQDMADFAIL. The polyclonal antibodies were raised by multiple monthly injections of New Zealand rabbits with the peptides (performed by Research Genetics, Huntsville, AL). The specificity of the antisera had been previously verified by immunoblotting with membrane preparations from cloned $P2X_{1\text{-}7}$ receptors expressing CHO K1 cells. The antibodies recognized only one protein of the expected size in the heterologous expression systems, and were shown to be receptor-subtype specific (33). $P2X_5$ and $P2X_7$ receptor antibodies were provided by Roche Bioscience (Palo Alto, CA) and were kept frozen at a stock concentration of 1 mg/ml.

Polyclonal anti-$P2Y_1$ and $P2Y_2$ receptor antibodies were obtained from Alomone Laboratories (Jerusalem, Israel), and corresponded to the third extracellular loop of the $P2Y_1$ (AA 242–258) and $P2Y_2$ receptors (AA 227–244). Antibodies were kept frozen at a stock concentration of 0.6 mg/ml ($P2Y_1$, $P2Y_2$).

Immunohistochemistry

Tissue was frozen in isopentane pre-cooled 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. For immunostaining of cryostat sections, the avidin-biotin technique was used according to a protocol developed by Llewellyn-Smith et al. (34,35). 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. 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, Poole, UK). This was 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 antirabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, PA) 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 visualize the reaction product. Sections were washed three times with PBS after each of the above steps, except after the 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 the primary antibody omitted from the staining procedure and the primary antibodies preabsorbed with the corresponding peptides. The results were analyzed using a Zeiss Axioplan high-definition light microscope (Oberkochen, Germany) mounted with a Leica DC 200 digital camera (Heerbrugg, Switzerland). Immunohistochemical analysis provided qualitative data.

Optical densitometry

To obtain semi-quantitative data, optical density measurements (36) of immunopositive epidermal keratinocytes were performed for sections immunostained for $P2X_5$, $P2Y_1$ and $P2Y_2$ receptors. To standardize the immunostaining for each antibody, all sections were stained at the same time. Two images of the regenerating epidermis per section were captured at ×40 magnification using a BX60 Olympus Microscope (Olympus, Japan) mounted with a spot digital camera. The images were captured at the wound edge, where keratinocyte proliferation was evident but not affected by inflammatory infiltrate or wound debris. The optical density was then calculated using Image-Pro Plus software (Media Cybernetics, Inc.) using the formula:

$$\text{Optical density} = -\log[(\text{intensity} - \text{black})/(\text{incident} - \text{black})]$$

where 'intensity' was the intensity expressed in pixels at the point of study, 'black' was the intensity when no light went through the material, and 'incident' was the maximum intensity of the incident light. This formula allowed a standard optical density curve to be plotted so that intensity could be converted to optical density and expressed in arbitrary units. A calibration of intensity was performed for both the camera and the images to correct for background before measuring the optical density of each image under study. The optical density of an average of 20 keratinocytes (only cells with a visible nucleus were included) per section was measured, and the mean optical density of the immunostained keratinocytes per section was plotted and statistical analyses were performed using GraphPad Prism 3.0 software (Graph Pad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test were carried out between groups for each staining.

Results

$P2X_5$ receptor protein expression, as measured by optical densitometry, was significantly increased ($P < 0.01$) in keratinocytes of the regenerating epidermis in the control wounds compared with keratinocytes in the unwounded control epidermis (Figs 1a,b and 2). Expression was particularly increased in migratory keratinocytes at the proliferating wound edge (Fig. 1b). $P2X_5$ receptor protein expression was significantly increased ($P < 0.001$) in keratinocytes in the NGF-treated control wounds (Figs 1c and 2). $P2X_5$ receptor protein expression was also increased in keratinocytes of denervated wounds ($P < 0.001$) (Figs 1d and 2). However, NGF treatment had no statistically significant effect on the expression of $P2X_5$ receptors in the epidermis of the denervated wounds (Fig. 2). So, NGF increased the expression of $P2X_5$ receptors in the epidermis of the normally innervated wounds but NGF did not have an effect on the expression of $P2X_5$ receptors in the epidermis of the denervated wounds.

$P2X_7$ receptor protein expression was absent in the regenerating epidermis of control wounds compared with the unwounded control epidermis, where the receptor was expressed in the stratum corneum (Fig. 3a,b). $P2X_7$ receptor protein expression was absent in the epidermis of five out of the six rats with denervated wounds (Fig. 3c). $P2X_7$ receptor protein expression was also absent in the epidermis of both the NGF-treated control wounds and the NGF-treated denervated wounds. Optical density measurements were not performed

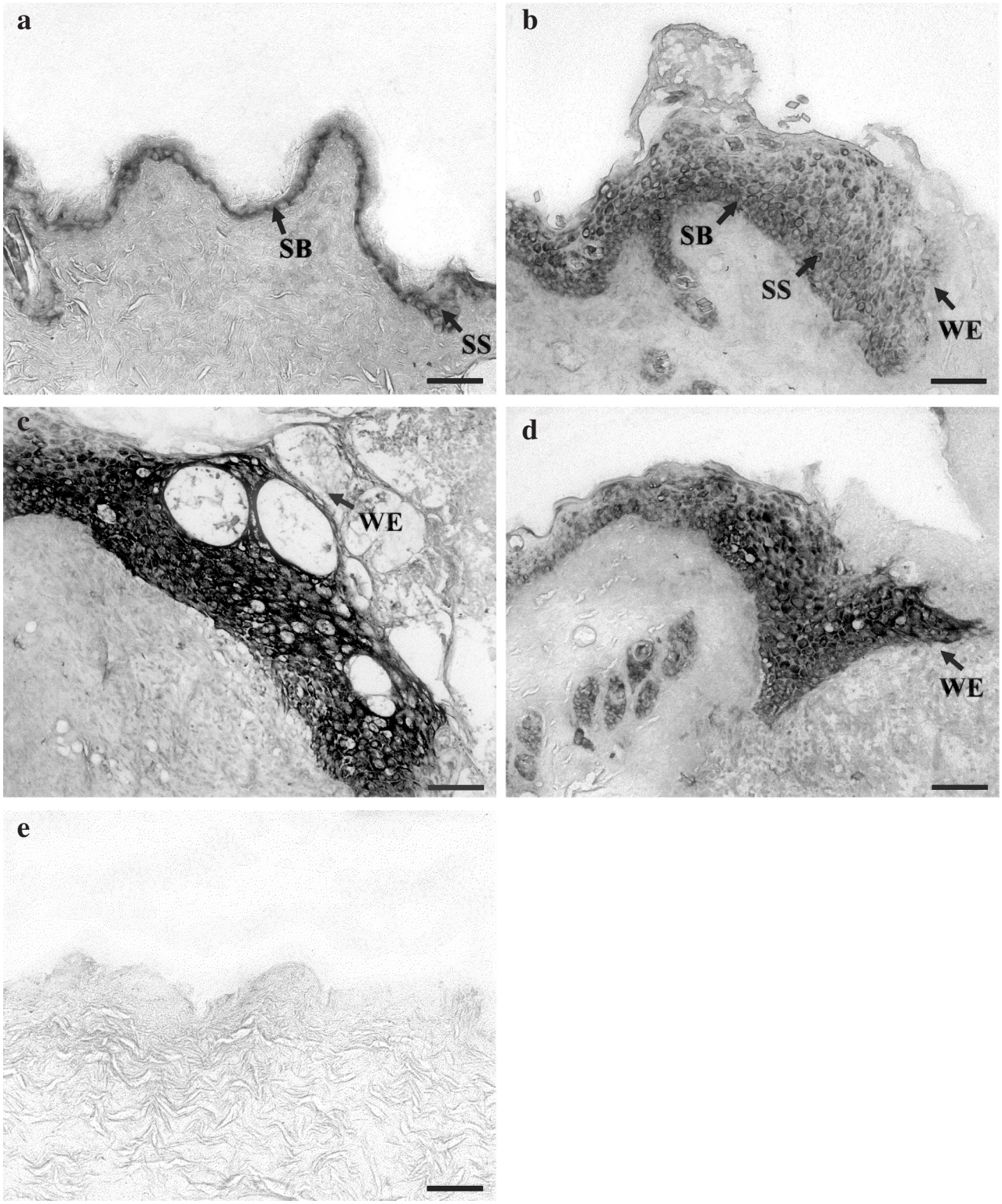

Figure 1. Comparison of $P2X_5$ receptor immunostaining in the epidermis of normal skin, and in the regenerating epidermal wound edge of innervated and denervated excisional wounds and innervated wounds treated with nerve growth factor (NGF). (a) Normal control skin from the contralateral abdomen. $P2X_5$ receptors were found in the basal layer (SB) and in the stratum spinosum (SS) of the epidermis. Scale bar = 50 μm. (b) Innervated control wound edge (WE). $P2X_5$ receptor expression was increased in migratory keratinocytes at the proliferating wound edge. Scale bar = 100 μm. (c) Innervated control NGF-treated wound edge (WE). Nerve growth factor increased the expression of $P2X_5$ receptors in normally innervated wounds. Scale bar = 100 μm. (d) Denervated wound edge (WE). $P2X_5$ receptor expression was also increased at the edge of denervated wounds. Scale bar = 100 μm. (e) Preabsorption of normal control skin: the immunoreaction was abolished after preabsorption of the $P2X_5$ receptor antibody with the corresponding peptide, confirming the specificity of the immunoreaction. Scale bar = 50 μm.

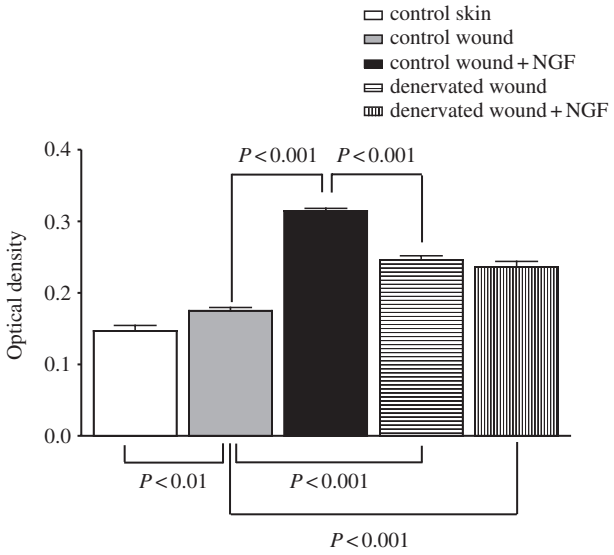

Figure 2. Histogram showing the optical density (arbitrary units) of $P2X_5$ receptor immunostaining in epidermal keratinocytes of different preparations (listed top right) in order to quantify the amount of immunostaining.

because of the very low expression of this receptor in the majority of the sections.

$P2Y_1$ receptors were found in the basal layer of the epidermis in the unwounded control skin, but in the regenerating epidermis of the control wound edges, $P2Y_1$ receptors were expressed throughout the basal and suprabasal layers (Fig. 4a,b). There was no statistically significant difference between the levels of receptor expression in keratinocytes of the control wounds compared with the control skin (Fig. 5). However there was a significant increase ($P < 0.001$) in $P2Y_1$ receptor protein expression in keratinocytes of the regenerating epidermis of the NGF-treated control wounds (Figs 4c and 5). There was also a significant increase ($P < 0.001$) in receptor protein expression in epidermal keratinocytes of the denervated wounds (Figs 4d and 5). In contrast, NGF treatment of the denervated wounds caused a significant reduction ($P < 0.001$) in the expression of $P2Y_1$ receptors in keratinocytes (Fig. 4e) compared with the untreated denervated wounds (Fig. 5). In the control skin (Fig. 4a) there was weak staining of $P2Y_1$ receptors in the dermis, immediately adjacent to the overlying epidermis. In the NGF-treated innervated control wounds (Fig. 4c) as well as the NGF-treated denervated wounds (Fig. 4e) there was heavy positive staining of $P2Y_1$ receptors in the wound matrix underneath the epidermis. This staining was absent when the primary antibody was omitted and in preabsorption controls of the $P2Y_1$ receptor antibody with the corresponding peptide, which were performed on sections of control skin (Fig. 4f), NGF-treated innervated control wounds (Fig. 4g) as well as NGF-treated denervated wounds (Fig. 4h), suggesting that the dermal staining was specific.

$P2Y_2$ receptors were also found in the basal layer of normal, unwounded epidermis, and the distribution of $P2Y_2$ receptors was also altered in the epidermal wound edge of healing skin, where they were found throughout the basal and suprabasal layers (Fig. 6a,b). The level of $P2Y_2$ receptor protein expression, however, was unchanged in keratinocytes in the regenerating epidermis of the control wounds compared with keratinocytes from the unwounded control skin (Figs 6a,b and 7). There was no significant difference between $P2Y_2$ receptor labelling in the epidermal keratinocytes of the NGF-treated control wounds (Fig. 6c) compared with the untreated control wounds (Fig. 7). There was a significant decrease ($P < 0.001$) in $P2Y_2$ receptor protein expression in keratinocytes of the denervated wounds (Figs 6d and 7), but there was a significant increase ($P < 0.01$) in the expression of $P2Y_2$ receptors in the epidermis of the NGF-treated denervated wounds (Fig. 6e) compared with the untreated denervated wounds (Fig. 7). These results are summarized in Table 1.

Both the omission of the primary antibody and preabsorption with corresponding peptides were performed as controls. The immunoreaction was abolished after preabsorption of the $P2X_5$ (Fig. 1e), $P2X_7$ (Fig. 3d), $P2Y_1$ (Fig. 4f) or $P2Y_2$ (Fig. 6f)-receptor antibodies with the corresponding peptides, confirming the specificity of the immunoreactions.

Discussion

This study showed, using immunohistochemistry, that during wound healing the purinergic receptor protein expression patterns in the epidermis were altered. It would have been of interest to compare the protein expression with receptor mRNA expression by performing *in situ* hybridization, although it is known that mRNA expression and protein expression of receptors do not necessarily coincide.

Keratinocytes proliferate during wound healing, become activated and change to a migratory phenotype (37). Previous work has proposed that $P2Y_1$ and $P2Y_2$ receptors are involved in keratinocyte proliferation (2,19). In this study, the level of expression of both $P2Y_1$ and $P2Y_2$ receptors was unchanged in the epidermis of control wound edges compared with normal skin. However, the distribution of the receptors within the epidermis was altered, with both receptors expressed in suprabasal as well as basal keratinocytes at the

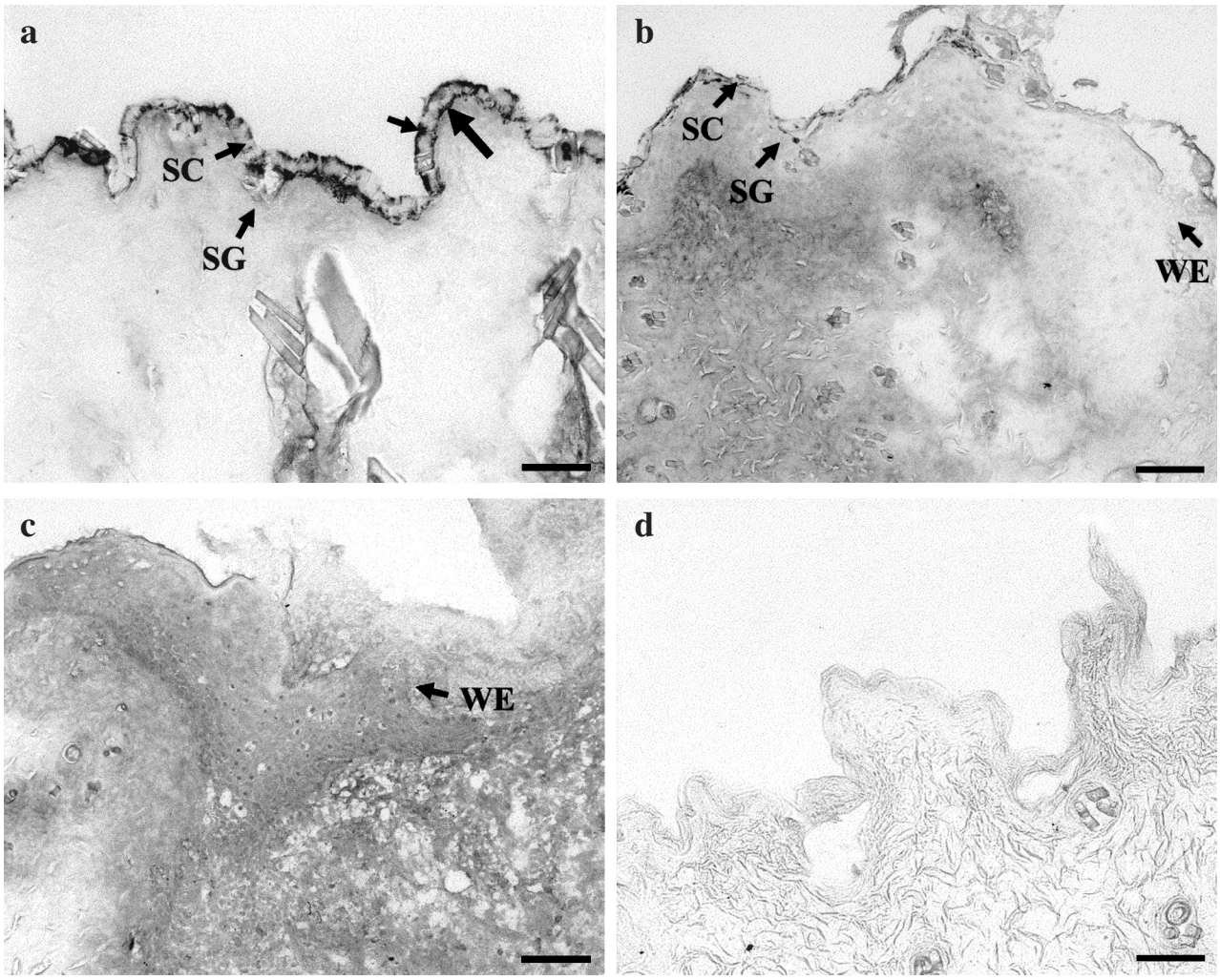

Figure 3. Comparison of $P2X_7$ receptor immunostaining in the epidermis of normal skin, and in the regenerating epidermal wound edge of innervated and denervated excisional wounds. (a) Normal control skin from the contralateral abdomen. $P2X_7$ receptors were found at the junction (large arrow) of the stratum granulosum (SG) and the stratum corneum (SC), and were also found on the outer edge of the stratum corneum (small arrow). Scale bar = 50 μm (b) Innervated control wound edge (WE). $P2X_7$ receptor expression was absent in control wounds compared with control skin. Scale bar = 100 μm (c) Denervated wound edge (WE). $P2X_7$ receptor expression was absent. Scale bar = 100 μm (d) Preabsorption of normal control skin: the immunoreaction was abolished after preabsorption of the $P2X_7$ receptor antibody with the corresponding peptide, confirming the specificity of the immunoreaction. Scale bar = 50 μm.

epidermal wound edge. This could represent part of the change of phenotype that keratinocytes undergo in order to become migratory during the wound healing process.

The expression of $P2X_5$ receptors in keratinocytes was significantly increased ($P < 0.01$) during wound healing. $P2X_5$ receptors are found in proliferating and differentiating keratinocytes in rat epidermis (1), but are thought to be more involved in keratinocyte differentiation (2). The expression of both $P2X_5$ and $P2Y_1$ receptors was significantly increased ($P < 0.001$) in keratinocytes of the regenerating epidermis of denervated wounds compared with control wounds, whereas the expression of $P2Y_2$ receptors was significantly decreased ($P < 0.001$). This could be explained by either the different nature of the agonists for these receptors or the role of NGF. ATP and ADP act as agonists at $P2X_5$ and $P2Y_1$ receptors, respectively, but uridine 5^1-triphosphate (UTP) is a potent agonist at $P2Y_2$ receptors. Thus, the receptors might be involved in different processes within the denervated epidermis. The difference in purinergic receptor expression in denervated wounds could be related to the role of NGF. NGF is involved in an autocrine loop within the epidermis to promote keratinocyte proliferation and has a trophic role in cutaneous innervation (38). The main cellular source of NGF in the skin is basal keratinocytes (39). It is possible that in a denervated wound there might be an extra requirement for NGF because of the need to supply trophic support to nerve fibres

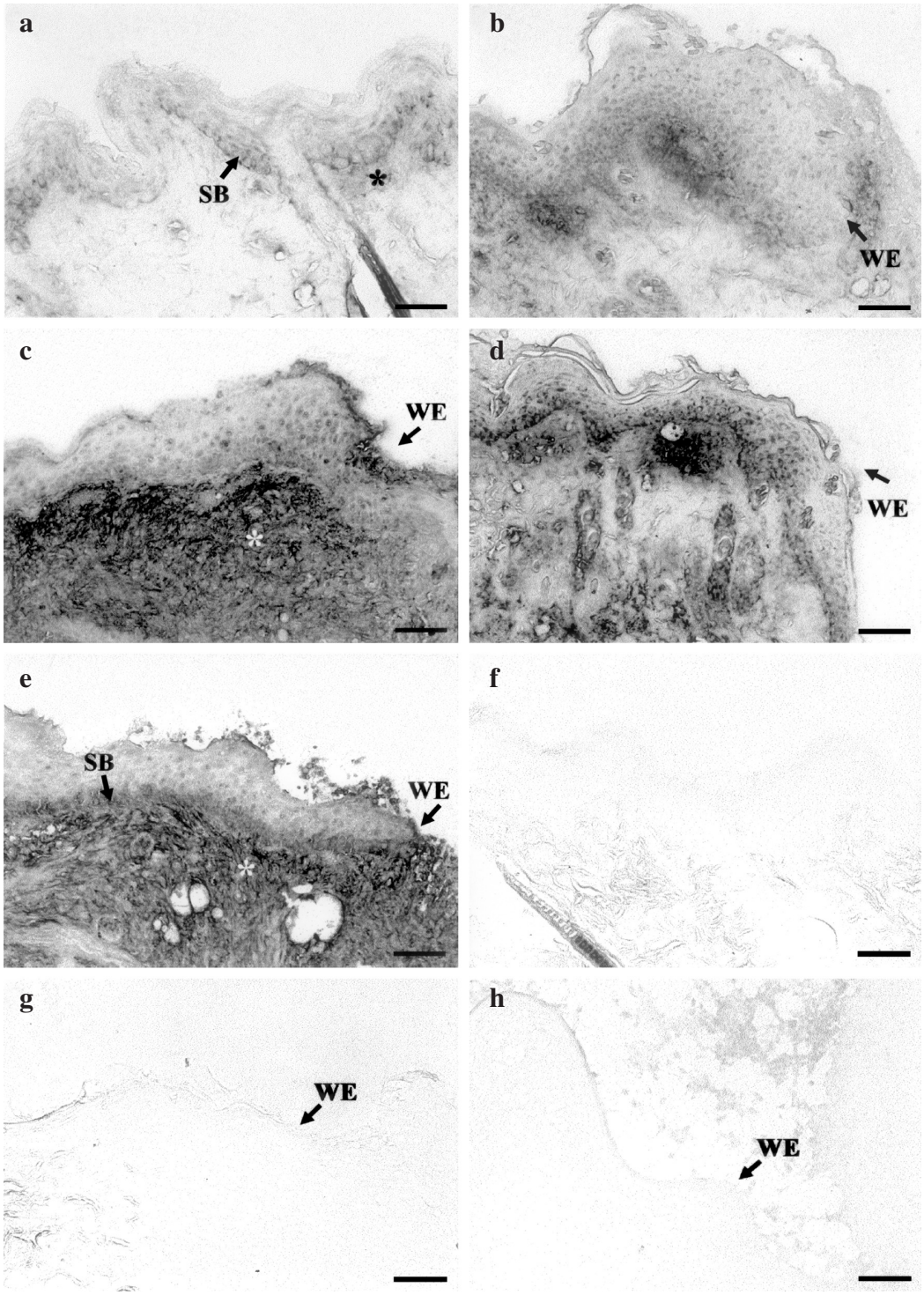

Figure 4. Comparison of $P2Y_1$ receptor immunostaining in the epidermis of normal skin, and in the regenerating epidermal wound edge of innervated and denervated excisional wounds and in both innervated and denervated wounds treated with nerve growth factor (NGF). (a) Normal control skin from the contralateral abdomen. $P2Y_1$ receptors were found in the basal layer (SB) of the epidermis in normal skin and also weakly in the underlying dermis (*). Scale bar = 50 μm. (b) Innervated control wound edge (WE). $P2Y_1$ receptors were also present throughout the suprabasal layers of the epidermis. Scale bar = 100 μm. (c) Innervated control NGF-treated wound edge (WE). $P2Y_1$ receptor expression was increased in the epidermis. Note heavy positive staining in wound matrix (white asterisk). Scale bar = 100 μm (d) Denervated wound edge (WE). $P2Y_1$ receptor expression was increased in the epidermis. Scale bar = 100 μm. (e) Denervated NGF-treated wound edge (WE). $P2Y_1$ receptor expression was reduced in the basal layer (SB) of the epidermis compared with untreated denervated wounds. Note heavy positive staining in wound matrix (white asterisk). Scale bar = 100 μm. (f–h) Preabsorption controls: (f) normal control skin; (g) innervated control NGF-treated wound edge; (h) denervated NGF-treated wound edge: the immunoreaction was abolished after preabsorption of the $P2Y_1$ receptor antibody with the corresponding peptide, confirming the specificity of the immunoreaction. Scale bars = 50 μm.

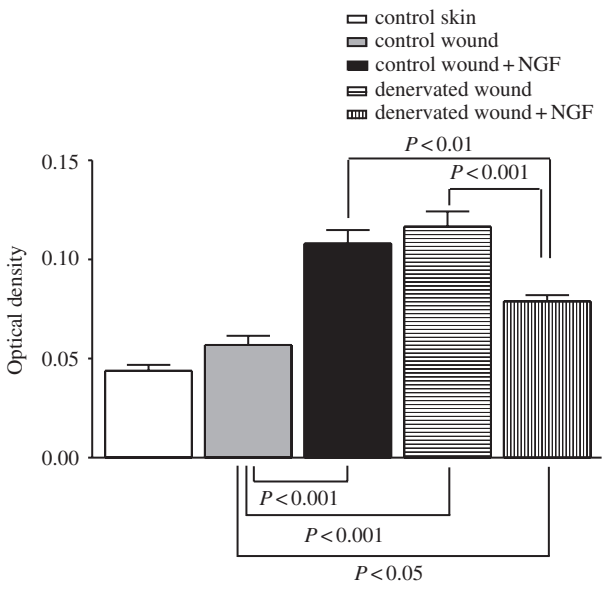

Figure 5. Histogram showing the optical density (arbitrary units) of $P2Y_1$ receptor immunostaining in epidermal keratinocytes of the different preparations (listed top right) in order to quantify the amount of immunostaining.

growing back into the denervated area. This need could place an extra burden on keratinocytes to synthesize NGF and so one could speculate that in a denervated wound, $P2X_5$ and $P2Y_1$ receptor expressions might be increased and $P2Y_2$ receptor expression might be decreased when keratinocytes become activated to synthesize NGF.

NGF accelerates the rate of normal wound healing (40,41). In epidermal keratinocytes from the NGF-treated control wounds there was a statistically significant increase ($P < 0.001$) in $P2X_5$ receptor expression compared with the untreated control wounds. It seemed that an intact nerve supply was necessary to up-regulate the expression of $P2X_5$ receptors with NGF because there was no statistically significant change in the level of $P2X_5$ receptor expression in keratinocytes of the regenerating epidermis of NGF-treated denervated wounds compared with the untreated denervated wounds.

Denervated rat skin has been found to show delayed wound healing (30). NGF has been shown to have a normalizing effect on the rate of healing of denervated wounds in rats (31). The protein expression of both $P2Y_1$ and $P2Y_2$ receptors was 'normalized' in keratinocytes of the regenerating epidermis of NGF-treated denervated wounds. $P2Y_1$ receptor protein expression in keratinocytes was significantly decreased ($P < 0.001$) in the epidermis of NGF-treated denervated wounds compared with the untreated denervated wounds, reducing the level of epidermal expression towards that seen in control wounds. However, in both the NGF-treated innervated control wounds and the NGF-treated denervated wounds there was heavy positive staining of $P2Y_1$ receptors in the wound matrix underneath the epidermis. In control skin there was weak staining of $P2Y_1$ receptors in the dermis, immediately adjacent to the overlying epidermis. This staining was absent after preabsorption of the $P2Y_1$ antibody with the corresponding peptide, suggesting that the dermal staining was specific in both control skin and the NGF-treated innervated and denervated wounds. $P2Y_1$ receptors are thought to be mitogenic in endothelial cells (18), and are expressed on platelets (6), immune cells (42,43) and fibroblasts (44). These cell types would certainly be present in the underlying wound matrix. It appears that NGF-treatment up-regulated the expression of $P2Y_1$ receptors in the wound matrix, and perhaps this might enhance wound healing. With respect to $P2Y_2$ receptors, there was no significant difference between the level of expression of $P2Y_2$ receptors in keratinocytes of the regenerating epidermis of the NGF-treated denervated wounds and the control wounds. $P2Y_2$ receptors were significantly increased in epidermal keratinocytes ($P < 0.01$) in the NGF-treated denervated wounds compared with the untreated denervated wounds. This might also represent a 'normalization' of $P2Y_2$ receptor expression in the NGF-treated denervated wounds towards the pattern of expression seen in the untreated control wounds.

During the proliferative phase of epidermal wound healing, keratinocyte proliferation is increased and apoptosis is reduced (45,46), so that the regenerating epidermis is thickened. $P2X_7$ receptors are strongly linked to apoptosis (14,15), and it has been proposed that $P2X_7$ receptors are involved in the specialized apoptotic process of terminal differentiation of keratinocytes (1,2). $P2X_7$ receptor protein expression disappeared in regenerating keratinocytes at the normal wound edge, whereas the receptors were found in intact epidermis. This reduction in $P2X_7$ receptors may be linked to the reduced apoptosis found in healing epidermis. $P2X_7$ receptors were absent in both the untreated and NGF-treated experimental wound-healing preparations. Autocrine NGF is known to prevent keratinocyte apoptosis (38). The fact that $P2X_7$ receptor expression was absent in both the NGF-treated control and denervated wounds would fit with the anti-apoptotic role of NGF, but as $P2X_7$ receptors were absent in the untreated wounds the receptors would not have been further down-regulated by NGF.

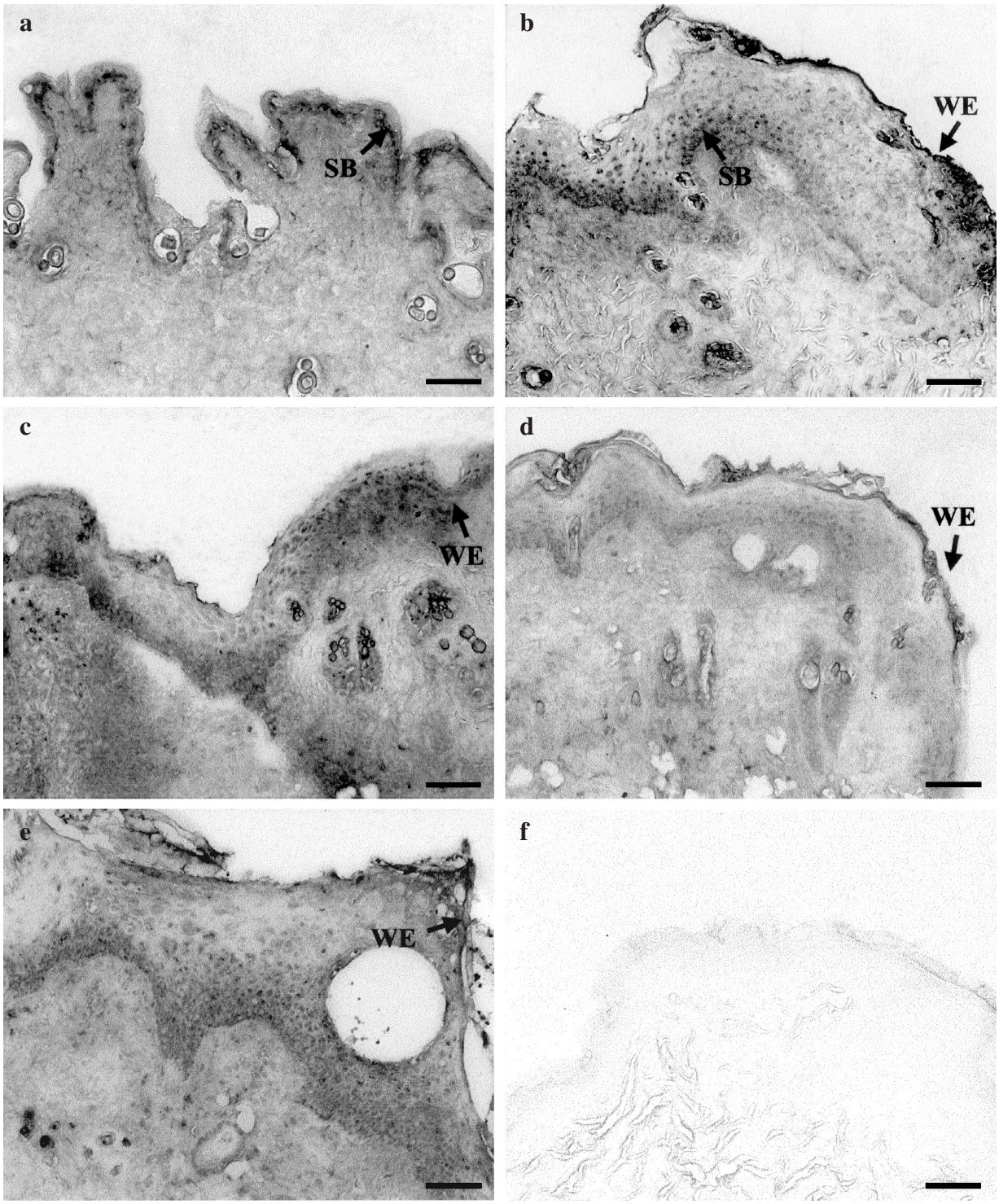

Figure 6. Comparison of $P2Y_2$ receptor immunostaining in the epidermis of normal skin, and in the regenerating epidermal wound edge of innervated and denervated excisional wounds and in both innervated and denervated wounds treated with nerve growth factor (NGF). (a) Normal control skin from the contralateral abdomen. $P2Y_2$ receptors were found in the basal layer (SB) of normal epidermis. Scale bar = 50 μm. (b) Innervated control wound edge (WE). $P2Y_2$ receptors were also found throughout the suprabasal layers of the epidermis. Scale bar = 100 μm. (c) Innervated control NGF-treated wound edge (WE). $P2Y_2$ receptor expression in the epidermis was unchanged compared with untreated control wounds. Scale bar = 100 μm. (d) Denervated wound edge (WE). $P2Y_2$ receptor expression in the epidermis of denervated wounds was reduced compared with control wounds. Scale bar = 100 μm. (e) Denervated NGF-treated wound edge (WE). $P2Y_2$ receptor expression was increased in the epidermis compared with untreated denervated wounds. Scale bar = 100 μm. (f) Preabsorption of normal control skin: the immunoreaction was abolished after preabsorption of the $P2Y_2$ receptor antibody with the corresponding peptide, confirming the specificity of the immunoreaction. Scale bar = 50 μm.

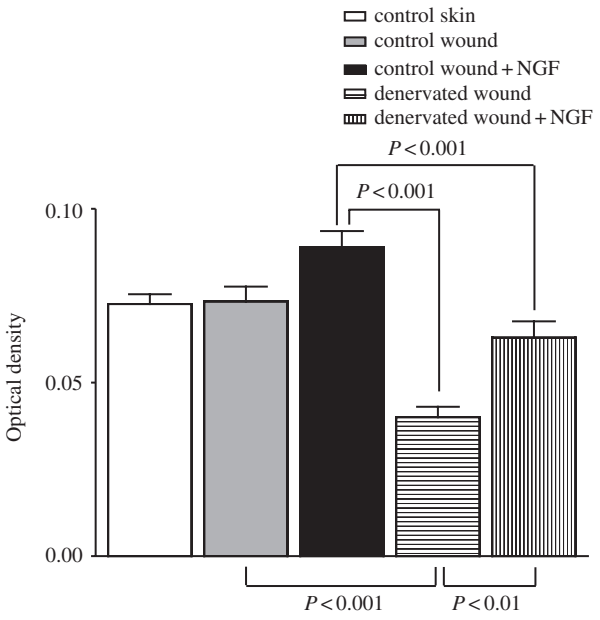

Figure 7. Histogram showing the optical density (arbitrary units) of $P2Y_2$ receptor immunostaining in the epidermal keratinocytes of different preparations (listed top right) in order to quantify the amount of immunostaining.

NGF is part of an autocrine system regulating epidermal homeostasis (38). It could be possible that NGF participates in the re-epithelialization of the wound by up-regulating the expression of $P2X_5$ and $P2Y_1$ receptors and down-regulating the expression of $P2X_7$ receptors in the epidermis. This suggests that these receptors might be involved in the balance between the number of keratinocytes being produced in the basal layer and the number of cells being shed at the cell surface.

The relationship between NGF, skin innervation and purinergic receptors still remains unclear, particularly whether there is a direct or indirect effect of the growth factor on the expression of the purinergic receptors. Further studies, both *in vitro* and *in vivo*, will be needed to elucidate these points. However, from the results of our study it appears that a relationship exists between these two components, as identified by the addition of NGF to the experimental wound. This correlation is also highlighted by the difference in results between normal and denervated skin. Indeed the latter experimental situation might reflect a similarity to what has been found in diabetic neuropathies, where the absence of skin innervation is linked to a decrease of NGF in epidermal keratinocytes. Hence, the addition of NGF in the experimental situation may re-establish the lost equilibrium between NGF and surrounding structures.

In conclusion, $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptor expression in the epidermis was altered during wound healing. $P2Y_1$ receptor expression was significantly increased in keratinocytes of the regenerating epidermis of the denervated wounds but $P2Y_2$ receptor expression was significantly decreased. NGF treatment enhanced $P2X_5$ and $P2Y_1$ receptor expressions in epidermal keratinocytes of the innervated wounds. NGF treatment reduced the expression of $P2Y_1$ receptors but enhanced the expression of $P2Y_2$ receptors in keratinocytes from denervated wounds compared with untreated denervated wounds. $P2X_7$ receptors were absent in all experimental wound-healing preparations. Further work is needed to elucidate whether purinergic receptors may have a functional role in re-epithelialization. It would be of interest to correlate purinergic receptor expression with the degree of wound closure over several time points. Purinergic receptors could represent new targets for wound-healing research, and functional studies involving specific agonists and antagonists may lead to new approaches to wound re-epithelialization.

Table 1. Summary of purinergic receptor sub-type expression in epidermal keratinocytes of normal control skin and in the regenerating epidermis of control wounds, nerve growth factor (NGF)-treated control wounds, denervated wounds and NGF-treated denervated wounds

	Purinergic receptor expression in epidermal keratinocytes				
Receptor sub-type	Control skin	Control wound	Control wound + NGF	Denervated wound	Denervated wound + NGF
$P2X_5$	+	++	++++	+++	+++
$P2X_7$	+	0	0	0	0
$P2Y_1$	+	+ (altered distribution)	+++	+++	++
$P2Y_2$	+	+ (altered distribution)	+	–	+

+: level of receptor expression in control epidermis; ++, +++, ++++: increased receptor expression compared with control epidermis; –: reduced receptor expression compared with control epidermis; 0: no staining.

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