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


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 P2X5, P2X7, P2Y1 and P2Y2 receptors, and immunostaining of keratinocytes was quantified using optical densitometry.

In normal rat epidermis, P2Y1 and P2Y2 receptors were found in the basal layer where keratinocytes proliferate; P2X5 receptors were associated with proliferating and differentiating epidermal keratinocytes in basal and suprabasal layers; P2X7 receptors were associated with terminally differentiated keratinocytes in the stratum corneum. In the regenerating epidermis of denervated wounds, P2Y1 receptor protein expression was significantly increased in keratinocytes (P < 0.001) but P2Y2 receptor protein expression was significantly decreased (P < 0.001). Conversely, NGF treatment of denervated wounds, reduced expression of P2Y1 receptors (P < 0.001) in keratinocytes but enhanced expression of the P2Y2 receptors (P < 0.01) compared with untreated denervated wounds. In innervated wounds, NGF treatment enhanced P2X5 (P < 0.001) and P2Y1 receptor protein (P < 0.001) expression in keratinocytes. P2X7 receptors were absent in all experimental wound healing preparations. P2X5, P2X7, P2Y1 and P2Y2 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.

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...
(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). P2X5 receptors are found in proliferating and differentiating keratinocytes in rat epidermis (1), but are thought to be more involved in keratinocyte differentiation (2). P2X5 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, P2X5 receptors are sequentially expressed during development (11). P2X5 receptors have been implicated in the regulation of osteoblastic differentiation and proliferation (12) and in triggering the differentiation of skeletal muscle satellite cells (13).

P2X7 receptors are strongly linked to apoptosis (14,15). The P2X7 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 P2X7 receptors are involved in the apoptotic process of terminal differentiation of keratinocytes because P2X7 receptors are associated with dying keratinocytes in the stratum corneum, and BzATP, a potent P2X7 receptor agonist, can induce a significant decrease in keratinocyte number in culture (1,2).

P2Y1 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). P2Y2 receptor mRNA has been localized in human epidermal basal cells via in situ hybridization. UTP, a P2Y2 receptor subtype agonist, has also been shown to cause proliferation of keratinocytes (19) and HaCaT cells (20). P2Y2 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 trisphosphate 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 P2X5, P2X7, P2Y1 and P2Y2 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 40μg/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).
previously verified by immunoblotting with membrane prepara-
tics, Huntsville, AL). The specificity of the antisera had been
zealand rabbits with the peptides (performed by Research Genet-
ants were raised by multiple monthly injections of New
obtained from Alomone Laboratories (Jerusalem, Israel), and
ated by Llewellyn-Smith et al. (34,35). Air-dried sections were
collection on gelatin-coated slides and air-dried at
heterologous expression systems, and were shown to be
receptor subtype specific (33). P2X3 and P2X7 receptor ant-
bodies were provided by Roche Bioscience (Palo Alto, CA) and
kept frozen at a stock concentration of 1 mg/ml.
Polyclonal anti-P2Y1 and P2Y2 receptor antibodies were
obtained from Alomone Laboratories (Jerusalem, Israel), and
corresponded to the third extracellular loop of the P2Y1 (AA
Endogenous peroxidase was blocked for 10 min with 50% metha-
fixed for 2 min in 4% formaldehyde in 0.1 M phosphate buffer,
with the primary antibodies diluted to 1:100 or 1:200
tion product. Sections were washed three times with PBS after
1% NHS in PBS for 30 min, followed by ExtrAvidin peroxidase
1% NHS in PBS for 30 min, followed by ExtrAvidin peroxidase
ting wound edge (Fig. 1b). P2X5 receptor pro-
test was absent in the epidermis of five out of the
Optical density measurements were not performed
immunostaining for each antibody, all
sections were stained at the same time. Two images of the regen-
epidermis was also absent in the
Results
P2X5 receptor protein expression, as measured by
optical densitometry, was significantly increased
(P < 0.01) in keratinocytes of the regenerating epi-
dermis in the control wounds compared with kerat-
inocytes in the unwounded control epidermis (Figs 1a, b and 2).
Expression was particularly increased in migratory keratinocytes at the prolif-
erating wound edge (Fig. 1b). P2X5 receptor protein
expression was significantly increased
(P < 0.001) in keratinocytes in the NGF-treated control
wounds (Figs 1c and 2). P2X5 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
P2X5 receptors in the epidermis of the denervated
wounds (Fig. 2). So, NGF increased the expression of
P2X5 receptors in the epidermis of the normally
innervated wounds but NGF did not have an
effect on the expression of P2X5 receptors in the epidermis of the
denervated wounds.
P2X7 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). P2X7 receptor protein
expression was absent in the epidermis of five out of the
six rats with denervated wounds (Fig. 3c). P2X7 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 P2X₅ 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. P2X₅ 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). P2X₅ 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 P2X₅ receptors in normally innervated wounds. Scale bar = 100 μm. (d) Denervated wound edge (WE). P2X₅ 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 P2X₅ receptor antibody with the corresponding peptide, confirming the specificity of the immunoreaction. Scale bar = 50 μm.
because of the very low expression of this receptor in the majority of the sections.

P2Y1 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, P2Y1 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 P2Y1 receptor protein expression in keratinocytes of the regenerating epidermis of the NGF-treated control wounds (Figs 4c and 5). There was no significant difference between P2Y2 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 P2Y2 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 P2Y2 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 P2X5 (Fig. 1e), P2X7 (Fig. 3d), P2Y1 (Fig. 4f) or P2Y2 (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 P2Y1 and P2Y2 receptors are involved in keratinocyte proliferation (2,19). In this study, the level of expression of both P2Y1 and P2Y2 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
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 P2X5 receptors in keratinocytes was significantly increased \((P < 0.01)\) during wound healing. P2X5 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 P2X5 and P2Y1 receptors was significantly increased \((P < 0.001)\) in keratinocytes of the regenerating epidermis of denervated wounds compared with control wounds, whereas the expression of P2Y2 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 P2X5 and P2Y1 receptors, respectively, but uridine 5’-triphosphate (UTP) is a potent agonist at P2Y2 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 3. Comparison of P2X7 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. P2X7 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). P2X7 receptor expression was absent in control wounds compared with control skin. Scale bar = 100 μm (c) Denervated wound edge (WE). P2X7 receptor expression was absent. Scale bar = 100 μm (d) Preabsorption of normal control skin: the immunoreaction was abolished after preabsorption of the P2X7 receptor antibody with the corresponding peptide, confirming the specificity of the immunoreaction. Scale bar = 50 μm.
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.
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 P2X5 receptor expression compared with the untreated control wounds. It seemed that an intact nerve supply was necessary to up-regulate the expression of P2X5 receptors with NGF because there was no statistically significant change in the level of P2X5 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 P2Y1 and P2Y2 receptors was ‘normalized’ in keratinocytes of the regenerating epidermis of NGF-treated denervated wounds. P2Y1 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 P2Y1 receptors in the wound matrix underneath the epidermis. In control skin there was weak staining of P2Y1 receptors in the dermis, immediately adjacent to the overlying epidermis. This staining was absent after preabsorption of the P2Y1 antibody with the corresponding peptide, suggesting that the dermal staining was specific in both control skin and the NGF-treated innervated and denervated wounds. P2Y1 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 P2Y1 receptors in the wound matrix, and perhaps this might enhance wound healing. With respect to P2Y2 receptors, there was no significant difference between the level of expression of P2Y2 receptors in keratinocytes of the regenerating epidermis of the NGF-treated denervated wounds and the control wounds. P2Y2 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 P2Y2 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. P2X7 receptors are strongly linked to apoptosis (14,15), and it has been proposed that P2X7 receptors are involved in the specialized apoptotic process of terminal differentiation of keratinocytes (1,2). P2X7 receptor protein expression disappeared in regenerating keratinocytes at the normal wound edge, whereas the receptors were found in intact epidermis. This reduction in P2X7 receptors may be linked to the reduced apoptosis found in healing epidermis. P2X7 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 P2X7 receptor expression was absent in both the NGF-treated control and denervated wounds would fit with the anti-apoptotic role of NGF, but as P2X7 receptors were absent in the untreated wounds the receptors would not have been further down-regulated by NGF.
Figure 6. Comparison of P2Y2 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. P2Y2 receptors were found in the basal layer (SB) of normal epidermis. Scale bar = 50 μm. (b) Innervated control wound edge (WE). P2Y2 receptors were also found throughout the suprabasal layers of the epidermis. Scale bar = 100 μm. (c) Innervated control NGF-treated wound edge (WE). P2Y2 receptor expression in the epidermis was unchanged compared with untreated control wounds. Scale bar = 100 μm. (d) Denervated wound edge (WE). P2Y2 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). P2Y2 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 P2Y2 receptor antibody with the corresponding peptide, confirming the specificity of the immunoreaction. Scale bar = 50 μm.
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 P2X5 and P2Y1 receptors and down-regulating the expression of P2X7 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, P2X5, P2X7, P2Y1 and P2Y2 receptor expression in the epidermis was altered during wound healing. P2Y1 receptor expression was significantly increased in keratinocytes of the regenerating epidermis of the denervated wounds but P2Y2 receptor expression was significantly decreased. NGF treatment enhanced P2X5 and P2Y1 receptor expressions in epidermal keratinocytes of the innervated wounds. NGF treatment reduced the expression of P2Y1 receptors but enhanced the expression of P2Y2 receptors in keratinocytes from denervated wounds compared with untreated denervated wounds. P2X7 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.

Acknowledgements

The support of Roche Bioscience, Palo Alto, California, USA, who provided P2X5 and P2X7 receptor antibodies, is gratefully acknowledged. Aina Greig is the recipient of a Research Fellowship from The Wellcome Trust and The Simpson Surgical Research Fellowship from The Royal College of Surgeons of England, UK.

References


