Measurement of NF-κB in normal and reconstructed human skin *in vitro*

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The survival of grafted donor skin for the treatment of burn injuries depends on several factors including wound bed vascularisation and the intensity of acute inflammation shortly after injury. However, acceptance rates approximate 50% at best and therefore a clinical need exists for improvement. The aim of the study was to develop a method for assessing the inflammatory response of cells in skin tissue based on activation of the NF-kappa *B* (NF-κ*B*) transcription factor complex, thereby providing a basis for analysing the inflammatory component and anti-inflammatory strategies for tissue-engineered treatments. We have extended a standard method of measuring NF-κB in monolayer cultures that relies on determining translocation of the p65 subunit from the cytoplasm to the nucleus. Normal human skin and tissue engineered skin was analysed using an immunofluorescence microscopy technique, that revealed base line NF-κB activation in the epidermis and dermis were different. It was possible to determine the activation of NF-κB in skin tissue, enabling correlation that NF-κB measurement is a sensitive indicator of cellular responses in 3-D tissue. The approach will provide a basis for early responses of skin cells in determining the efficacy of anti-inflammatory delivery via tissue-engineered scaffolds for burn injuries.

Introduction

After a decade of research into the development of tissue engineered skin for permanent wound cover for patients who have suffered extensive full-thickness skin loss, the focus is now turning towards improving the survival of grafted skin on the patient. Tissue engineered skin is most commonly based on autologous keratinocytes and fibroblasts introduced to either a bovine collagen matrix, as demonstrated to perform well in badly burned children by Boyce *et al*. [1], or to a sterilised donor allodermis such as that produced from our laboratory [2, 3]. Irrespective of the nature of the reconstructed skin, cells within the 3-D structure have to survive an acute period of hypoxia and inflammation when introduced to the wound bed.

The problem is that grafted tissue-engineered skin lacks an intrinsic vasculature. The only cells present in the most advanced of reconstructed skin tissue are keratinocytes for the synthesis of a superficial epidermis and fibroblasts for dermal collagen production. Until successful neo-vasculature occurs, the donor graft has to survive extended periods of hypoxia. The early wound bed also contains a very high level of soluble proinflammatory cytokines, which initiate the local inflammatory response. Inflammation is essential for preventing infection during injury and is also a vital part of wound repair, accelerating cellular migration, proliferation and dermal remodelling [4, 5]. However, it also causes problems for healthy autologous donor cells, compromising their ability to survive short periods of acute inflammation. Hence, the intensity of inflammation at the wound site contributes to rejecting the donor material.

Thus grafted material may be lost during the inflammatory phase of wound healing even when autologous cells are used. The ''take rate'' of cultured epithelial autografts on patients can be disappointing overall [6], and inflammation and the inability of cells to survive the hostile wound bed are therefore important. This has been overlooked until now. In designing any reconstructed skin we suggest that modulating local excessive inflammation in the wound environment (while maintaining a permissive level for triggering repair and preventing infection) may be a useful approach for cell and tissue survival, improving graft acceptance rates. In response to this, it is therefore important to develop methods in parallel for assessing the response of cells to acute inflammatory signals in tissue-engineered skin.

Human keratinocyte and fibroblast cells (in common with many other body cells) respond to proinflammatory

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cytokines with a rapid activation of NF-κB [7]. NF-κB is a genetic intracellular transcription factor that regulates the early synthesis of genes principally involved in the immune and inflammatory response. It is positioned in the cytoplasm in cells that have not been exposed to an inflammatory stress, but is activated rapidly in response to a variety of stimuli including cytokines (e.g. tumour necrosis factor-α (TNF-α)), viral infection, lipopolysaccharide, ultraviolet exposure and pH induced by hypoxia [8–12]. Activation leads to the synthesis of key membrane-associated cell adhesion molecules (e.g. intercellular adhesion molecule-1 (ICAM-1)), necessary for lymphocyte interaction [13]. As such NF-κB activation is an early central indicator of cellular responses to inflammation, as well as associated stress.

The aim of this study was therefore to develop a method for assessing the response of cells to inflammatory and associated stimuli in normal and reconstructed human skin tissue, based on measuring the activation of the NF-κB transcription factor. We compared the effect of adding a pro-inflammatory cytokine (TNF-α) on NF-κB activity in cultured human dermal fibroblasts in monolayer (principally for the purpose of validating the method) and then extended the approach for assessing normal and reconstructed human skin. This study therefore reports on establishing a baseline method for developing approaches directed at improving cellular responses to cytotoxic stress in reconstructed skin tissue, but will also be applicable to measurement in other tissues.

Materials and methods

Materials were obtained from the following manufacturers: phosphate-buffered saline (PBS) tablets (Oxoid, Unipath, Hampshire, UK); Dulbecco's modified Eagle's medium (DMEM) (ICN Flow, Thame, Oxfordshire, UK); Ham's F12 medium, glutamine, amphotericin B, penicillin and streptomycin (Gibco Europe, Life Technologies, Paisley, UK), foetal calf serum (FCS) (Advanced Protein Products, Brierley Hill, West Midlands, UK); trypsin (Difco Laboratories, Detroit, MI, USA); collagenase A (Boehringer-Mannheim, Lewes, East Sussex, UK); bovine serum albumin, cholera toxin, epidermal growth factor (EGF), adenine, insulin, sodium chloride, transferrin, triiodothyronine, ethylenediamine tetraacetic acid (EDTA) and trypan blue (Sigma, Poole, Dorset, UK). TNF-α (Boehringher-Mannheim, Lewes, UK); paraformaldehyde, Triton X100, ammonium chloride (BDH Chemicals Ltd, Poole, UK); anti-NF-κB/p65 (C-20) rabbit polyclonal IgG antibody, normal rabbit IgG (Santa Cruz Biotechnology, Ca, USA); biotinylated goat anti-rabbit IgG, fluorescein streptavidin, diaminophenolindole (DAPI, Vector Laboratories Inc., Ca, USA); ProLong Antifade kit (Eugene, Oregon, USA).

Cell culture

Normal human keratinocytes and fibroblasts were isolated from skin obtained from routine operations (abdominoplasty and breast reduction) according to local Ethical Committee guidelines (NHS Trust, Sheffield, UK). Keratinocytes were freshly isolated from the dermal/epidermal junction after trypsinisation (4 °C, 18 h) following the method of Goberdhan *et al*. [14]. Keratinocytes were cultured in Green's medium (DMEM and Ham's F12 medium in a 3 : 1 (v/v) ratio supplemented with 10% (v/v) FCS, 10 ng/ml EGF, 0.4 μg/ml hydrocortisone, 10^{-10} mol/l cholera toxin, 1.8×10^{-4} mol/l adenine, 5 μg/ml insulin, 5 μg/ml transferrin, 2×10^{-3} mol/l glutamine, 2×10^{-7} mol/l triiodothyrionine, 0.625 μg/ml amphotericin B, 100 IU/ml penicillin and 100 μg/ml streptomycin). Fibroblasts were established as described previously [15]. Briefly, remaining dermis left after trypsinisation for keratinocyte extraction was washed in PBS, finely minced and incubated in 0.5% (w/v) collagenase A at 37 °C for 16 h. Fibroblasts obtained from this enzyme digest were cultured in DMEM supplemented with 10% (v/v) foetal calf serum, 2×10^{-3} mol/l glutamine, 0.625 μg/ml amphotericin B, 100 IU/ml penicillin and 100 μg/ml streptomycin and were used between passages 4 and 9 for experimentation.

Normal human skin

Normal human skin was obtained from routine operations as above. Samples prior to experimentation were washed thoroughly with PBS and stored at 4 °C in PBS. Tissue was prepared such that samples contained an epidermal layer, papillary and reticular dermis. Samples were placed for one to three days at an air/liquid interface on stainless steel grids in Green's media at 37 °C in a 5% (v/v) CO_2 humidified atmosphere for experimentation.

Acellular de-epidermalised dermis (DED)

Split thickness skin was dehydrated with increasing concentrations of glycerol and then sterilised using a standard ethylene oxide method [3]. Skin was then rehydrated in PBS and incubated in 1 M sodium chloride solution (4 h) to remove the epidermis. The remaining dermis was rendered acellular by hypotonic lysis in distilled water.

Skin composite preparation

Reconstructed skin containing fibroblasts and keratinocytes was produced using DED as described previously [3]. Briefly, stainless steel rings of 1 cm^2 internal diameter were applied with pressure to the reticular surface of 1.8 cm^2 pieces of DED in six well plates. Where required, 1×10^5 fibroblasts in 0.5 ml culture medium were introduced to the reticular surface for 18 h prior to the addition of keratinocytes (1×10^6 in 0.5 ml keratinocyte culture medium) to the papillary surface for 18 h, submerged prior to raising to an air/liquid interface on stainless steel grids and cultured for 15 days in Green's medium. Composite medium was replenished three times per week.

Experimental conditions

TNF-α (2.0 ng/ml) was added to monolayers of fibroblasts and reconstructed skin for 30 min. The action of

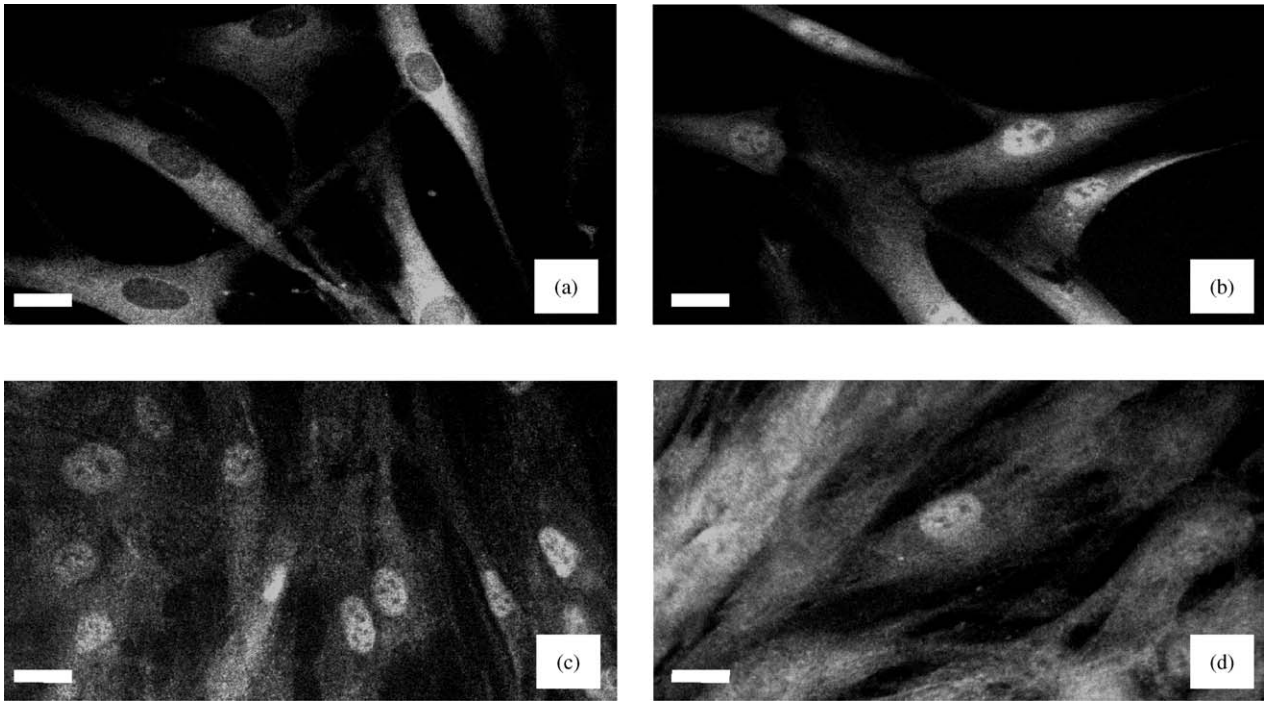

Figure 1 Cultured human dermal fibroblasts immunolabelled for the p65 subunit of NF-κB. (a) Control unstimulated cells; (b) stimulated with TNF-α (200 units/ml) for 1 h; (c) incubated in a 37 °C/100% (v/v) air environment for 60 min and (d) incubated in culture medium at pH 8.0 for 60 min (37 °C/5% CO_2/95% air). Bar = 20 μm.

culturing fibroblasts in a reduced carbon dioxide environment and in culture medium varying in pH (from 6.3 to 8.5) for up to 80 min was investigated as parameters responsible for activating NF-κB. After experimentation, medium was removed, and the samples were washed with phosphate buffered saline (PBS) three times. Fibroblasts were fixed with 2% (v/v) paraformaldehyde for 10 min, washed again with PBS (× 3) and permeablised using 0.1% (v/v) Triton X100 for 20 min, washed with PBS (× 3) and then neutralised with 50 mM ammonium chloride in PBS for 10 min. After experimental treatment, normal human skin or reconstructed skin was frozen in isopentane (− 150 °C) and oriented cryosection blocks made using OCT. Frozen sections (5 μm) were processed for immunolabelling studies.

Immunofluorescent labelling of NF-κB/p65

Cultured dermal fibroblasts (fixed as above) or 5 μm skin sections were incubated with 5% (w/v) bovine serum albumin diluted in PBS at room temperature (RTP) for 60 min, and then incubated with anti-NF-κB/p65 (C-20) rabbit polyclonal IgG antibody for 60 min at RTP (1 : 100 v/v, in blocking buffer/PBS) and washed with PBS (× 3). Samples were then incubated with a biotinylated goat anti-rabbit IgG (1 : 1000 (v/v)) for 60 min at RTP and washed with PBS (× 3), followed by incubation with fluorescein streptavidin (1 : 100 (v/v) in PBS) and 4′, 6-diamino-2-phenylindole (DAPI (1 : 1000 (v/v) in PBS)) for 30 min. After further washing with PBS (× 3) microscope slides were mounted using ProLong antifade. Negative control samples were processed by substituting the primary antibody with a preimmune isotype IgG antibody.

NF-κB image analysis

NF-κB is inactive in unstimulated proliferating cells. This is readily observed as cytoplasmic localisation by immunolabelling for the NF-κB/p65 subunit. Activation of NF-κB (e.g. by TNF-α stimulation) is observed as a rapid translocation to the nucleus. Therefore, a computer software image analysis method was used (Openlab v.3.0.2, Improvision, UK) to ''score'' mean NF-κB activity in a population of cells, either in a cultured monolayer or in a tissue section, based on cellular localisation. Fluorescence micrographs of immunolabelled samples were taken using a Leica DM-IRB inverted fluorescent microscope using epifluorescent illumination at $\lambda_{ex} = 495$ nm, $\lambda_{em} = 515$ nm (for fluorescein isothiocyanate (FITC)/NF-κB visualisation) and $\lambda_{ex} = 358$ nm, $\lambda_{em} = 461$ nm (for DAPI/nuclei visualisation). The nucleus of each cell in a microscope field of view was initially located using the DAPI signal alone. The peripheral outline was registered (dia-1) corresponding to the exact cross sectional area of the nucleus. A second peripheral contour was then made outside of the dia-1 area (dia-2) that described a cytoplasmic region for that cell, but falling within the cellular membrane. Using the FITC (NF-κB) signal, dia-1 versus dia-2 intensity was calculated using image analysis (Openlab v3.0.2). Thus, NF-κB activation could be calculated based on the ratio of nucleus versus cytoplasmic localisation.

Statistical analysis

Student's unpaired *t*-test was used to analyse differences between control unstimulated and cytokine/stress stimulated samples ($*p < 0.05$; $**p < 0.01$; $***$ $p < 0.001$).

Results

Effect of proinflammatory cytokine TNF-α and cell culture stress on NF-κB activation in cultured dermal fibroblasts

Cultured human dermal fibroblasts were stimulated with TNF-α (200 units/ml) for 1 h and then immunolabelled for the p65 transcriptionally active subunit of NF-κB. Unstimulated control cells demonstrated exclusive labelling in the cytoplasm, indicating that the transcription factor was inactive under these conditions (Fig. 1(a)). However, cells that were stimulated with TNF-α contained nuclear expression of NF-κB, indicating the potential of fibroblasts to respond to proinflammatory stimuli rapidly (Fig. 1(b)). We then investigated the sensitivity of this technique for other cellular stress activating factors. The culture of cells in a monolayer is a very common and straightforward practice; in comparison the culture of tissues is less common. We were interested in the association between cell culture pH and acidosis resulting from conditions that commonly arise in culture, as it is known that pH can trigger the inflammatory NF-κB pathway. We investigated this using cultured fibroblast cells incubating samples in a 37 °C/100% (v/v) air environment (as opposed to a standard 95% air/5% CO_2) for different periods of time. NF-κB activation was analysed using an imagine analysis method that relies upon nuclear versus cytoplasmic localisation. Unstimulated fibroblasts and TNF-α stimulated fibroblasts were used as negative and positive control samples (described above), respectively. Fig. 1(a) shows the location of NF-κB in control cells, while Fig. 1(c) shows cells incubated in an ''unbuffered'' environment for 60 min. Analysis of the intracellular location of NF-κB revealed that incubation of fibroblasts in an unbuffered environment was associated with a rapid and progressive activation of NF-κB. The degree of activation increased from 10 to 55% after 80 min, with as little as a 20-min unbuffered incubation increasing activity two-fold (Fig. 2(a), $p < 0.05$).

Relationship between cell culture incubation, medium pH and NF-κB

We then investigated whether the activation of NF-κB arising out of non-ideal culture conditions arose directly to changes in medium pH. Freshly prepared, fibroblast-conditioned Greens medium (separated from the cells) and medium (in direct contact with cells) were exposed to the same conditions as above (37 °C/100% (v/v) air) for 0 to 60 min and medium pH was measured. Removal of fibroblasts from a standard 5% (v/v) CO_2/bicarbonate buffered environment led to a rapid increase in the pH of cell culture medium, as shown in Fig. 2(b). The initial pH of the culture medium was 7.3, but rose to 7.8 after a 60-min removal from the 5% CO_2 environment. A similar rise in pH was seen (from 7.65 to 7.82) if cell-free medium was used. We then investigated whether a direct change of culture medium pH could activate fibroblast NF-κB activity. Cultured fibroblast cells were placed in medium adjusted to a range of pH's (6.3, 6.8, 7.3, 7.7, 8.0 or 8.5) for 60 min and incubated in a standard 5% CO_2/95% air incubator. Results demonstrated that a relatively small change in pH, either above or below 7.3, was

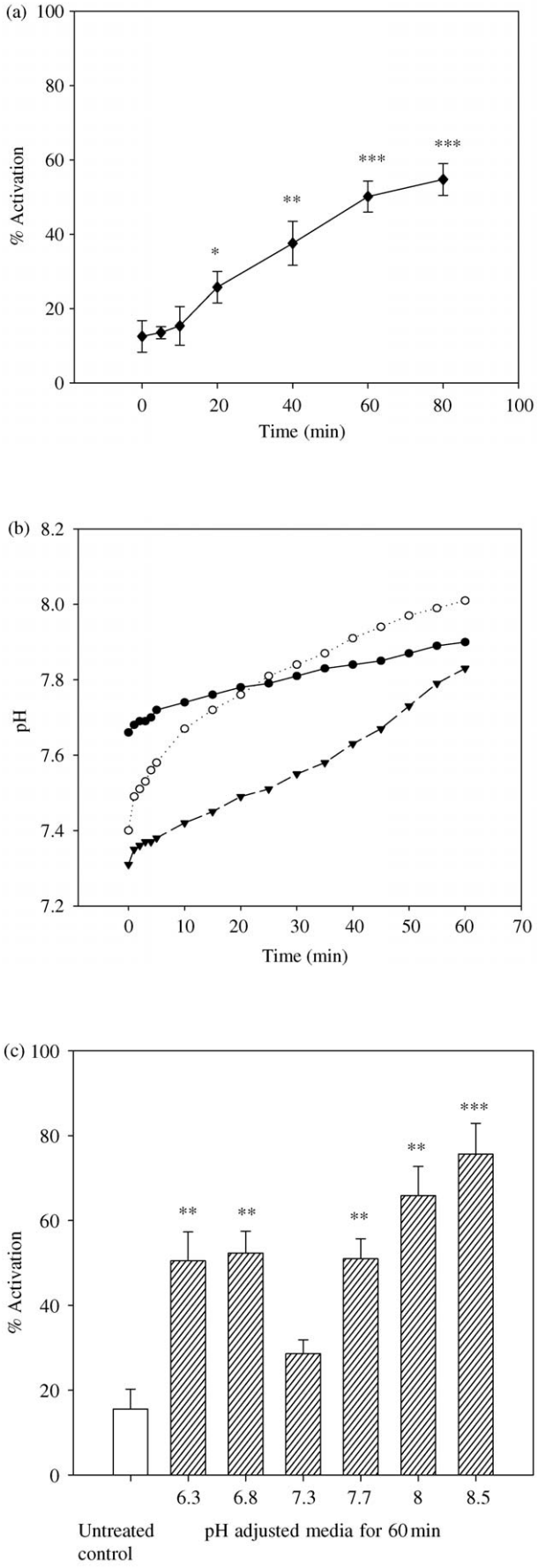

Figure 2 (a) Cultured human fibroblast NF-κB activity increased when cells were exposed to 100% (v/v) air (at 37 °C) for up to 80 min. (b) Freshly prepared Greens medium (•), fibroblast-conditioned Greens medium (○) and Greens medium in direct contact with fibroblasts (▼) increased in pH when transferred from a 37 °C/5% CO_2/95% air to 37 °C/100% air over 60 min. (c) Incubation of cultured dermal fibroblasts with pH corrected Green medium (6.3–8.5) for 60 min increased NF-κB activity. Mean values ± SD ($n = 3$) shown for all experiments.

associated with a significant increase in the activation of NF-κB ($p < 0.01$) compared with cells maintained at a physiological pH of 7.3 (Figs. 1(d) and 2(c)).

Assessment of NF-κB activity in normal and reconstructed human skin

Using the same image analysis technique for determining NF-κB activity for cultured fibroblasts, we determined activation in normal and reconstructed human skin. Freshly frozen sections of tissue were analysed by immunolabelling for the NF-κB/p65 subunit. Using this approach it was possible to analyse different areas of the skin and we therefore interpreted NF-κB activation in three regions of skin: (i) the superficial upper keratinocyte layer (U); (ii) the lower basal keratinocyte layer (L) and (iii) the dermal fibroblast layer (F). As we were interpreting data from predominantly two cell types in a tissue it was first of all necessary to identify the nuclei of all cells initially using DAPI. Fig. 3 shows the position of keratinocyte nuclei in the upper (U) and lower (L) regions, and also the nuclei of fibroblasts in the dermis (F) (Fig. 3(b), (d) and (f)). The DAPI image was then used as a template for determining the nuclei position in the tissue sections. As samples were dual labelled for DAPI and NF-κB the use of fluorescent Pinkle filters enabled the intracellular location of NF-κB alone to be identified in the same tissue section. Fig. 3(a), (c) and (e) illustrate the location of NF-κB in the upper and lower epidermal keratinocytes and in the dermal fibroblasts, respectively. Between one hundred and two hundred cells were analysed individually to calculate a relative mean intracellular position of NF-κB for a population of cells in each of the regions above. Upon quantifying NF-κB using this approach we found immediately that cells in the different regions of skin varied considerably in the extent of NF-κB activation. In unstimulated normal human skin, the apparent resting levels of NF-κB activity in the lower epidermal keratinocytes and dermal fibroblasts was relatively high, while the superficial keratinocytes had lower activity. Fig. 4(a) shows that NF-κB activity in the U, L and F regions were 22, 44 and 61% active for patient sample 1 and 38, 64 and 75% for patient sample 2. We observed that the use of a DAPI image initially to identify nuclei position enabled a very precise micrograph image for discriminating between cellular position and extracellular components. Thus, it was possible to exclude high levels of FITC autofluorescence when interpreting NF-κB activation associated with keratins contained in the stratum corneum (Fig. 3(a)) and also collagen fibrils within the dermis (Fig. 3(e)).

NF-κB activity was extended to analysing cells in a tissue-engineered reconstructed human skin. It was observed that NF-κB activity in the superficial keratinocyte layer was 71% active, while the lower basal keratinocyte cells were only 26% active (Fig. 4(b)). To validate the response of cells cultured within a reconstructed human skin to proinflammatory cytokines we incubated the tissue engineered skin, cultured at a static air–liquid interface with TNF-α for 30 min. We found that NF-κB activity in the upper superficial keratinocyte layer increased from 71 to 90%, while keratinocytes in the lower epidermal layer increased NF-κB activity from 26 to 62% ($p < 0.05$). It was not possible to accurately determine the dermal fibroblast activity in the tissue-engineered skin due to the relatively small number of cells present.

Discussion

The NF-κB transcription factor plays a coordinating role in several biological processes. Principally, it is involved in controlling the first stages of acute inflammation in body tissues [8–12]. The aim of this study was to develop a method for assessing early NF-κB activity in cells contained in normal and reconstructed human skin *in vitro*. The ability to measure NF-κB in tissue could prove to be a sensitive and rapid indicator of early cellular responses to inflammatory and cytosolic stress, in particular for tissue-engineered applications where healthy donor cells are frequently introduced to an inflammatory recipient site.

We report on a method for measuring NF-κB activation in normal and reconstructed human skin using immunofluorescence microscopy of cryosections. The basis of the method is similar to that previously reported by our group for keratinocyte cells, whereby translocation of the p65 subunit of NF-κB from cytosol to nucleus is assessed visually in monolayers of cells [7]. However, visual interpretation was not thought to be a reliable approach for assessment in tissue sections of skin, where sample depth can complicate analysis. We therefore developed a computer imaging software method that enabled a more accurate interpretation of nuclear versus cytoplasmic events, enabling a more accurate quantification of NF-κB in sections of skin tissue *in vitro*.

To validate the method we analysed cultured human dermal fibroblasts. Incubating cells with TNF-α strongly activated NF-κB, demonstrated as a rapid and almost complete translocation from the cytoplasm to the nucleus, as expected. But we also observed that NF-κB could be activated more subtly via experimental procedures encountered during control experimentation. We investigated the influence of such contributing factors individually on dermal fibroblasts in monolayer and found that NF-κB activation arose when cells were cultured at 37 °C in an ''unbuffered'' 100% air environment after a short period of time. Indeed, removal of samples from the tissue culture incubator for periods greater than 20 min led to a significant activation of NF-κB.

It has been reported previously that hypoxic endothelial cells demonstrate a $>$ 10-fold increase in sensitivity to inhibitors of proteasome activation, and combinations of hypoxia and lipopolysaccharide (LPS) enhances proteasome-dependent cytoplasmic-to-nuclear localisation of NF-κB [12]. Furthermore, hypoxic endothelial cells are also more sensitive to agents such as LPS in the upregulation of intercellular adhesion molecule-1 (ICAM-1) [12], due to metabolic acidification. In our study, we did not examine whether acute changes in NF-κB lead to activation of downstream indicators of inflammation (such as upregulation of ICAM-1 or integrin expression). However, it is widely reported

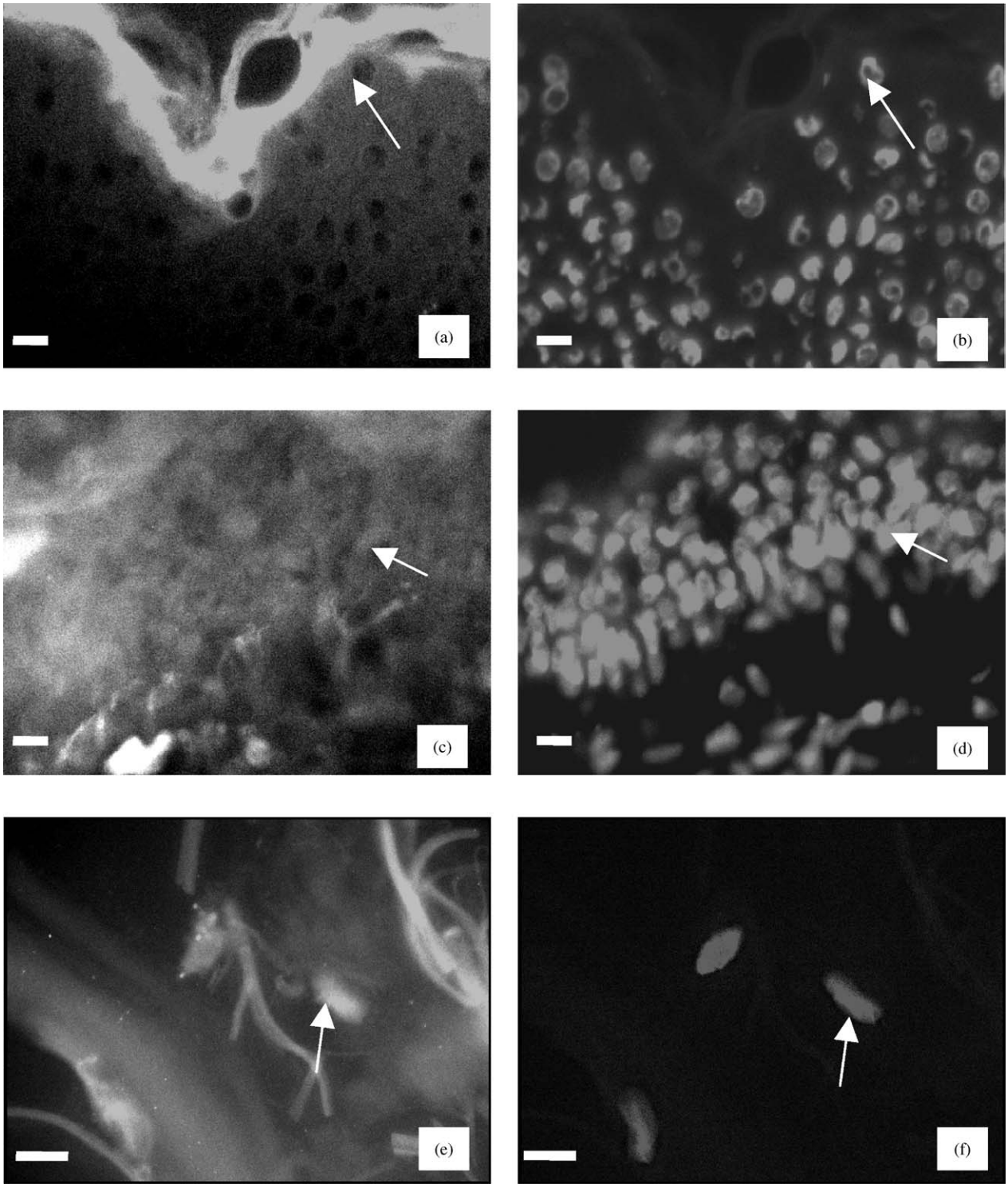

Figure 3 Intracellular location of the p65 subunit of NF-κB in (a) the upper superficial epiderimis (keratinocytes); (c) the lower basal epidermis (keratinocytes) and (e) dermal fibroblasts of normal human skin. Sections were dual labelled and corresponding nuclei position is identified by DAPI fluorescence (b, d and f). Arrows indicate the position of nuclei (stained with DAPI (b, d and f)) and NF-κ B/p65 (a = inactive, c and e = active). Note that keratin (a) and collagen fibril (e) FITC autofluorescence can be excluded on the basis of nuclei position. Bar = 20 μm.

that NF-κB activity is strongly associated with upregulation of ICAM-1 in many cells, including fibroblasts [16]. In the above study, activation of the proteasome was correlated with hypoxia-evoked decreases in both extracellular and intracellular pH. The mechanism that elicits an increased proteasome activity via hypoxia is not fully understood but a correlation with metabolic acidification is thought to be important. NF-κB activation in a low pH environment was confirmed in our study. Interestingly, we also found that an increase in pH could lead to activation.

Thus NF-κB can be acutely activated by the addition of TNF-α, but also by a change in pH (due to acidosis or alkalosis). Removal of cells from a 5% CO_2/95% air environment to 100% air will reduce culture medium carbon dioxide tension, decreasing the carbon dioxide/bicarbonate buffer potential accounting for a change in pH. Such a pH change was observed within 20 min when transferring fibroblast cultures from 5% CO_2/95% air to 100% air. The explanation of a pH change as a single causative factor for increasing NF-κB activity was directly confirmed by incubating

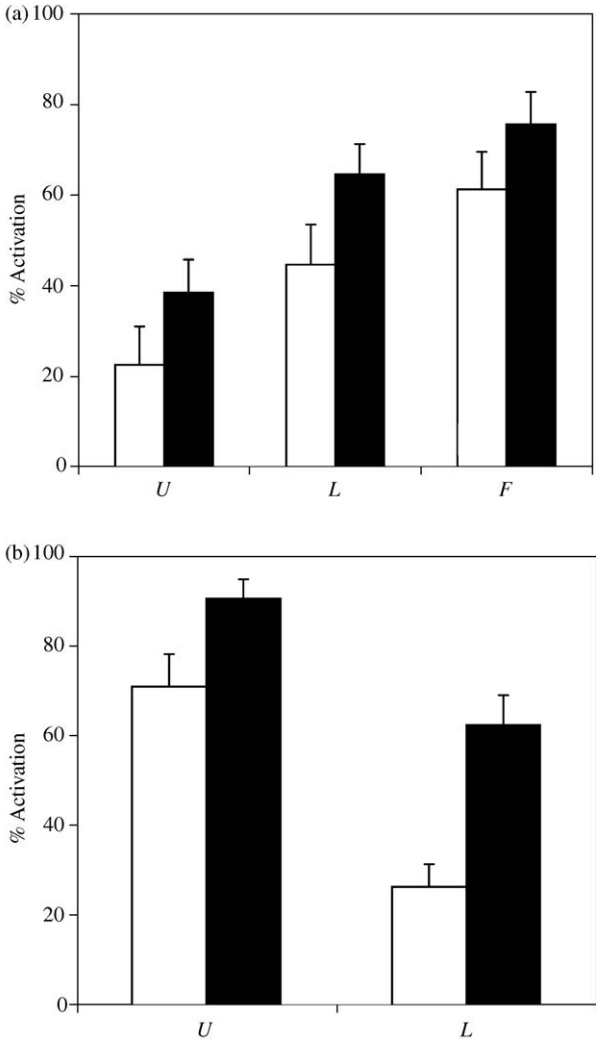

Figure 4 (a) Relative activation of NF-κB in keratinocytes of the upper epidermis (U), the lower epidermis (L) and in dermal fibroblasts (F) from normal human skin. Data shown for patient sample 1 (white bars) and patient sample 2 (black bars). (b) Relative activation of NF-κB in keratinocytes of the upper epidermis (U) and lower epidermis (L) of control (white bars) and TNF-α stimulated (black bars) reconstructed human skin. Mean values ± SD ($n = 3$) shown for all experiments.

cells in culture medium adjusted to a pH above or below 7.3.

NF-κB activity was then measured using image analysis in the upper superficial keratinocyte layer, the lower basal keratinocyte layer and the dermal fibroblast layer of normal human skin from two independent patient samples. In sections of normal human skin, the apparent control levels of NF-κB activity in the three regions were relatively high compared to cultured dermal fibroblasts. The NF-κB activity of the upper keratinocyte layer was significantly lower than that of the lower keratinocyte and dermal fibroblast layer. It is not obvious why this relatively high resting level of activation was present. But it is entirely feasible that it arises due to the trauma of the surgical procedure for retrieval. In support of this the NF-κB activity in the keratinocytes of the tissue engineered reconstructed skin was significantly lower. Furthermore, the cells in the reconstructed skin could be activated after a short incubation time with the cytokine TNF-α.

In summary, the present study demonstrates that a method used to determine NF-κB activity in cultured fibroblasts can in principle be used to determine activity in skin tissue *in vitro*, by using a fluorescence imaging technique to assess nuclear translocation. However, the study also shows that activation of NF-κB is acutely sensitive to culture conditions and pH. We conclude that it is possible to measure NF-κB activity in skin tissue and that activation is an extremely sensitive indicator of inflammatory stress, but also to procedures commonly encountered during laboratory culture and experimentation. It is important for investigators to be aware of possible artefact if using this technique for detection of early inflammatory responses. In light of this we suggest that this approach is a useful method for assessing the response of cells in tissue-engineered constructs and as a measure for determining the effectiveness of interventional therapeutic delivery.

Acknowledgments

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