Exploring cellular interactions relevant to wound healing

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Abstract

The specific involvement of individual cell types in wound repair is generally well understood. How cells interact with each other at the wound site and what effect this has on their healing-related functions, however, is not so clear.

To begin exploring the influence cell-cell interactions have on wound healing, this article examines how inflammatory T cells effect skin fibroblast and keratinocyte function. Our studies show that T cells can reduce collagen production by fibroblasts and induce programmed cell death (apoptosis) in keratinocytes, with both of these outcomes having the potential to impair healing.

Given that a number of different cell types are present in a wound, the challenge is to identify the cell-cell interactions that are beneficial and those that are detrimental to healing so they can be manipulated appropriately to promote repair.

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Introduction

The inflammatory response is an integral part of wound healing; the creation of a wound initiates a cascade of events leading to the rapid influx of inflammatory cells into the wound site. Neutrophils are the first blood-borne cells to infiltrate the wound over the immediate 24 hours post-wounding, followed by leukocytes, which differentiate into activated macrophages as they migrate through the adjacent tissue to the wound. This activation induces the release of cytokines that help stimulate the proliferative phase of repair, resulting in matrix remodelling events aimed at restoring tissue integrity and function. In skin, healing is completed when epidermal keratinocytes restore the epidermis via re-epithelialisation and the tightly regulated balance between proliferation and terminal differentiation is re-established to maintain the structural integrity and homeostatic function of the epidermis.

Whilst these events in the wound healing cascade are reasonably well delineated in isolation, the influence of direct cell-cell interactions between inflammatory cells and dermal fibroblasts or epidermal keratinocytes is not as well understood.

In particular, how primary immune cells like T cells modify the wound healing response is still somewhat controversial (recently reviewed by Park & Barbul [1]). Most studies have either characterised the T cell phenotypes present in various types of wounds and at different times during healing, or assessed the effects of T cell depletion on wound healing as measured by wound breaking strength. In general, depletion of $CD4^+$ T cells (T helper cells) has little effect on wound breaking strength, whilst depletion of $CD8^+$ T cells (cytotoxic T cells) is associated with increased breaking strength and greater collagen production [1]. To date, there is no literature describing the effects T cells have on keratinocytes in the context of wound healing.

In this article, we report studies undertaken to start elucidating the direct interactions between T cells and fibroblasts and keratinocytes that may influence aspects of wound healing. These studies involved establishing *in vitro* co-culture models

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combining T cells with primary skin fibroblasts or the HaCat keratinocyte cell line.

T cell – fibroblast interactions

Despite playing a central role in the tissue restitution phase of wound healing, dermal fibroblasts have long been considered passive bystanders during the inflammatory response. Of critical importance, however, is the resolution of inflammation, with fibroblasts increasingly implicated as modulators of the transition from acute, resolving inflammation to chronic, dysregulated inflammation. Fibroblast activation is now recognised to result in the rapid production of signalling molecules such as cytokines, chemokines and prostanoids that direct immune cell function.

Of particular relevance is the release from fibroblasts of putative 'survival factors' like interferon β (IFNβ) that inhibit T cell apoptosis, the primary mechanism by which activated immune cells are cleared from the site of inflammation [2]. Stromal derived factor 1 (SDF-1), also released from fibroblasts, has been implicated as a pro-retention agent promoting the accumulation of T cells in inflamed tissue and preventing the resolution of acute inflammatory responses [3].

Non-healing wounds (ulcers), skin photodamage resulting from prolonged sun-exposure and atopic eczema are all skin conditions characterised by persistant inflammation where the inappropriate accumulation of immune cells is accompanied in the long-term by dermal degeneration and skin atrophy. This is consistent with the studies described that propose an inhibitory role of $CD8^+$ T cells on fibroblast function, but inconsistent with reports suggesting the persistence of T cells in a wound prolongs the scarring response [2].

To investigate the direct effects T cells have on fibroblast function, we developed an *in vitro* model of skin inflammation involving the co-culture of Jurkat T cells with primary skin fibroblasts. Jurkats were stimulated for 48 hours prior to co-culture using phorbol 12-myristate 13-acetate (PMA; 10ng) with activation confirmed by the increased expression of the IL-2 receptor (CD25) by the T cells (data not shown). Co-culturing unactivated or activated T cells with fibroblasts at a ratio of 16:1 for 48 hours markedly reduced the collagen I immunostaining of fibroblasts compared to controls (Figure 1), with this result confirmed using a collagen synthesis assay (proline incorporation). Figure 2 shows that the total collagenous protein synthesised by the fibroblasts was significantly reduced by the T cell co-culture ($p<0.05$).

To determine if the inhibitory effect of the T cells was mediated by cytokines released from the T cells or due

Figure 1. Fibroblast/T cell co-culture: immunostaining of fibroblasts for Collagen I.

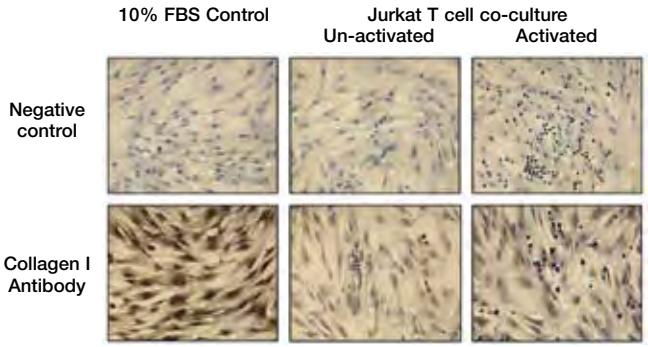
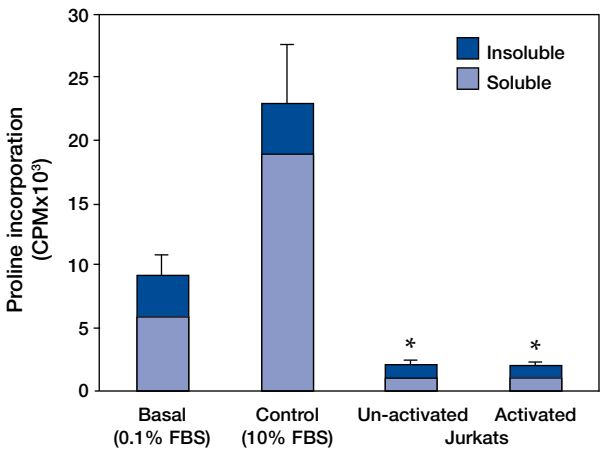


Figure 2. Fibroblast/T cell co-culture: fibroblast collagen synthesis.



to direct cell-cell interactions between the two cell types, fibroblasts were incubated with conditioned media collected from T cell cultures or with T cells fixed with formaldehyde (to preserve their cell surface molecules). Figure 3 shows that incubating fibroblasts for 48 hours with media collected from both un-activated (UA) and activated (A) Jurkat T cells significantly inhibited fibroblast proliferation ($p<0.05$) compared to untreated fibroblasts (C) and fibroblasts incubated with PMA (included as a control because PMA is present in the T cell conditioned media).

Conversely, the T cell conditioned media appeared to induce a small increase in collagen synthesis, although this was not found to be significant (Figure 4). The matrixmetalloproteinase (MMP) activity found in the fibroblast media at the end of the culture period was also analysed by zymography, with Figure 5 showing no change in the levels of the gelatinases (MMP-2 and MMP-9) when fibroblasts were incubated with T cell conditioned media (un-activated or activated). Collagenase activity (MMP-1 like) was, however, increased in cells incubated with the conditioned media from activated T cells (Figure 6).

Figure 3. Fibroblast/T cell conditioned media: fibroblast proliferation.

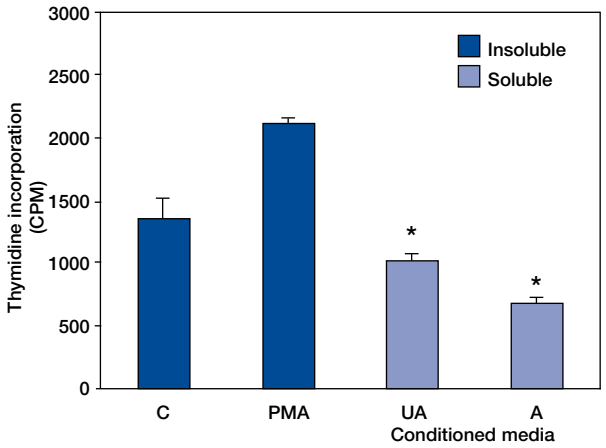
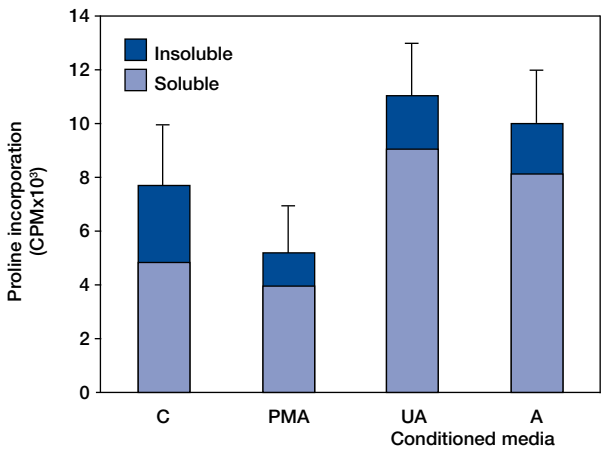


Figure 4. Fibroblast/T cell conditioned media: fibroblast collagen synthesis.



Un-activated or activated T cells were fixed with formaldehyde and, after washing, added to fibroblast cultures (ratio 16:1) for 48 hours; the effects on fibroblast proliferation and collagen synthesis were then determined. This experiment aimed to assess the effect of direct cell-cell interactions between the fibroblasts and T cells without the confounding presence of T cell produced cytokines (as present in live T cell co-cultures). Figure 7 shows that whilst both un-activated and activated T cells significantly inhibited fibroblast growth, activated T cells restricted fibroblast proliferation to levels observed in cells maintained under basal (non-proliferative) conditions (i.e. cultured with 0.1% FBS only). Incubating the fibroblasts with fixed T cells, both activated and un-activated, totally inhibited collagen production by the fibroblasts (Figure 8), with this result consistent with those obtained when live T cells were co-cultured with the fibroblasts (Figure 1).

Overall, both fixed and live Jurkats adhered readily to the fibroblasts, with the responses observed generally more

Figure 5. Fibroblast/T cell conditioned media: MMP activity – gelatin.

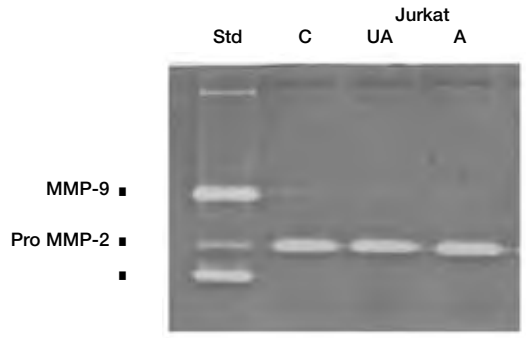


Figure 6. Fibroblast/T cell conditioned media: MMP activity – casein.

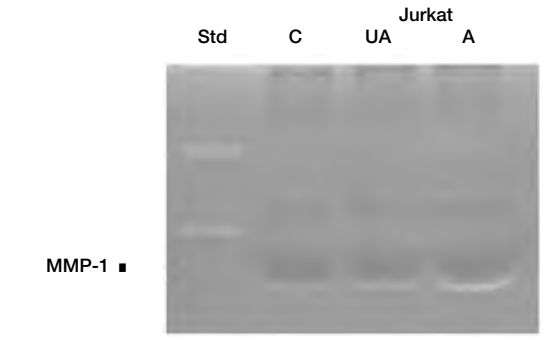
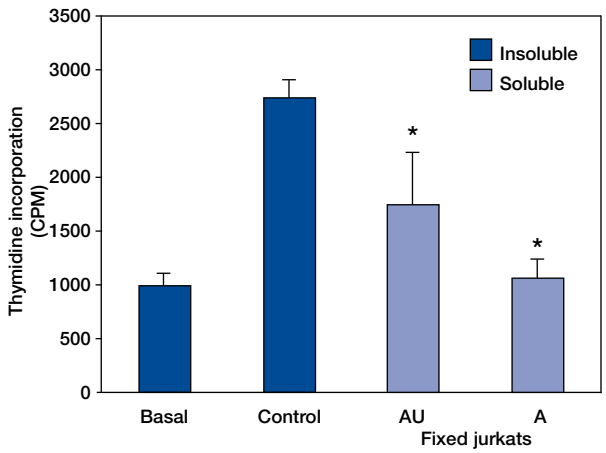
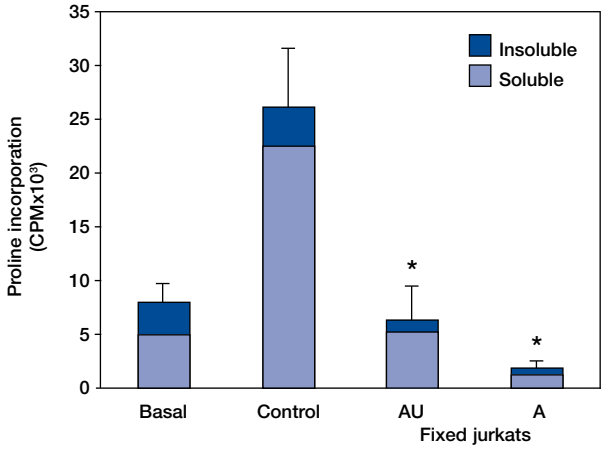


Figure 7. Fibroblast/Fixed T cell co-culture: fibroblast proliferation.



pronounced when the T cells were pre-activated. Our results show that fibroblast proliferation was inhibited by co-culture with fixed Jurkats and Jurkat conditioned media. Culturing fibroblasts with both fixed and live Jurkats, but not T cell conditioned media, inhibited collagen synthesis, indicating direct interactions between the cells were likely to be responsible for this effect rather than soluble cytokines secreted by the T cells. T cell cytokines, however, appeared to be responsible for increasing MMP production by the fibroblasts, as both conditioned media and co-culture with

Figure 8. Fibroblast/Fixed T cell co-culture: fibroblast collagen synthesis.



live Jurkats increased activity levels, whereas co-culture with fixed cells did not influence MMP activity (not shown).

These findings indicate that direct fibroblast/T cell interactions via expressed T cell surface molecules reduce the proliferative and synthetic capacity of fibroblasts, while secreted T cell cytokines appear responsible for promoting a proteolytic environment. As such, the retention and increased survival of T cells in inflamed skin may contribute to the gradual decline in collagen content observed with skin photodamage and help promote the persistence of chronic, non-healing ulcers.

T cell – keratinocyte interactions

The primary role of epidermal keratinocytes is to maintain skin barrier function. This is achieved by keratinocytes undergoing keratinisation, a tightly regulated process requiring the cells to terminally differentiate and accumulate keratin and other proteins that are required to generate the protective outer layer of the skin or stratum corneum.

Dysregulation of terminal differentiation or premature keratinocyte cell death can adversely effect the integrity of the epidermis and lead to a breakdown of the protective barrier and poor re-epithelialisation during wound healing. Premature keratinocyte cell death can occur when keratinocytes are induced to undergo programmed cell death or apoptosis.

Apotosis of keratinocytes can be induced by a variety of stimuli; however, the effects of ultra-violet (UV) radiation are best understood, given UV is the main environmental carcinogen responsible for the formation of keratinocyte derived skin carcinomas [4, 5]. UV induction of apoptosis is the primary mechanism by which keratinocytes are protected from the mutagenic effect of sunlight; this appears to be mediated by the intrinsic caspase 9 pathway rather than activation of death receptors like Fas and TRAIL [6].

In inflammatory skin disorders such as atopic eczema, T cells have been identified as inducers of keratinocyte apoptosis. Infiltration of the epidermis by T cells results in pathological apoptosis of keratinocytes leading to spongiosis, the histological hallmark of the epidermis in eczema, and subsequent breakdown of barrier function [7]. Unlike UV-stimulated apoptosis, T cell induced keratinocyte apoptosis is primarily directed through Fas (CD95) and facilitated by interferon gamma (IFNγ) stimulated upregulation of both Fas and Fas ligand (FasL) by keratinocytes [8, 9].

Both $CD4^+$ and $CD8^+$ cells are present in the inflammatory infiltrate of acute inflammatory lesions and have the potential to induce keratinocyte apoptosis [9]. As these T cells have also been described in wounds during both the healing and maturation phases [10, 11], it can be hypothesised that pathological T cell induced keratinocyte apoptosis may contribute to delayed healing or failure to heal in chronic wounds. That is, T cells interacting with keratinocytes may promote apoptosis, leading to impaired re-epithelialisation and restitution of the protective epidermal barrier, or, indeed, leading to persistant breakdown of the epidermis.

To begin examining the mechanisms by which T cells directly effect keratinocytes, an *in vitro* system mimicing the induction of keratinocyte apoptosis by T cells (as reported in atopic eczema) was established. This model uses the HaCat keratinocyte cell line and Jurkat T cells and has been adapted from work by others who used this system to examine the effect of keratinocytes on T cell apoptosis [8].

By measuring early apoptotic events, such as the exposure of annexin V on the surface of cells induced to die using flow cytometry, we have found that activated Jurkat T cells (treated with PMA 10ng for 48 hours) induced a 3-4 fold increase in HaCat apoptosis when these cells were co-cultured for 24 hours (Figure 9). Un-activated T cells and the conditioned media from either unactivated or activated T cells had no effect on HaCat apoptosis (Figure 9). Consistent with T cells inducing Fas-mediated apoptosis of keratinocytes, activated Jurkats were found to highly express FasL (Figure 10) and Fas expression by HaCats was markedly upregulated by T cell co-culture (Figure 11).

As such, this model exhibited the characteristics of T cell induced keratinocyte apoptosis associated with skin inflammation in eczema. The results also suggest that the direct interaction of activated T cells with keratinocytes was necessary for inducing apoptosis, as co-culturing HaCats with unactivated cells or T cell conditioned media had no effect on the keratinocytes. Thus it would appear that, should activated T cells infiltrate the epidermis or come into direct contact with

Figure 9. Jurkat co-culture induced HaCat apotosis: Annexin V positive (apoptotic) cells measured by flow cytometry.

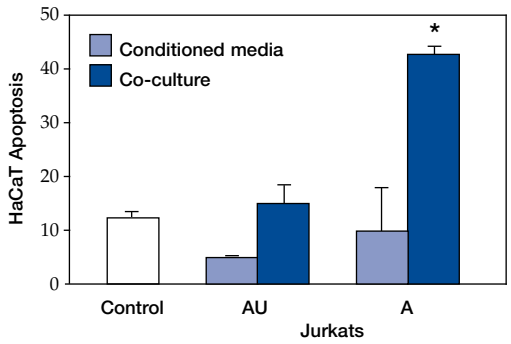


Figure 10. FasL expression by PMA activated Jurkats: measured by flow cytometry.

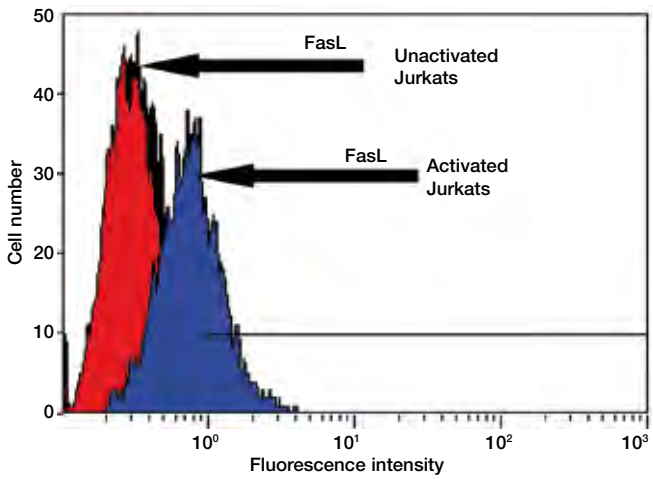
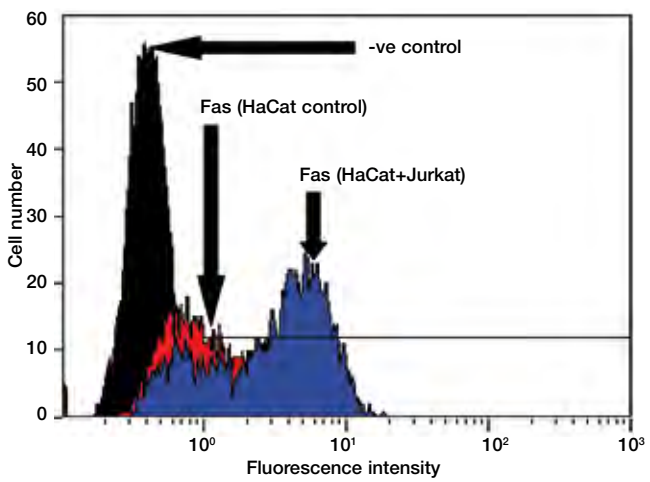


Figure 11. Jurkat induced Fas expression by HaCats: measured by flow cytometry.



keratinocytes, the keratinocytes could be placed at risk of being induced to undergo apoptosis, with a breakdown in the epidermal barrier a potential pathological outcome.

Although it remains to be established whether T cell mediated keratinocyte apoptosis actually impairs wound healing and in particular re-epithelialisation, the current data suggest this hypothesis may have merit. Moreover, known anti-apoptotic agents have been shown to enhance wound re-epithelialisation. Insulin-like growth factors I and II (IGF-I&II) are peptide growth factors that exert metabolic and mitogenic effects on different cell types. These growth factors have been shown to promote wound re-epithelialisation [12], with IGF-I also identified as a potent survival factor capable of protecting cells from a variety of apoptotic stimuli [13].

Conclusion

Future studies will help us determine the ability of agents with the capacity to ameliorate keratinocyte cell death in order to potentially improve healing outcomes where re-epithelialisation is impaired.

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