CHAPTER 7

7 EFFECTS OF WGFE ON T-LYMPHOCYTE INDUCED KERATINOCYTE APOPTOSIS

7.1 Introduction

Keratinocyte apoptosis is the proposed mechanism by which epidermal integrity is impaired in atopic eczema. The studies presented in Chapter 6 demonstrated the ability of recombinant TGFβ_1 and IGF-I to ameliorate T-lymphocyte induced keratinocyte apoptosis. The studies in this chapter examine the effect of whey growth factor extract (WGFE), a milk-derived growth factor extract containing TGFβ and IGF-1, on T-lymphocyte induced keratinocyte apoptosis. These studies were divided into the following specific aims:

1. Establish the effects of whey growth factor extract (WGFE) on primary CD4+ T-lymphocyte induced keratinocyte apoptosis and how these relate to the equivalent TGFβ and IGF-I content.

WGFE contains high levels of several growth factors including IGF-I, IGF-II, TGFβ_2, fibroblast growth factor and platelet-derived growth factor as shown in Table 7.1 (Belford *et al.* 1997, Francis *et al.* 1995, Rogers *et al.* 1996).

Table 7-1

Growth Factor	**Concentration in WGFE (ng/ml)**
IGF-I	22.7
IGF-II	23.9
IGFBP (total)	70
PDGF	4
Betacellulin	2.59
aFGF	0.2
bFGF	0.7
TGFβ	2-5
TGFβ (acid activated)*	72-250

*TGFβ exists in milk as a high molecular weight complex that is activated under acidification to separate TGFβ from latency-associated peptide (LAP) and yield the bioactive low molecular weight factor.

A number of reports suggest that WGFE has anti-inflammatory properties and can stimulate the growth and repair of skin cells (Belford et al. 1995, Francis et al. 1995, Francis et al. 1997, Penttila et al. 2001, Rayner et al. 2000). The experiments in this Chapter assess the effect of WGFE, on keratinocyte apoptosis induced by T-lymphocytes. Moreover, in order to determine if the growth factor content of WGFE is

associated with effects on keratinocytes in co-culture, comparative studies were performed using the equivalent content of TGFβ and IGF-I present in WGFE.

2. Examine the effect of WGFE on Fas expression and differentiation by keratinocytes co-cultured with T-lymphocytes.

In Chapter 5 it was shown that T-lymphocyte induced keratinocyte apoptosis was mediated by Fas with IFNγ released into the co-culture media implicated in the upregulation of keratinocyte Fas expression (Chapter 5 Section 5.3.5 to 5.3.8). However recombinant $TGF\beta_1$ and IGF-I appear to protect keratinocytes from T-lymphocyte induced apoptosis via a Fas independent manner. The growth factors appeared to protect keratinocytes from T-lymphocyte induced apoptosis by preventing T-lymphocyte induced keratinocyte differentiation, as defined by a decrease in α6 expression (Section 6.3.6 and 6.3.8). To investigate whether WGFE could have the same effect on keratinocytes as the recombinant growth factors, WGFE was added to co-cultures and its effect on keratinocyte α6 expression was investigated.

7.2 Methods

7.2.1 WGFE

The experiments in this chapter were performed using laboratory scale preparations of WGFE (batch # D14348). WGFE was analysed and provided by TGR BioSciences Pty. Ltd. The procedures used to extract the growth factor fraction from whey have been described in detail by GL. Francis et al, 1995 and GL. Francis et al, 1997. WGFE was used at concentrations between 0.1-2mg/ml. The TGFβ and IGF-I content of WGFE used in these experiments is shown in Table 7-2. Some experiments were performed in parallel using equivalent concentrations of recombinant TGFβ 1 and IGF-1.

An IGF-I enriched fraction of WGFE was generated by dialysis against a 50KDa membrane and collecting the permeate (analysed and provided by TGR-BioSciences Pty. Ltd.) This permeate; UFO2N010 contained no TGFβ and high levels of IGF-I and was used in some experiments with NHEKs (Table 7-2). The concentrations of UFO2N010 used in these experiments was calculated to give final IGF-I concentrations between 1-20 ng/ml.

Table 7-2

STREAM	**PROTEIN** (mg/mg)	**TGFβ** (ng/mg)	**IGF-1** (ng/mg)
WGFE – Native: batch D14348	37.96	16.99	4.09
WGFE - Permeate: UFO2N010	2.07	0	73.55

7.3 Results

7.3.1 WGFE prevented T-lymphocyte induced HaCaT apoptosis

As shown in Figures 7.1 c, d and e, WGFE at concentrations of 0.1, 0.5 and 1 mg/ml respectively, reduced HaCaT cell shrinkage, membrane blebbing and the amount of HaCaT detachment from culture plate induced by T-lymphocytes compared to untreated co-cultures (Figure 7.1 b). In fact, HaCaT co-cultures treated with 0.5 and 1 mg/ml WGFE appeared to have a similar appearance to control HaCaTs shown in Figure 7.1 a. Consistent with these morphological observations, WGFE (0.1-1 mg/ml) decrease the number of HOESCHT 33342 positive HaCaT nuclei found in T-lymphocyte co-cultures (Figure 7.1 h, i and j respectively) compared to co-culture controls (Figure 7.1 g).

The effect of WGFE on T-lymphocyte induced HaCaT apoptosis was quantified by Annexin V and PI staining. The combined results from four replicate experiments demonstrates that WGFE (0.5 and 1 mg/ml) significantly decreased the total number of apoptotic HaCaTs ($p<0.05$ Figure 7.2 a). To ensure the effect of WGFE was not attributed to T-lymphocyte apoptosis during co-culture, T-lymphocytes were stained with Annexin V/PI at the end of the experiments. Figure 7.2 b demonstrates that WGFE had no effect on T-lymphocyte apoptosis in co-culture as 100% of the T-lymphocytes were Annexin V/PI negative.

Figure 7.1

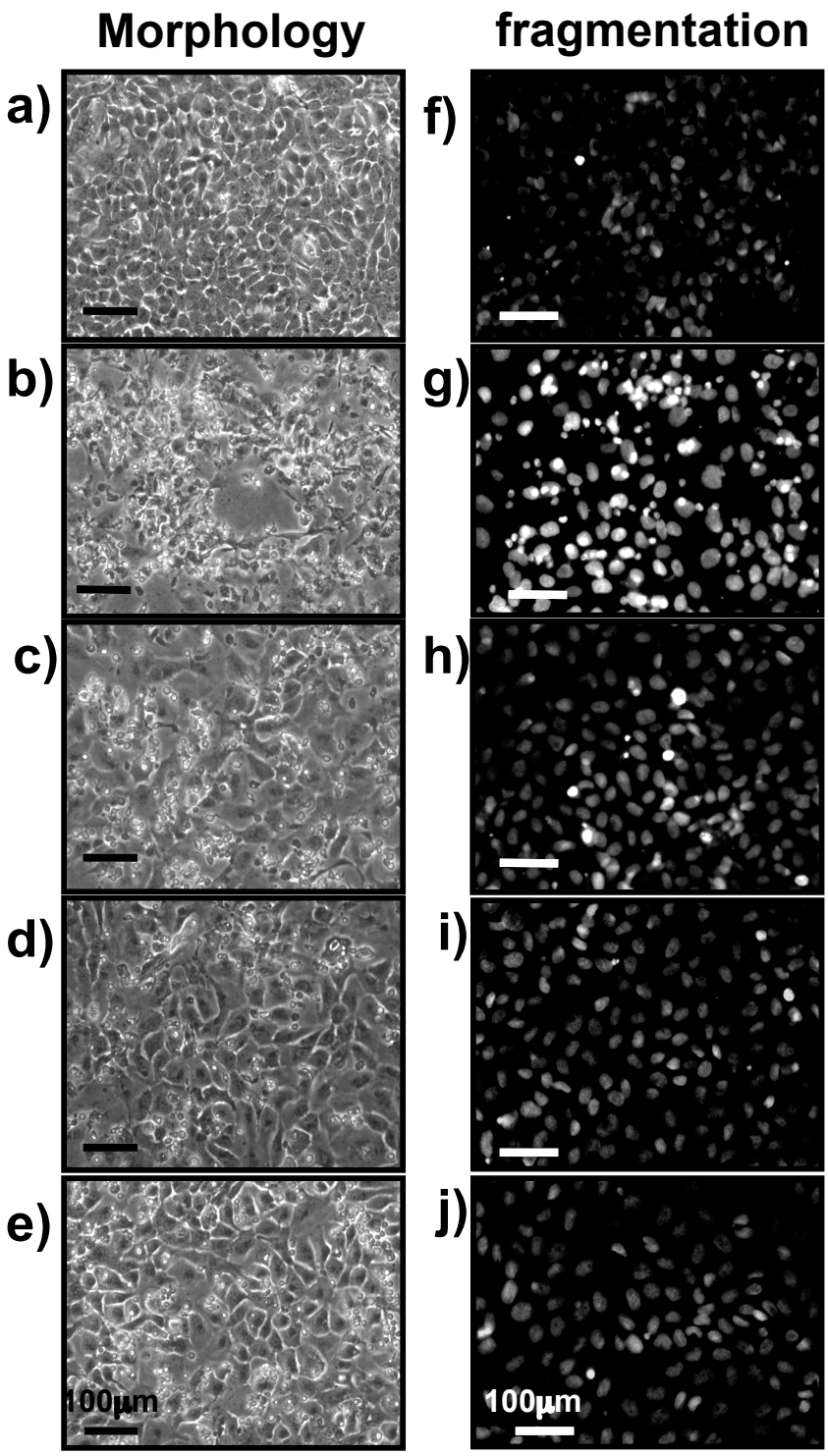


Figure 7.1 Effect of WGFE on T-lymphocyte induced HaCaT apoptosis. HaCaT morphology was assessed prior to flow cytometric analysis of apoptosis (a) Control HaCaTs, (b) HaCaTs co-cultured with $5X10^5$ T-lymphocytes, (c) HaCaT T-lymphocyte co-cultures incubated with 0.1mg/ml WGFE, or (d) 0.5mg/ml WGFE, or (e) 1mg/ml WGFE. Chromatin condensation and DNA fragmentation was assessed in parallel cultures using the HOESCHT 33342 fluorescent stain of (f) Control HaCaTs, (g) HaCaTs co-cultured with $5X10^5$ T-lymphocytes, (h) HaCaTs T-lymphocyte co-cultures with 0.1mg/ml WGFE, or (i) 0.5mg/ml WGFE, or (j) 1mg/ml WGFE. Scale bar represents 100µm for the phase contrast images and the HOESCHT 33342 images.

Figure 7.2

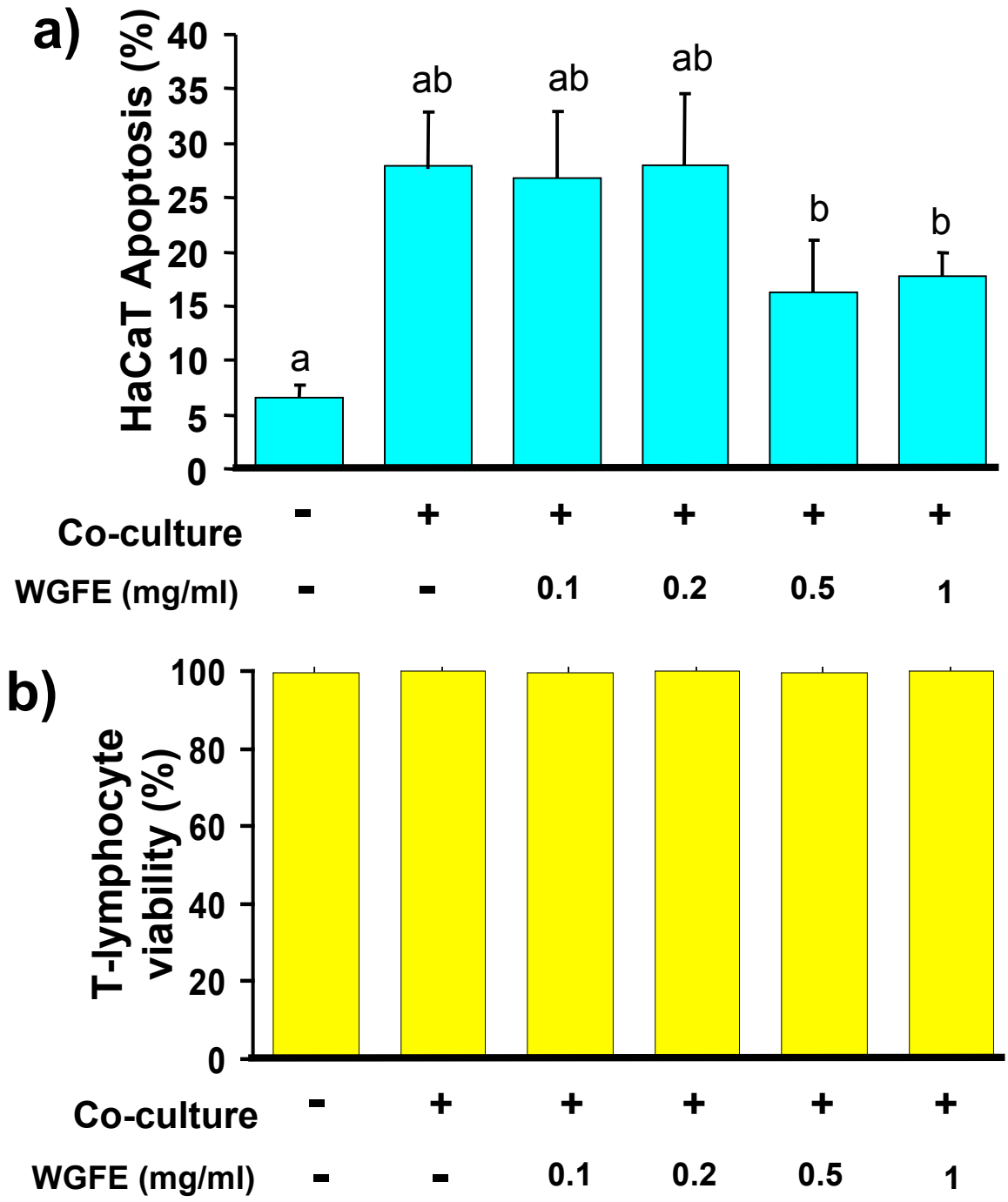


Figure 7.2 Effect of WGFE on T-lymphocyte induced HaCaT apoptosis and T-lymphocyte survival in co-culture. (a) Apoptosis was quantified by Annexin V and PI staining of control HaCaTs, HaCaTs co-cultured with T-lymphocytes and HaCaTs co-cultured with T-lymphocytes together with WGFE (0.1-1mg/ml) for 48hrs. (b) T-lymphocyte viability was determined on T-lymphocytes from cultures described in (a) by exclusion of Annexin V and PI staining. Bar graphs show the parameters measured as a percentage of the total cell population with the data presented as mean±SEM from 4 experiments. The data was analysed using one-way analysis of variance (ANOVA) and post-hoc t-test with significance ($p<0.05$) between treatments shown by the matching symbols ($^{a, b}$)

7.3.2 Effect of WGFE on IFNγ release and Fas expression

The results presented in the previous Section demonstrate that WGFE significantly prevented T-lymphocyte induced apoptosis of HaCaTs. The following studies aimed to investigate the effect of WGFE on IFNγ release and the Fas pathway.

Figure 7.3 demonstrates that the increase in IFNγ levels normally associated with co-culture was prevented by WGFE (0.1-1 mg/ml; $p<0.05$). The effect was comparable to results obtained with recombinant TGFβ and IGF-I (Section 6.3.4). Having established that 0.5 and 1 mg/ml WGFE prevented T-lymphocyte induced HaCaT apoptosis, the effect of these concentrations on IFNγ induced HaCaT Fas expression was examined. IFNγ (100 ng/ml) induced Fas expression by HaCaTs (pink histogram MFI from 12 to 2; Figure 7.3 b), was markedly down regulated by both 0.5 and 1 mg/ml WGFE concentrations (Figure 7.3 c and d respectively).

T-lymphocyte induced keratinocyte apoptosis was shown to be mediated by a Fas dependent mechanism (Chapter 5 Section 5.3.5 to 5.3.8). To determine whether WGFE prevented T-lymphocyte induced HaCaT apoptosis by modulating Fas expression, the effect of 0.5 and 1 mg/ml WGFE on T-lymphocyte induced HaCaT Fas expression was investigated. Figure 7.4 a shows the upregulation of Fas associated with T-lymphocyte co-culture (yellow histogram) and panels in Figure 7.4 b and c show that this was not affected by either 0.5 or 1 mg/ml WGFE (blue and purple histogram; respectively).

In parallel wells the effect of TGFβ and IGF-I, at concentrations present in WGFE (Table 7-2), on T-lymphocyte induced HaCaT Fas expression was also examined. The results were similar to those obtained with WGFE, as 8.5ng/ml TGFβ and 2ng/ml IGF-I equivalent to 0.5m/ml WGFE, or 17ng/ml TGFβ and 4ng/ml IGF-I equivalent to 1mg/ml WGFE, did not affect T-lymphocyte induced HaCaT Fas expression (pink and orange histogram; Figure 7.4 d and e respectively).

7.3.3 WGFE prevents T-lymphocyte induced early differentiation

Evidence in Chapter 5 clearly demonstrates that T-lymphocytes induced early differentiation of HaCaTs (shown by a decrease in α6 expression) which correlated with apoptosis (Section 5.3.9). The effect of WGFE on HaCaT α6 expression after co-culture was investigated. The results in Figure 7.5 demonstrate that WGFE (0.5 and 1 mg/ml) prevented the shift from α6-bright to dim associated with T-lymphocyte co-culture (light blue and purple histogram; Figure 7.5 c and d respectively). The distribution of the histograms were similar to those of control HaCaTs (Figure 7.5 a).

The effect of TGFβ and IGF-I concentrations equivalent to those present in WGFE on HaCaT α6 expression was also investigated. Consistent with the effect observed in WGFE treated co-cultures, the recombinant TGFβ and IGF-I combinations also prevented the T-lymphocyte induced, loss of α6 expression with the cell remaining α6-bright (pink and orange histogram respectively, Figure 7.5 e and f respectively).

Figure 7.3

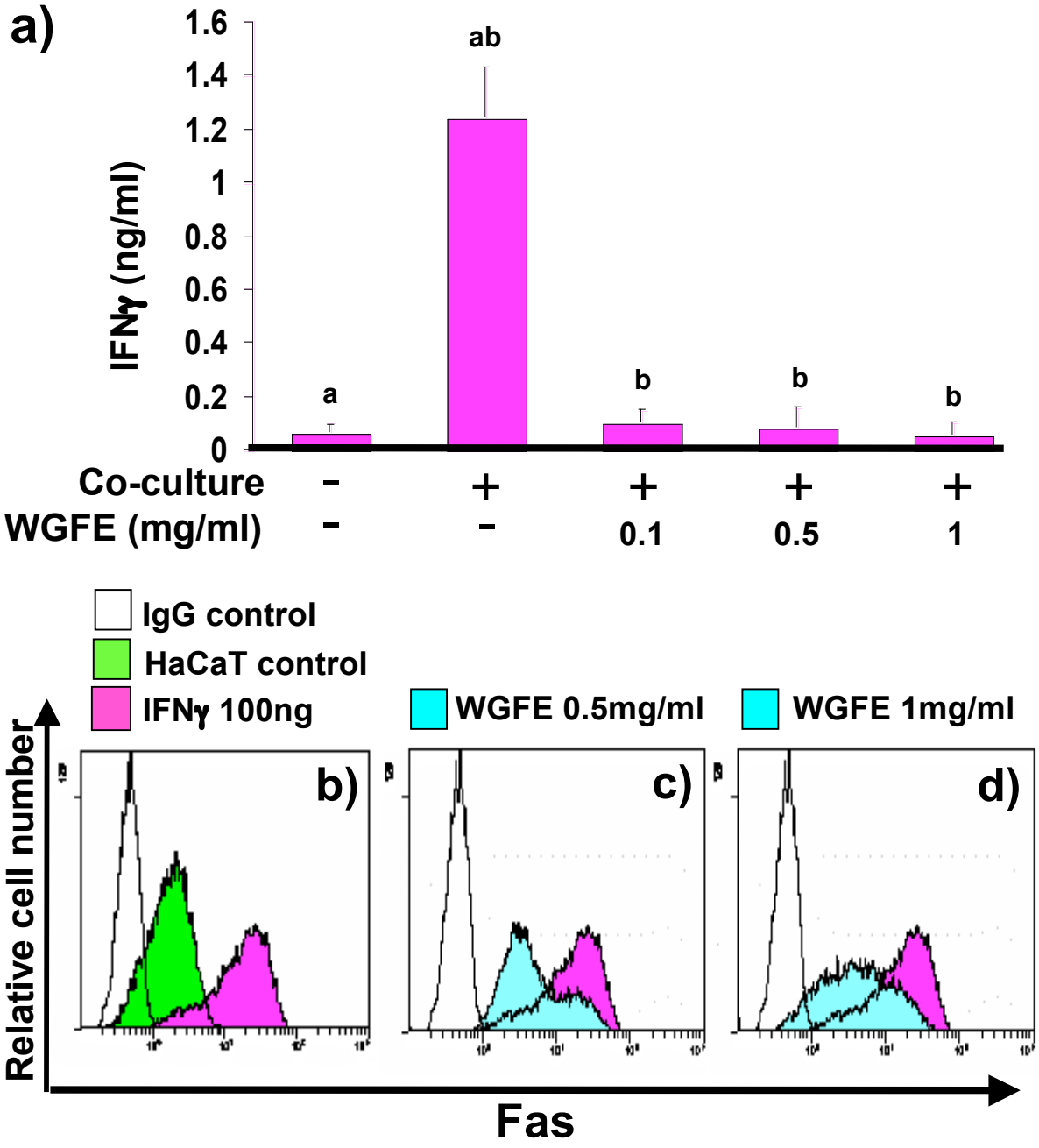


Figure 7.3 Effect of WGFE on IFNγ release in co-culture and IFNγ induced HaCaT Fas expression. Levels of IFNγ in conditioned media from HaCaT controls, HaCaTs co-cultured with T-lymphocytes and HaCaTs co-cultured with T-lymphocytes and WGFE (0.1-1 mg/ml). Values represent the mean release of IFNγ ± SEM from 4 independent experiments. The data was analysed using one-way analysis of variance (ANOVA) and post-hoc t-test with significance ($p<0.05$) between treatments shown by the matching symbols ($^{a, b}$). Compiled histograms showing changes in surface Fas expression by (b) HaCaT control; green histogram or HaCaTs treated with 100ng IFNγ; pink histogram present in each panel, (c) HaCaTs treated with IFNγ + WGFE (0.5mg/ml) blue histogram and (d) HaCaTs treated with IFNγ + WGFE (1mg/ml) blue histogram. The unfilled histogram is the negative control (isotype-matched Ab).

Figure 7.4

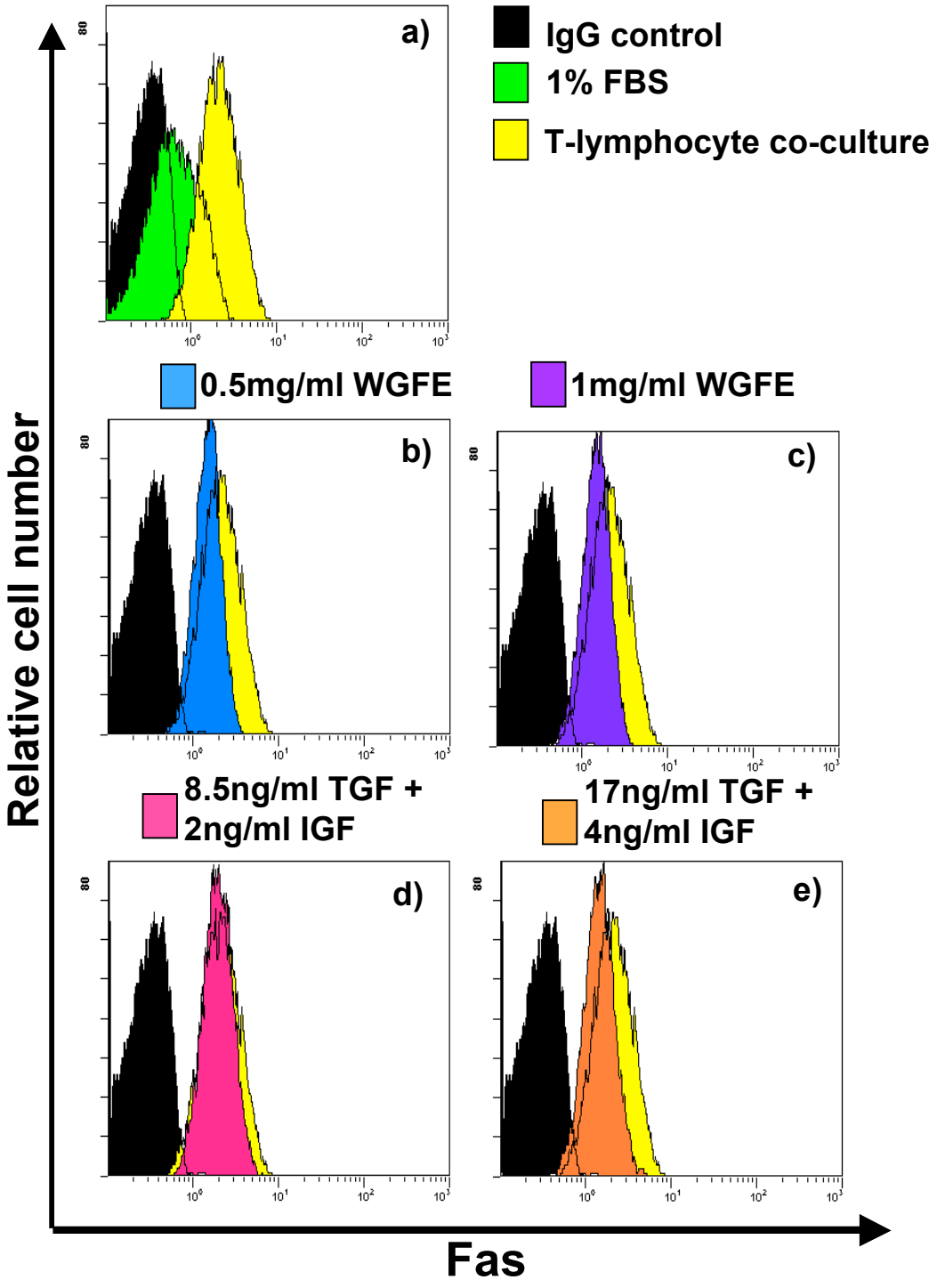


Figure 7.4. Effect of WGFE on T-lymphocyte induced HaCaT Fas expression. Compiled histograms showing the change in surface Fas expression by (a) HaCaT control; green histogram and HaCaTs co-cultured with T-lymphocytes (yellow histogram) present in each panel. (b) HaCaTs T-lymphocyte co-culture + 0.5mg/ml WGFE; blue histogram (c) HaCaTs T-lymphocyte co-culture + 1mg/ml WGFE; purple histogram. (d) HaCaTs T-lymphocyte co-culture + TGFβ:IGF-I (8.5:2 ng/ml) pink histogram. (e) HaCaTs T-lymphocyte co-culture + TGFβ:IGF-I (17:4 ng/ml); orange histogram. The black histogram is the negative control (isotype-matched Ab).

Figure 7.5

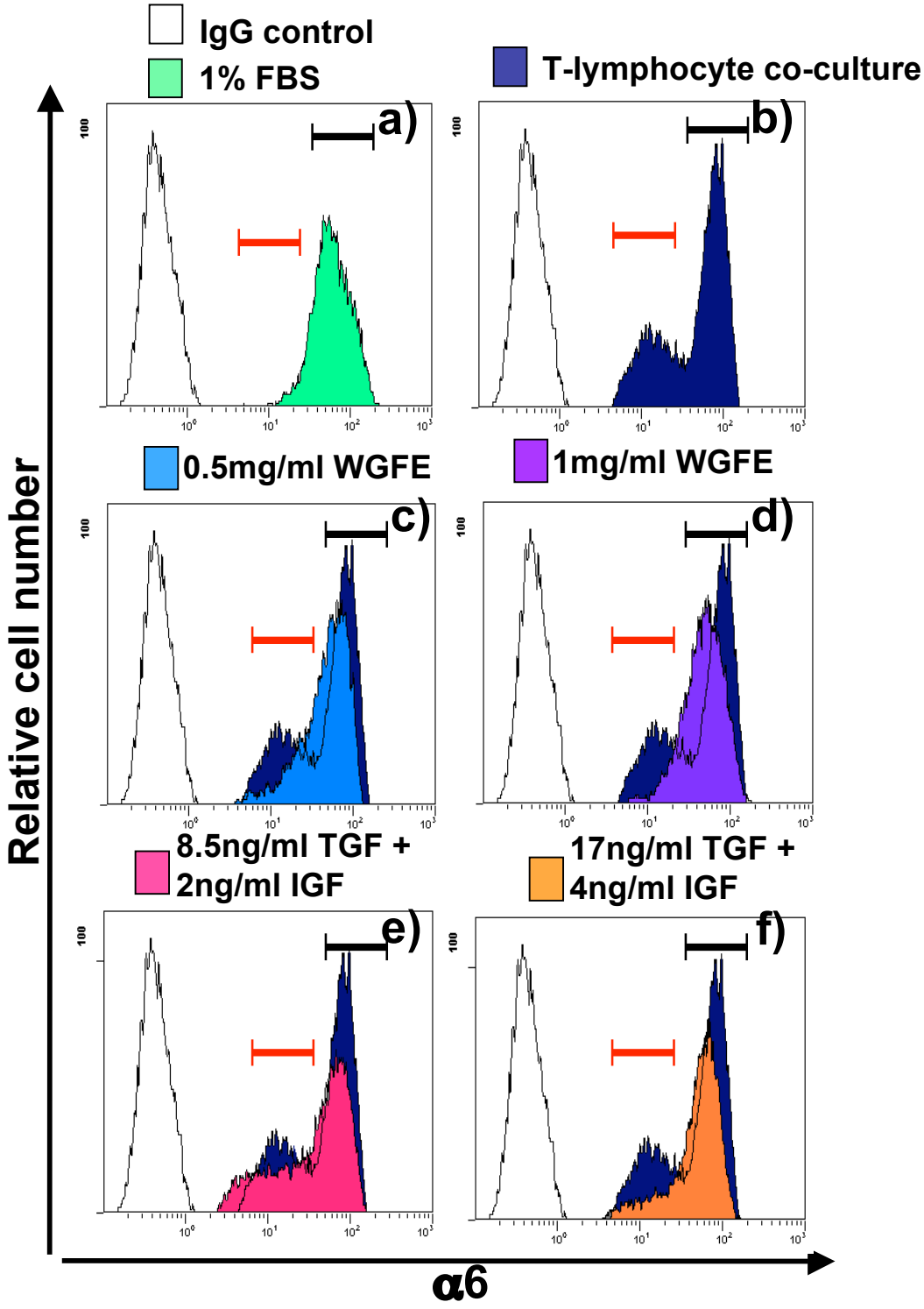


Figure 7.5. Effect of WGFE on T-lymphocyte induced HaCaT α6 dim expression. Expression of α6 integrin by (a) HaCaT controls green histogram, (b) HaCaTs T-lymphocyte co-cultures dark blue histogram (present in each panel), (c) HaCaTs co-cultured with T-lymphocytes + 0.5mg/ml WGFE; light blue histogram, (d) HaCaTs co-cultured with T-lymphocytes + 1mg/ml WGFE; purple histogram, (e) HaCaTs co-cultured with T-lymphocytes + TGFβ:IGF-I (8.5:2ng/ml) pink histogram, and (f) HaCaTs co-cultured with T-lymphocytes + TGFβ:IGF-I (17:4ng/ml) orange histogram. The unfilled histogram is the negative control (isotype-matched Ab).

7.3.4 WGFE did not prevent T-lymphocyte induced apoptosis of NHEKs

The WGFE results presented in this chapter indicate that WGFE protects HaCaTs from T-lymphocyte induced apoptosis. To determine if the same effect could be obtained using primary keratinocytes, NHEK co-cultured with pre-activated CD4+ T-lymphocytes were treated with WGFE (primary co-culture model described in Chapter 5 Section 5.2.2). NHEK morphology studies demonstrate that WGFE (0.5 and 1 mg/ml) did not decrease cell shrinkage and cell detachment from the culture plate associated with T-lymphocyte co-culture (Figure 7.6 c and d). These observations were supported by Annexin V and PI staining experiments, which show that WGFE (0.5 and 1 mg/ml) had no effect on the number of apoptotic NHEKs induced by T-lymphocyte co-cultures (Figure 7.6 e).

7.3.5 Effect of IGF-I enriched WGFE (UFO2N010) on T-lymphocyte induced NHEK apoptosis

It was previously demonstrated in Chapter 6 (Section 6.3.7) that TGFβ did not protect NHEKs from T-lymphocyte induced apoptosis, whereas IGF-I did. Given that WGFE contains a high concentration of TGFβ, preliminary studies were performed to examine the effect of a WGFE fraction; UFO2N010 which contained a high concentration of IGF-I but no TGFβ (Table 7-2).

NHEKs co-cultured with T-lymphocytes and treated with UFO2N010 to give IGF-I levels of 1, 10 and 20 ng/ml were found to have decreased levels of T-lymphocyte induced apoptosis as measured by Annexin V and PI staining (Figure 7.7 a). Figure 7.7 d shows that UFO2N010 used at a concentration to give 10 ng/ml IGF-I was able to prevent the T-lymphocyte induced cell shrinkage and detachment from the culture plate associated with untreated T-lymphocyte co-cultures (Figure 7.7 c). UFO2N010 treated NHEKs had similar morphological characteristics as the control cells shown in Figure 7.7 b. UFO2N010 also decreased the number of HOESCHT positive NHEKs induced by T-lymphocytes (Figure 7.7 g) compared to untreated NHEK-T-lymphocyte co-cultures (Figure 7.7 f).

7.3.6 UFO2N010 prevents T-lymphocyte induced early differentiation of normal human epidermal keratinocytes

It was demonstrated in Chapter 6 Section 6.3.8, that recombinant IGF-I suppressed T-lymphocyte induced early differentiation of NHEKs, as IGF-I (100 ng/ml) maintained α6-bright expression, preventing the T-lymphocyte mediated shift to α6-dim (Section 6.3.7). The effect of UFO2N010 on NHEK α6 expression associated with T-lymphocyte co-cultures was investigated and the result shown in Figure 7.8. UFO2N010 at a concentration equivalent to 10 ng/ml IGF-1 totally prevented the shift from α6-bright to α6-dim expression (purple histogram; Figure 7.8 b). The number of cells retaining their bright α6 expression levels in UFO2N010 treated co-cultures was similar to NHEK controls (orange histogram; MFI 95; Figure 7.8 a and b)

Figure 7.6

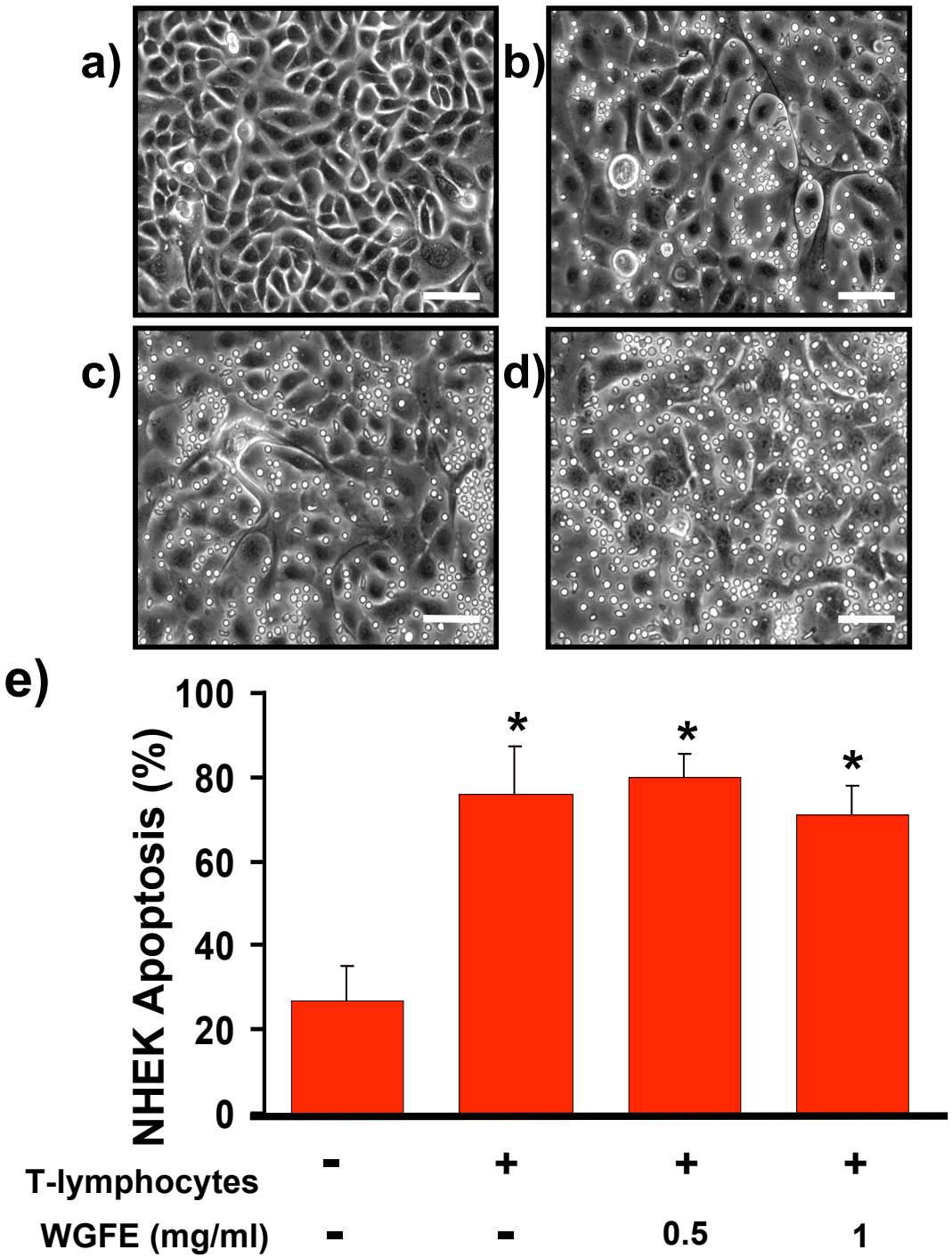


Figure 7.6 Effect of WGFE on T-lymphocyte induced NHEK apoptosis. NHEK morphology was assessed on cultures and imaged by phase contrast microscopy prior to flow cytometric analysis of apoptosis. (a) Control NHEK, (b) NHEK co-cultured with T-lymphocytes for 48hrs, (c) NHEK co-cultured with T-lymphocytes + 0.5mg/ml and (d) NHEKs co-cultured with T-lymphocytes + 1mg/ml WGFE. Scale bar represents 100μm. (e) Apoptosis of samples represented in (a-d) was quantified by Annexin and PI staining. Values represent the mean % apoptosis ± SEM from 3 independent experiments. The data was analysed using one-way analysis of variance (ANOVA) and post-hoc t-test with significance compared to HaCaT controls are shown as *$p<0.05$.

Figure 7.7

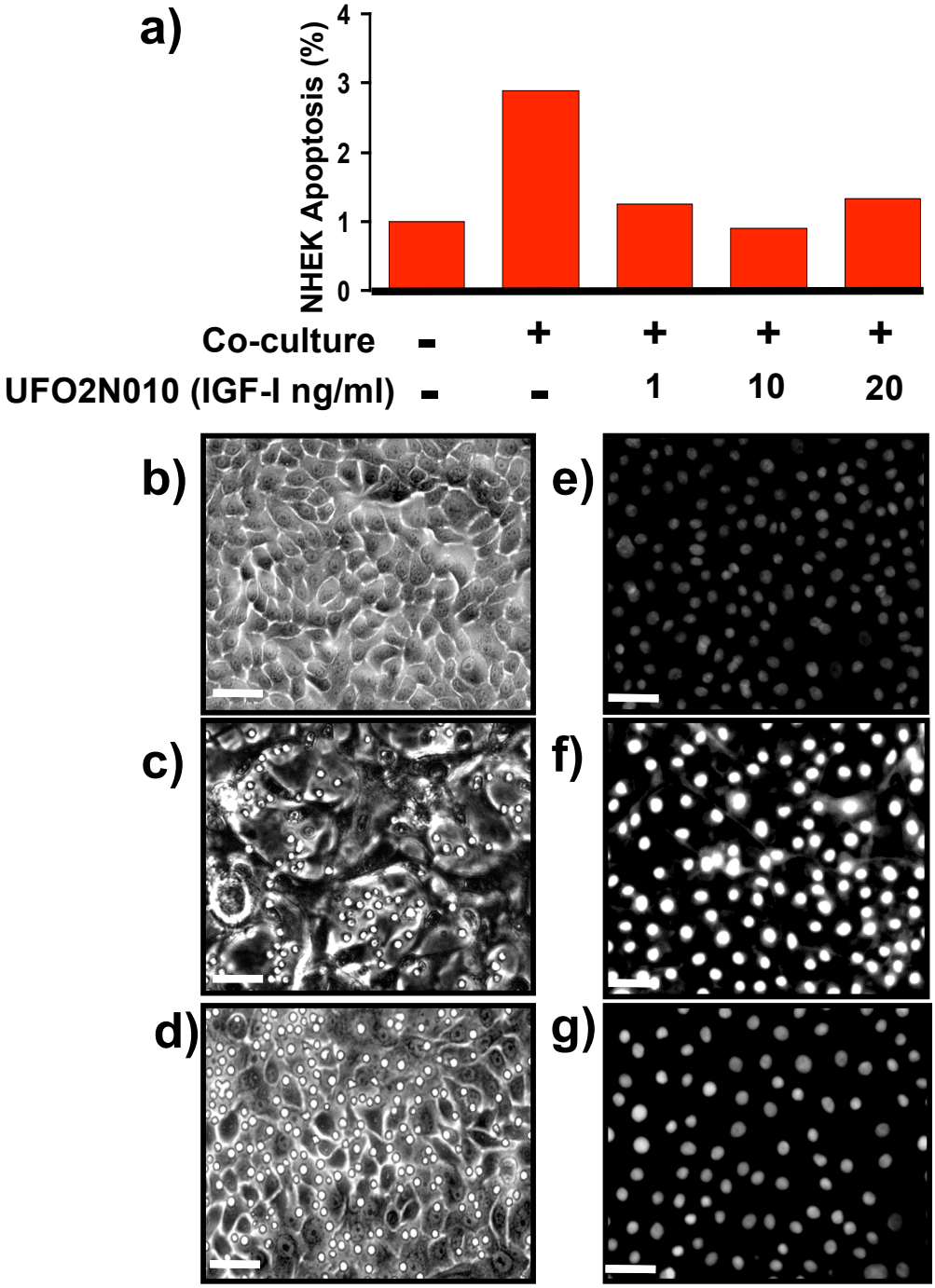


Figure 7.7 Effect of UFO2N010 on T-lymphocyte induced NHEK apoptosis. (a) NHEKs were co-cultured with T-lymphocytes together with UFO2N010 (to give IGF-I concentration 1, 10, 20ng/ml) for 48hrs. Cells were stained with Annexin V and PI and analysed by flow cytometry. Values represent the percentage of Annexin V positive cells. NHEK morphology was imaged by phase contrast microscopy prior to flow cytometric analysis of (b) Control NHEK (c) NHEK T-lymphocyte co-cultures and (d) NHEK T-lymphocyte co-cultures + UFO2N010 (10ng/ml IGF-1). Chromatin condensation and DNA fragmentation was assessed in parallel cultures using the HOESCHT 33342 fluorescent stain of (e) Control NHEK (f) NHEK T-lymphocyte co-cultures and (g) NHEK T-lymphocyte co-cultures + UFO2N010 (10ng/ml IGF-1). Scale bar represents 100μm for all images.

Figure 7.8

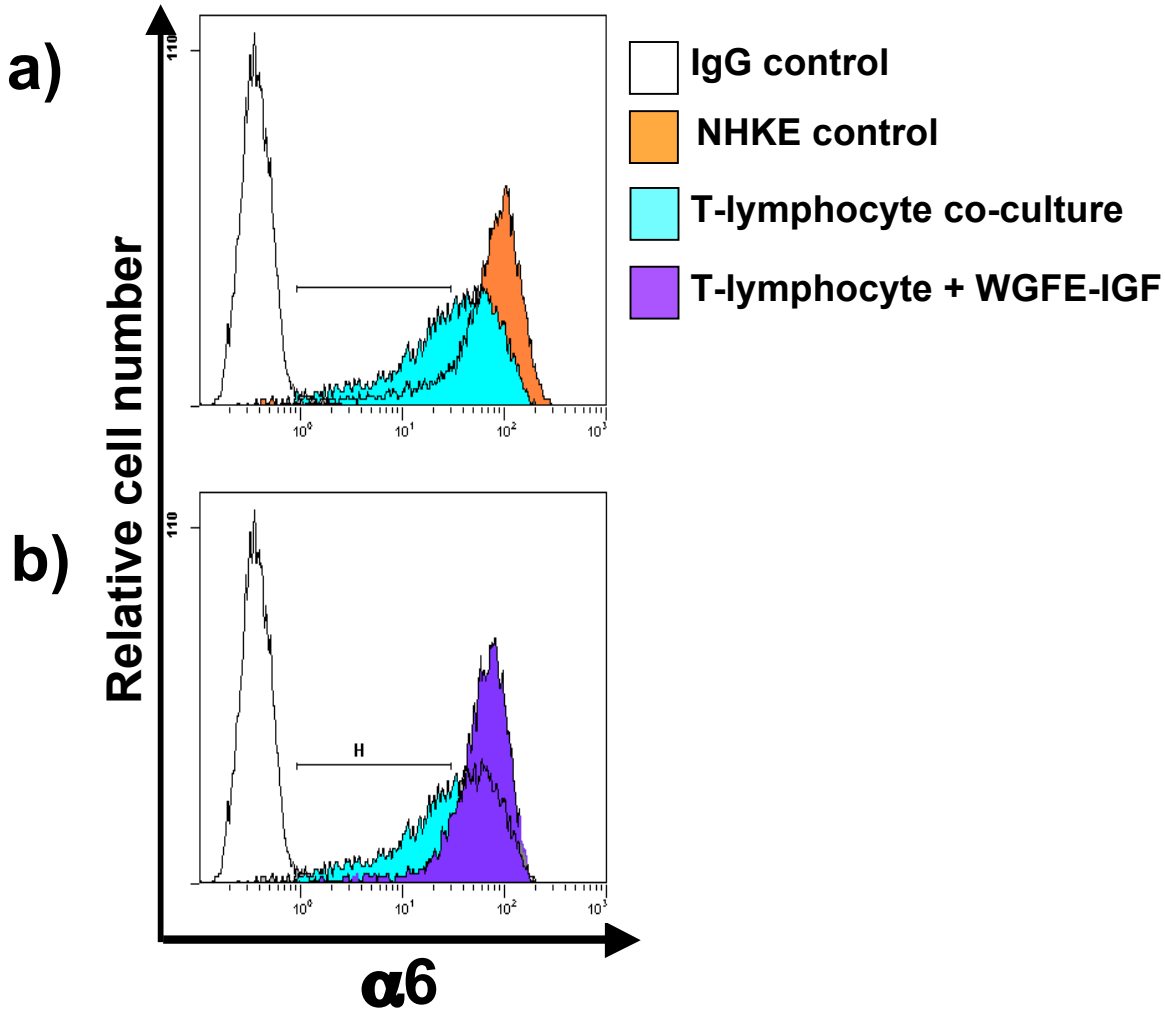


Figure 7.8. Effect of UFO2N010 on T-lymphocyte induced NHEK α6 dim expression. Surface expression of α6 integrin on (a) NHEK controls orange histogram and NHEKs T-lymphocyte co-cultures aqua histogram, (b) NHEKs co-cultured with T-lymphocytes + UFO2N010 (10ng/ml IGF-1) blue histogram. The unfilled histogram is the negative control (isotype-matched Ab).

7.4 Summary

The results presented in this chapter demonstrate that WGFE appears to be able to protect keratinocytes from T-lymphocyte induced apoptosis. Consistent with work performed using recombinant growth factors in Chapter 6, protection from apoptosis was shown to be independent of T-lymphocyte death. Even though WGFE was able to reduce the elevated IFNγ levels in the co-culture media as well as IFNγ induced Fas expression, WGFE did not change T-lymphocyte induced HaCaT Fas expression. This observation is consistent with results obtained with recombinant growth factors (Chapter 6). In fact, it was demonstrated in this Chapter that WGFE prevented T-lymphocyte induced early differentiation, an event found to be associated with HaCaT apoptosis in Chapter 6. The effect of WGFE on keratinocyte differentiation appears to be due to the TGFβ and IGF-I content as equivalent concentrations to those found in WGFE had a similar response.

NHEKs however, did not respond to WGFE in the same way as the HaCaTs. Given that it has been previously shown TGFβ did not protect NHEKs from T-lymphocyte induced apoptosis (Section 6.3.7), WGFE may not have reduced T-lymphocyte mediated apoptosis because of its high concentration of TGFβ. Removal of TGFβ and the enrichment of IGF-I in UFO2N010, resulted in the protection of NHEKs from T-lymphocyte induced apoptosis. The mechanism mediating the prevention of keratinocyte apoptosis appeared consistent between HaCaTs and NHEKs as UFO2N010 prevented the induction of keratinocyte differentiation associated with T-lymphocyte co-culture.

From the results presented in this chapter, the following conclusions can be drawn:

- Growth factor extracts derived from whey (WGFE) are as effective as recombinant growth factors in protecting HaCaT keratinocytes from T-lymphocyte induced apoptosis.
- WGFE itself does not protect NHEKs from T-lymphocyte induced apoptosis however, a WGFE fraction high in IGF-1; UFO2N010, had protective properties.
- IGF-I contained in WGFE appears to be the primary protective agent from T-lymphocyte induced apoptosis, and appears to work by preventing keratinocytes from undergoing early differentiation in response to the presence of T-lymphocytes.

CHAPTER 8

8 DISCUSSION

Atopic eczema (AE) is a chronic inflammatory skin disease resulting in complex dysregulation of the immune response to antigens in skin (Akdis *et al.* 2000). Current therapies are targeted at reducing the inflammation and reducing the symptoms of AE, however they do not cure the disease. As discussed previously, some of the more commonly used agents are associated with a number of adverse side-effects such as skin atrophy (Leung & Boguniewicz 2003) and recently there has been reports on potential risks of cancer with some of the more newly used treatments such as Elidel Cream (pimecrolimus; SDZ ASM 981) and Protopic Ointment (tacrolimus; FK506) (FDA Consumer magazine 2007) alone or in combination with topical corticosteroid {Ormerod *et al.* 2005}. However follow up case-control studies have reported contrary evidence regarding concerns of topical immunosuppressants including calcineurin inhibitors such as pimecrolimus or tacrolimus and the risk of lymphoma in patients with AE {Arellano *et al.* 2007}.

Although T-lymphocyte induced keratinocyte apoptosis plays a crucial role in the pathogenesis of the disease and is partly responsible for the development of lesions in AE (Trautmann *et al.* 2000a), there are no medications that specifically target keratinocyte apoptosis and promote survival. Keratinocyte apoptosis results in

breakdown of the epidermal barrier and propagation of immune dysregulation. As such, the identification of agents that can target multiple aspects of AE pathology, in particular keratinocyte apoptosis and inflammation, may be more useful for patients that do not respond to current treatments.

Previous studies have identified keratinocyte-T-lymphocyte co-cultures as good *in vitro* disease models for eczema and have been used to investigate various therapeutic treatments (Trautmann *et al.* 2001c). Consequently, the first aim of this thesis was to establish an *in vitro* cell model suitable for investigating the induction of keratinocyte apoptosis by T-lymphocytes. The second aim of this thesis was to use this model to examine whether growth factors, such as IGF-1, TGFβ and a milk derived growth factor extract containing TGFβ and IGF-1, could ameliorate T-lymphocyte induced keratinocyte apoptosis.

8.1 T-lymphocyte induced keratinocyte apoptosis

The results presented in this thesis demonstrated that major histocompatibility complex non-matched T-lymphocyte cultures induced keratinocyte apoptosis in co-culture. It was demonstrated here for the first time that mitogen activated Jurkat T-lymphocytes were capable of inducing apoptosis of HaCaTs, resulting in 29% of HaCaTs being apoptotic after a 48 hr co-culture. Similarly, mitogen activation of major histocompatibility complex non-matched primary CD4+ T-lymphocytes induced apoptosis in 28% of HaCaTs, whereas NHEKs were more sensitive to T-lymphocytes with 80% of NHEKs

being apoptotic after co-culture for 48 hr. This outcome corresponds to results obtained from primary keratinocyte co-culture system developed by Trautmann et al using autologous CD4+5RO+ (memory/effector) T-lymphocytes. The authors demonstrated that T-lymphocyte activation with anti-CD2, anti-CD3, and anti-CD28 mAb's resulted in 81.6% apoptotic keratinocytes after a 72 hr co-culture (Trautmann *et al.* 2000a). Also consistent with the system developed by Trautmman et al, unstimulated T-lymphocytes used in the studies described here, did not affect keratinocyte morphology. In contrast, keratinocytes co-cultured with activated T-lymphocytes underwent several morphological changes including cell shrinkage and rounding, membrane blebbing, chromatin condensation, increased nuclear fragmentation and finally cell detachment from the culture plate. These changes in cell morphology are consistent with other studies using several inducers of apoptosis such as UV radiation and IFNγ (Henseleit *et al.* 1996, Mammone *et al.* 2000a).

8.1.1 T-lymphocyte induced Fas mediated apoptosis of keratinocyte

A mechanism by which T-lymphocytes induce keratinocyte cell death in the co-culture cell system established in this thesis was proposed in Figure 5.19. Consistent with *in vivo* data from the skin of AE patients (Trautmann *et al.* 2000a), the results in Chapter 4 and 5 suggest a central role for Fas in mediating CD4+ T-lymphocyte induced keratinocyte apoptosis, with inhibition of apoptosis achieved by blocking Fas with an anti-Fas antibody. This result implies that the FasL-Fas interaction is responsible for T cell–mediated keratinocyte apoptosis. Similar inhibitory effect have been reported using the same concentrations of anti-Fas antibody, such that blocking Fas completely

inhibited keratinocyte apoptosis in co-cultures with stimulated CD45RO+ memory/effector T-lymphocytes (Trautmann *et al.* 2000a, Trautmann *et al.* 2001c, Trautmann *et al.* 2001b). The authors of these studies suggested that there were no apoptosis-inducing pathways other than Fas that mediated keratinocyte apoptosis induced by T-lymphocytes. Given that lesional AE skin is also defined histologically by the infiltration of CD8+ T-lymphocytes (Trautmann *et al.* 2000b), additional work was performed in this thesis to assess the effect of CD4-depleted mononuclear cells, a mixed population of cells consisting mainly of CD8+ T-lymphocytes (Appendix 7) on keratinocytes. Confirming the results obtained with CD4+ T-lymphocytes, the CD4 depleted mononuclear cells induced apoptosis of keratinocytes by upregulation of Fas expression thus increasing the susceptibility to apoptosis mediated by FasL (Arnold *et al.* 1999, Traidl *et al.* 2000), also shown to be upregulated by these population of T-lymphocytes upon activation.

8.1.2 T-lymphocyte induced keratinocyte apoptosis is mediated by IFNγ stimulated upregulation of keratinocyte Fas and subsequent activation of caspase 3

The Th1 cytokines IFNγ and TNFα released by T-lymphocytes in skin can upregulate Fas expression and increase keratinocyte susceptibility to apoptosis (Arnold *et al.* 1999, Traidl *et al.* 2000, Trautmann *et al.* 2000a). In co-culture however, only IFNγ levels were elevated in the conditioned media. The increased IFNγ in the co-culture system corresponds with studies that demonstrate expression of IFNγ is predominant in the chronic AE skin lesions (Grewe *et al.* 1994, Spergel *et al.* 1999). Furthermore, biopsies

of skin lesions elicited by patch testing AE patients with dust-mite antigens reveal that a majority of T-lymphocytes in the lesions express IFNγ (Thepen *et al.* 1996). In contrast to reports showing that soluble factors released into the conditioned media by activated T-lymphocytes can induce keratinocyte apoptosis by upregulation of Fas (Trautmann *et al.* 2000a), studies in this thesis demonstrated that soluble factors released by activated T-lymphocytes did not induce apoptosis of keratinocytes even though sFasL was detected in the conditioned media. An explanation for this may be that IFNγ released into the co-culture conditioned media was at a relatively low concentration (~1ng/ml), and hence not capable of increasing keratinocyte Fas expression above control levels or above the threshold level required to promote apoptosis signalling as previously described by Trautmann et al (Trautmann *et al.* 2000a). However, consistent with *in vitro* observations in HaCaTs by Henseleit U et al, (Henseleit *et al.* 1996), the addition of recombinant IFNγ alone at 100ng/ml was shown to be a strong inducer of keratinocyte Fas expression and apoptosis. Although TNFα induces Fas expression, it does not induce keratinocyte apoptosis, however together with IFNγ it has been shown to synergistically induce keratinocyte apoptosis (Konur *et al.* 2005). Since TNFα was shown to be released by activated T-lymphocytes but not detected in co-culture media, it may be speculated that any TNFα released may be utilised by keratinocytes and act in a paracrine manner with the IFNγ present to induce keratinocyte apoptosis in co-culture. However, further work would need to be performed in order to clarify the involvement of TNFα in co-culture induced HaCaT apoptosis.

The presence of IFNγ in co-culture was shown to potentiate co-culture induced Fas expression by keratinocytes, resulting in greater sensitivity to T-lymphocyte-mediated apoptosis. Previous work has demonstrated IFNγ to be a prerequisite for T-lymphocyte–mediated keratinocyte apoptosis (Trautmann *et al.* 2000a). The authors showed that blocking IFNγ with neutralising antibodies totally abrogated keratinocyte apoptosis induced by activated T-lymphocytes in co-culture. By using numerous cytokines and anti-cytokine neutralizing mAb's, the authors concluded that no cytokines other than IFNγ participated in this process (Trautmann *et al.* 2000a). Supporting this study, the results in this thesis, it appears that IFNγ is not only involved in maintaining the inflammatory response, but also has potential implications for inducing keratinocyte apoptosis in AE. These results would indicate that the release of IFNγ together with direct interaction between T-lymphocytes and keratinocytes would be required to induce keratinocytes apoptosis in the skin.

Consistent with studies demonstrating increased adhesion molecules expression in inflamed skin (Nickoloff *et al.* 1993, Singer *et al.* 1989), keratinocyte ICAM-1 expression was markedly increased with co-culture and further augmented by the addition of IFNγ. Together with a concomitant upregulation of LFA-1 by T-lymphocytes in co-culture, it can be speculated that the attachment of keratinocytes to T-lymphocytes could aid the delivery of the apoptosis signal by FasL expressed on the surface of T-lymphocytes. These observations are strengthened by previous reports that suggest that keratinocytes may be stimulated by IFNγ to serve as accessory cells by retaining T-

lymphocytes in the skin and contribute to the dysregulated inflammatory response in AE (Nickoloff *et al.* 1993).

The Fas and FasL pathway is involved in mediating keratinocyte apoptosis of UV-irradiated human skin (Aragane *et al.* 1998, Bang *et al.* 2002, Leverkus *et al.* 1997). The mechanism is still unclear, however as UVB light can induce direct activation of Fas (Aragane *et al.* 1998), or act indirectly by increasing endogenous FasL expression (Leverkus *et al.* 1997). Similar to UV radiation and the induction of apoptosis using sodium butyrate (Daehn *et al.* 2006, Denning *et al.* 1998), T-lymphocyte induced keratinocyte apoptosis appears to be mediated by caspase 3 and subsequent cleavage of PARP. Caspase 3 was responsible for mediating key apoptosis related morphological changes including chromatin condensation and DNA fragmentation as confirmed by the use of caspase 3 inhibitor; DMQD-CHO (Hirata *et al.* 1998, Zhu & Otterson 2003).

The findings from this thesis, together with the published literature presented, support the conclusion that the established co-culture model provides a proof-of-principle model for T-lymphocyte induced Fas mediated keratinocyte apoptosis. T-lymphocyte induced keratinocyte apoptosis is mediated by IFNγ stimulated upregulation of keratinocyte Fas that is consistent with AE *in vivo*. This resulted in subsequent activation of the caspase cascade by binding T-lymphocyte associated FasL.

Disease relevant studies could be performed in the future, to confirm the established system *in-vitro*. For example, it has been known for some time that colonization and

infection with Staphylococcus aureus and streptococci exacerbates AE and psoriasis. An early study performed by Leyden *et al.* reported that out of 50 AE patients containing chronic plaques, 90% of them were colonized with Staphylococcus aureus (Leyden *et al.* 1974). Other studies have shown that over half of the S. aureus strains isolated from AE skin secrete superantigenic toxins, including staphylococcal enterotoxins A and B (SEA, SEB), and toxic shock syndrome toxin-1 (TSST-1) (Leung *et al.* 1998, Manders *et al. 1998*). These superantigenic toxins not only activate T-lymphocytes, but have also been proposed to support the persistence of the disease by directly influencing keratinocytes to release TNFα, as previously shown in cultured HaCaTs and primary keratinocytes (Leung *et al.* 1995, Ezepchuk *et al.* 1996). Studies could investigate the effect of T-lymphocyte activation using toxins isolated from patients with AE and assess their effect on keratinocytes in co-cultures. It is worth recognising that although superantigenic stimuli like SEB, can significantly increase in FasL expression of CD4/ T-lymphocytes resulting in apoptosis of target EA.hy926 endothelial cells in co-culture, other apoptosis inducing factors may be activated by increasing expression of apoptosis-promoting protein TIA-1, granzyme B, or Apo-2 ligand, thereby mediating apoptosis by non-Fas/FasL pathways (Urayama *et al.* 1997).

8.2 Growth factors protected keratinocytes from T-lymphocyte induced apoptosis

The results from Chapter 6 demonstrated that a combination of insulin-like growth factor-1 (IGF-1) and transforming growth factor β_1 (TGFβ_1) was able to prevent T-lymphocyte induced HaCaT keratinocyte apoptosis. The HaCaT morphology seemed unaltered and the intercellular adhesions between cells and the monolayer appeared intact after co-culture with growth factors. This is particularly important as impairment of the barrier function is a major consequence in AE, despite this, there are currently no treatments for this disease that aim to protect or maintain the barrier function of the skin. Although the studies described in this thesis did not specifically examine the effect of T-lymphocytes and growth factors on keratinocyte barrier function, it is also worth noting that the data and observations presented indicate that IGF-I and TGFβ may have helped maintain the barrier function of the monolayer in culture. Future experiments investigating trans-epithelial resistance of the monolayer would be required to understand these effects more clearly.

It was demonstrated here that the cell survival and preservation of the monolayer was attributed to a direct effect on the keratinocytes as the growth factors did not affect the viability of T-lymphocytes in co-culture. Although TGFβ_1 is known to induce lymphocyte apoptosis (Bommireddy *et al.* 2003, Chung *et al.* 2000, Lomo *et al.* 1995, Sillett *et al.* 2001), the T-lymphocytes may have survived in co-culture due to the opposing effects of IGF-1, which has been shown to promote the survival of cord blood T-lymphocytes in culture (Tu *et al.* 2000).

In contrast to the effect of the IGF-I and TGFβ$_1$ combination on HaCaTs, preliminary studies with primary keratinocytes demonstrated that IGF-1 alone and not TGFβ$_1$, was capable of preventing T-lymphocyte induced apoptosis. Whilst HaCaTs share many features of differentiation with normal keratinocytes (Boukamp *et al.* 1988, Schoop *et al.* 1999), they are an immortalised and genetically abnormal cell line (Boelsma *et al.* 1999, Boukamp *et al.* 1988). Besides the inactivation of both p53 alleles in HaCaTs (Lehman *et al.* 1993), other apoptosis related differences between HaCaTs and primary keratinocytes have been reported, including evidence of a differential response to TGFβ (Chaturvedi *et al.* 1999). The authors proposed that these discrepancies may be attributed to defective NF-κβ signalling, which is involved in regulation of apoptosis and survival of keratinocytes (Chaturvedi *et al.* 2001, Qin *et al.* 1999).

8.2.1 Growth factors effects Fas

Despite the differential response between HaCaTs and NHEKs to TGFβ$_1$, the mechanism by which IGF-1 alone or together with TGFβ$_1$ protects keratinocytes from T-lymphocyte induced apoptosis were further addressed in this study. In the first instance, the effect of growth factors on IFNγ induced Fas levels was examined, given that it was demonstrated here and by others to be a key mediator of T-lymphocyte–induced keratinocyte apoptosis (Trautmann *et al.* 2000a). IGF-1 and TGFβ$_1$ were able to decrease IFNγ induced keratinocyte Fas expression and to suppress the release of IFNγ in co-cultures. This outcome suggested that treatment with growth factors may render keratinocytes to be less susceptible to T-lymphocyte induced FasL mediated apoptosis.

This was found not to be the case however, as IGF-1 alone or in combination with TGFβ_1 did not decrease T-lymphocyte induced keratinocyte Fas.

The inability of growth factors to suppress Fas expression induced by T-lymphocytes indicated that survival was potentially acting on targets downstream of Fas. The activation of growth factor receptors has been shown to affect the functional state and pattern of expression of survival proteins from the Bcl-2 family (Jost *et al.* 1999). For instance, there is convincing evidence suggesting that epidermal growth factor receptors (EGFR) activation increases keratinocytes survival by modulating Bcl-xL expression (Sibilia *et al.* 2000). Yet, although the specific intracellular pathway mediating the inhibition of apoptosis in growth factor treated keratinocytes were not investigated in this thesis, the means by which TGFβ or IGF-1 independently can prevent apoptosis of human epidermal keratinocytes has been the subject of previous work (Heron-Milhavet *et al.* 2001, Kuhn *et al.* 1999, Shin *et al.* 2001).

8.2.2 Potential pathways mediating keratinocyte survival induced by growth factors

Two potential pathways are proposed to explain how growth factors may protect keratinocytes from T-lymphocyte induced apoptosis and are illustrated in Figure 8.1. These pathways suggest a potential link between extracellular growth factor signals and intracellular mechanisms of keratinocyte survival. The first pathway involves the production of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3), which delivers signals from the cell surface to the cytoplasm to activate the kinase 3-

phosphoinositide-dependent protein kinase-1 (PDK1) (Xie *et al.* 2005). PDK1 in turn activates the kinase Akt also known as PKB (Protein Kinase-B) (Decraene *et al.* 2002, Shin *et al.* 2001). Once activated, Akt can promote cell survival by phosphorylation and inactivation of pro-apoptotic Bad protein, through binding to proteins of the 14-3-3 family (Hekman *et al.* 2006). Phosphorylated Bad causes a dissociation of the Bad/Bcl-xl complex, allowing cell survival (Hekman *et al.* 2006) (Figure 8.1 a). Similarly,

Figure 8.1

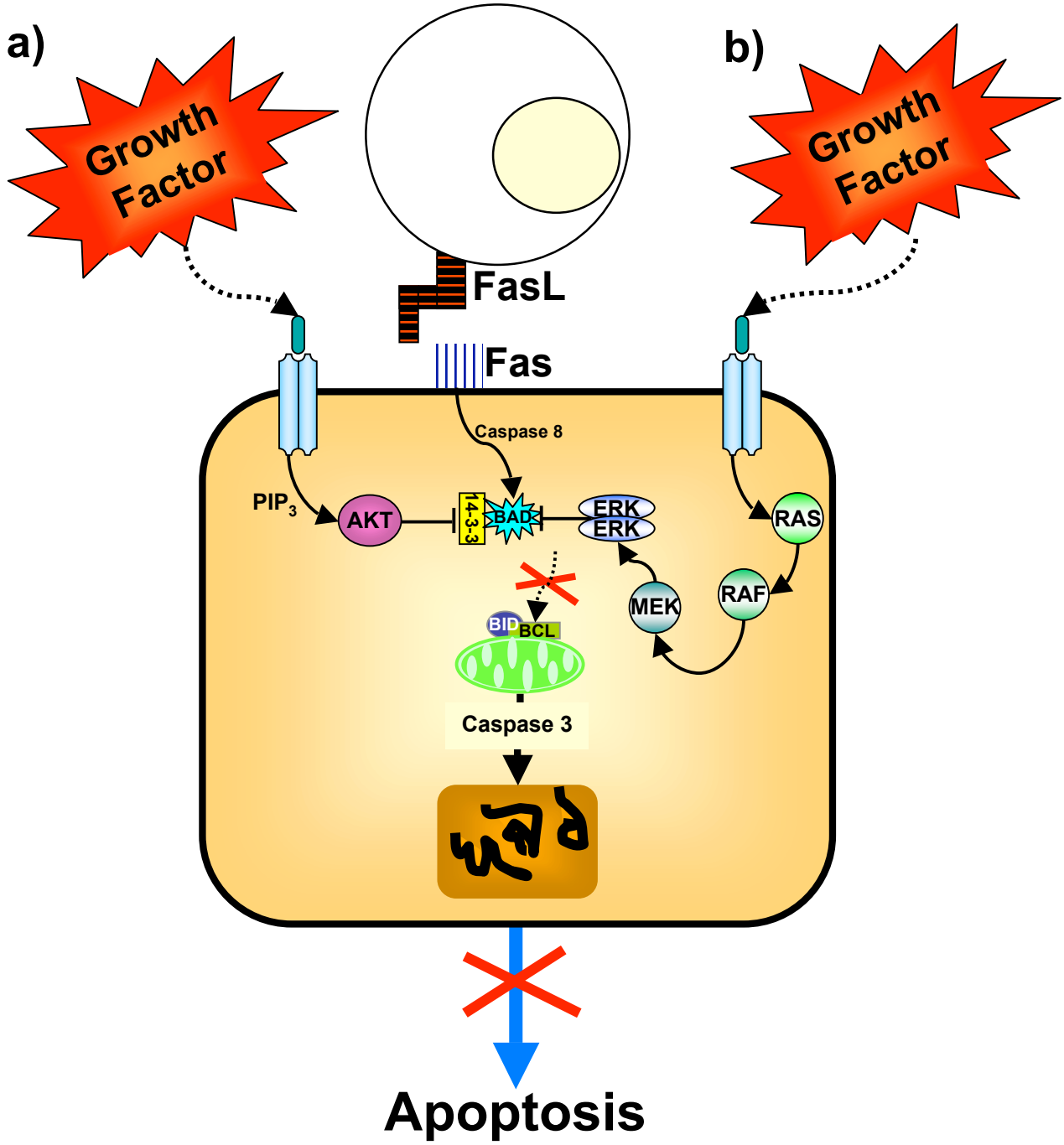


Figure 8.1. Proposed mechanism of growth factor effects in protecting keratinocytes from T-cell co-culture induced Fas mediated apoptosis .

phosphorylation of the protease caspase 9 or forkhead transcription factors by Akt could also inhibit the induction of apoptosis (Linseman *et al.* 2002) (Figure 8.1 a).

Figure 8.1 b illustrates the second potential mechanism for survival involving the mitogen-activated protein kinase (MAPK) pathway, which also plays an important role in growth factor mediated survival, as demonstrated in keratinocytes overexpressing IGF-IR (Heron-Milhavet *et al.* 2001). In this pathway, growth factor receptor activation results in subsequent Ras and Raf activation by a series of complex changes in phosphorylation, protein-protein interactions, and protein-lipid interactions (Yuryev & Wennogle 1998). Activated Raf, can in turn activate MEK. MEK phosphorylates the MAP kinase ERK (extracellular-signal-regulated kinase) into two residues resulting in activation of the pro-survival molecule CREB and inactivation of proapoptotic Bad (Bonni *et al.* 1999, Neithardt *et al.* 2006, Perkinton *et al.* 2002). Essentially, both pathways could influence the balance of pro-apoptotic and anti-apoptotic regulators in favour of keratinocyte survival.

8.3 T-lymphocyte induced early keratinocyte differentiation

8.3.1 Loss of α6 integrin by apoptotic keratinocytes

The results in Chapter 5 demonstrate that T-lymphocytes can initiate the onset of keratinocyte differentiation. The induction of keratinocyte differentiation was

determined by measuring changes in α6 integrin expression. Anchorage of basal keratinocytes by integrins to the basement membrane extracellular matrix proteins such as laminins, is essential for the structural integrity of the skin (Lanschuetzer *et al.* 2003). Integrins can further regulate the functional differentiation of epithelial cells in culture and *in vivo* (Adams & Watt 1993) and have previously been shown to be implicated in linking the extracellular matrix to keratin intermediate filaments (Borradori & Sonnenberg 1999, Stepp *et al.* 1990). Once keratinocytes begin to differentiate, they detach from the basement membrane after losing their integrin expression and migrate upwards through the suprabasal layers of the epidermis and eventually they are sloughed from the outer skin layers as dead squames (Li *et al.* 1998, Webb *et al.* 2004). The findings from this thesis demonstrated that keratinocytes in culture were normally high expressors of α6-integrin, however when co-cultured with T-lymphocytes, α6 expression decreased, consistent with the induction of early keratinocyte differentiation (Kaur & Li 2000, Li & Kaur 2005, Webb *et al.* 2004). Furthermore, co-staining experiments demonstrated that the apoptotic keratinocytes were also the differentiating, low expressors of α6-integrin. Thus, it may be postulated that T-lymphocytes cause cultured keratinocytes to lose resistance to apoptosis by loss of adhesion and induction of differentiation. This hypothesis is supported by the highly apoptotic basal keratinocytes observed by TUNEL staining, of the detached epidermis from α6-integrin deficient mice embryos (DiPersio *et al.* 2000). Similar to keratinocytes, melanocytes also bind to laminin molecules of the basement membrane via the integrins such as α3β1 and α6β1 (Krengel *et al.* 2005). UVB light has also been shown to induce apoptosis of melanocytes along with down-regulation of α6-integrin (Krengel *et al.* 2005). A

proposed relationship between apoptosis induction and loss of integrin adhesion was demonstrated by the respective authors as selective down-regulation of α6-integrin and no effect on other adhesion molecules such as E-, N-cadherin, α2-, α3-, α5-, αV-, β1-, β3-integrins or ICAM-1 was shown. This in turn altered the interaction between melanocytes and the basement membrane, resulting in apoptosis and cell detachment.

8.3.2 α6 integrin mediated survival of keratinocytes

It has been previously proposed by Norris *et al.* that the basal layer of the epidermis is resistant to apoptosis due to basal cells having highly developed anti-apoptotic defences (Norris *et al.* 1997). Undifferentiated basal keratinocytes have been shown to be resistant to UV induced apoptosis by constitutively expressing higher levels of anti-apoptotic members of the Bcl-2 family (Delehedde *et al.* 1999, Hendrix *et al.* 1998). It has also been suggested that basal keratinocytes can prevent apoptosis via a pro-survival function of integrins (Dowling *et al.* 1996, Norris *et al.* 1997). Interestingly, these defences appear to be decreased during differentiation (Norris *et al.* 1997).

The relationship between integrins, such as α6, and cell survival has been also demonstrated in other epithelial cells including normal breast epithelium and ovine granulosa cells where cell survival, growth and proliferation were shown to be α6 or α6β1 integrin dependent (Howlett *et al.* 1995, Le Bellego *et al.* 2005). Survival via α6 is mediated by binding to extracellular matrix molecules such as Laminin 5 (Ryan *et al.* 1999). A Laminin rich basement membrane but not collagen, was shown to be able to prevent primary mammary epithelial cells apoptosis (Farrelly *et al.* 1999, Pullan *et al.*

1996), indicating that survival signals were specific to the attachment of integrin receptors. These reports support the notion that keratinocytes need to adhere to the extracellular matrix for survival. In culture, keratinocytes have been shown to produce extracellular matrix proteins which serve as putative ligands for integrins (Larjava *et al.* 1993, Nickoloff *et al.* 1988). As consequence the adhesion of keratinocytes in culture to the endogenous extracellular matrix, is mediated by α6 and promotes keratinocyte proliferation and migration together with the formation of a confluent monolayer (Larjava *et al.* 1993). This is consistent with the migrating keratinocytes in wound healing (Larjava *et al.* 1993).

The novel finding that T-lymphocytes promoted the induction of keratinocyte terminal differentiation raises a number of questions. The key is whether the pathways mediating keratinocyte death and differentiation are independent, as previously suggested by Gandarillas et al (Gandarillas *et al.* 1999). Given that T-lymphocyte induced Fas mediated keratinocyte apoptosis was shown to occur most readily in α6-dim expressing cells being the early differentiating cells, these two pathways could be more closely related than currently thought. Perhaps α6 could be considered as a potential therapeutic target for treatment of inflammatory diseases. Although the detailed mechanism mediating these effects was beyond the scope of this study, future studies aimed at understanding the mechanisms resulting in death and differentiation of keratinocytes will help to answer some of these questions as well as confirm the role α6 integrin in this process. However based on the data presented, it can be postulated that T-lymphocytes may cause cultured keratinocytes to lose resistance to apoptosis by loss of integrin

expression and subsequent induction of differentiation. The onset of keratinocyte differentiation may result in keratinocytes being more susceptible to death induced by T-lymphocyte associated FasL and activation of the Fas death pathway. In light of these findings, it may be hypothesised that the increased keratinocyte susceptibility to death induced by T-lymphocytes *in vitro* may be due to both increased Fas expression and reduced anti-apoptotic defences as the cells are induced to differentiate.

8.4 Growth factor mediated keratinocyte survival

Studies performed in this thesis demonstrated that the decrease in T-lymphocyte induced keratinocyte apoptosis by IGF-1 and TGFβ_1 was associated with preservation of α6 integrin expression. This novel finding would suggest that these growth factors were able to reduce the susceptibility to T-lymphocyte induced apoptosis, potentially by preventing keratinocytes from entering terminal differentiation.

The importance of integrins in cell survival and the effect of IGF-1 and TGFβ_1 observed in the studies reported in this thesis implies that IGF-1 and TGFβ_1, and subsequent activation of their receptors, may cooperate together with integrins to mediate survival signalling in keratinocytes. This concept is supported by work performed in normal mammary epithelial cells, which demonstrate that tyrosine phosphorylation of insulin, IGF-I or IGF-II receptors, led to subsequent PI3K mediated survival (Farrelly *et al.* 1999). Furthermore, the authors demonstrated that PI3K followed by Akt activation

required specific adhesion to the basement membrane via α6 and β1 subunits. Thus integrins appear to mediate IGF-1 survival signals in epithelial cells. Based on this evidence and the work presented in this thesis, the protection of keratinocytes from T-lymphocyte induced apoptosis by growth factors appears to be facilitated by α6 mediated activation of the downstream survival signalling pathway. Despite the differential response between HaCaTs and NHEKs to the $TGF\beta_1$, it can also be concluded that keratinocytes undergo Fas-dependent apoptosis when released from the protection of integrin and are induced to differentiate by T-lymphocytes. However future studies aimed at understanding the mechanisms resulting in death and differentiation of keratinocytes will help confirm the role growth factors may have in keratinocyte survival.

The role of integrins in T-lymphocyte mediate apoptosis of keratinocytes and the mechanisms by which specific growth factors regulate keratinocyte survival via integrins remains unclear. However Figure 8.2 illustrates a potential mechanism for growth factor mediated survival of keratinocytes challenged by T-lymphocytes. The proposed model would involve growth factor receptor activation of the focal adhesion kinase (FAK) pathway (Jost *et al.* 1999, Manohar *et al.* 2004). FAK together with α6 integrin activation could mediate survival through interactions with signal transduction molecules such as PI3K and consequently stimulate the MEK/ERK signalling cascade (Eblen *et al.* 2002, Miyamoto *et al.* 1995). This would result in the subsequent inhibition of caspase-3 activity, and ultimately keratinocyte cell survival (Manohar *et al.* 2004) (Figure 8.2).

Figure 8.2

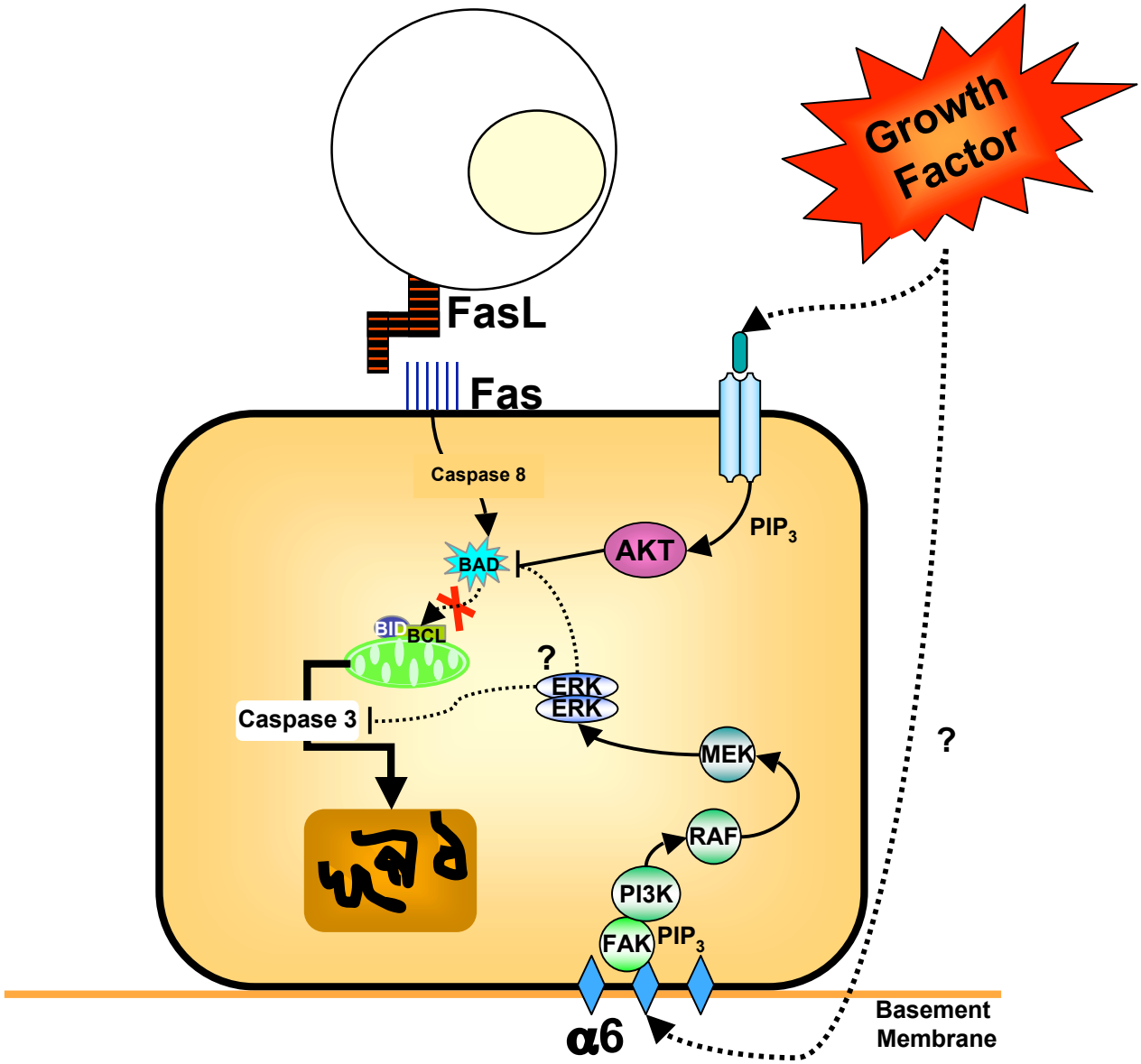


Figure 8.2. Involvements of integrin mediated survival pathway induced by growth factor for protecting keratinocytes from T-cell co-culture induced apoptosis.

8.5 Application of thesis outcomes and future work

The work presented in this thesis suggests that growth factor based treatments could help maintain the important barrier function of the skin by preventing T-lymphocyte induced keratinocyte apoptosis, which can result in increased invasion of allergens in AE. This outcome offers encouraging prospects for the development of growth factor based applications for treatment of inflammatory skin conditions.

8.5.1 Growth factor based therapies as potential treatments for inflammatory skin disorders

Although protection of keratinocytes in skin from T-lymphocyte induced apoptosis could be achieved by IGF-I alone, the use of anti-inflammatory agents would be necessary for the treatment of AE, since IGF-1 may also promote the survival of T-lymphocytes (Tu *et al.* 2000). Although TGFβ_1 showed no effect in protecting primary keratinocytes from T-lymphocytes, the combination of IGF-1 and TGFβ may still be beneficial in the clinical setting of AE, given the potential anti-inflammatory effects of TGFβ. It has been previously shown that TGFβ plays an important role in modulating immune cell function and immune homeostasis (Bommireddy *et al.* 2003, Christ *et al.* 1994, Kulkarni *et al.* 1993, Kulkarni & Karlsson 1993). It has been shown that TGFβ can reduce the airway hyperreactivity in acute lung injury by means of inhibiting T-lymphocyte proliferation and regulating Th2-induced effects (Hodge *et al.* 2002,

Schramm *et al.* 2003). Recently it has been demonstrated that the increased levels of IL-10 and TGF-β produced by T-regulatory cells (T_{Reg}) suppress IgE production and promote resolution of allergic inflammation by suppressing allergen-specific activation of Th1 and Th2 cells, mast cells, basophils, and eosinophils in asthma in AE (Akdis *et al.* 2005, Verhagen *et al.* 2006). Complementing these reports, TGFβ has been shown to suppress inflammation in skin by promoting apoptosis of activated T-lymphocytes, as well as decreasing FasL expression on T-lymphocytes undergoing re-stimulation (Arnold *et al.* 1999, Sillett *et al.* 2001). There is also evidence to suggest that TGFβ can inhibit T-lymphocyte differentiation by regulating the expression of GARA-3 on mature CD4+ cells (Gorelik *et al.* 2000), consequently preventing T-lymphocytes from secreting proinflammatory cytokines important in the early onset and development of chronic disease. In keratinocytes, $TGF\beta_1$ is able to inhibit the expression of thymus and activation regulated chemokine (TARC/CCL17), which is responsible for recruiting CLA^+CCR4^+ T-lymphocytes to the skin (Sumiyoshi *et al.* 2003, Zheng *et al.* 2002), resulting in decreased infiltration of T-lymphocytes into the skin. As such, TGFβ would be of most benefit during the acute inflammatory phase of the disease, to prevent the activation and recruitment of T-lymphocytes.

The work in this thesis suggests that a combination of IGF and TGFβ peptides could provide the greatest efficacy in AE by TGFβ preventing the initial infiltration of T-lymphocytes into the skin in the early phase of the disease, while IGF-I protects keratinocytes from T-lymphocyte induced apoptosis. The development of novel therapies containing IGF-I and TGFβ could augment the effectiveness of current

treatments for AE such as corticosteroids, or reduce the reliance on these therapies and thus reduce the frequency and severity of adverse side effects.

8.5.2 Milk derived growth factor based therapies as potential treatments for atopic eczema

The production of recombinant growth factors is a difficult and expensive process. Alternatively, milk-derived growth factor preparations containing IGF-I and TGFβ such as WGFE, may have an the advantage over recombinant proteins, due to the comparative ease and lower cost to produce, which makes it potentially more commercially viable. Results in Chapter 7 demonstrated that WGFE containing concentrations of growth factors equivalent to recombinant IGF-I and TGFβ, was indeed capable of protecting keratinocytes from T-lymphocyte induced apoptosis. Consistent with the results using recombinant growth factors, inhibition of keratinocyte apoptosis by WGFE was not attributed to changes in keratinocyte Fas expression. The mechanism of keratinocyte survival appears similar to that proposed for the recombinant growth factors, whereby activation of survival pathway by the growth factors present in the mixture, together with the involvement of an α6-integrin survival pathway, may result in the protection of the keratinocytes.

Cell based and clinical studies provide evidence to support the anti-inflammatory properties of WGFE and its ability to stimulate repair of skin (Penttila *et al.* 2001, Rayner *et al.* 2000, Varelias *et al.* 2006). Whilst the action of WGFE may be attributed largely to the IGF I & II and TGFβ content of the extract (Conlon & Tomas 2003), other

molecules present in WGFE may be mediating the action of WGFE (Belford *et al.* 1997). For instance, Betacellulin which accounts to about 50% of the growth promoting activity of WGFE (Dunbar *et al.* 1999), has also been demosntrated to have apoptotic inhibitory activity through enhanced Erk activation (Saito *et al.* 2004). As such, specific growth factor blocking antibody studies will need to be performed to verify whether the effect of WGFE seen here are attributed to the IGF-I and TGFβ present in the mixture.

Notwithstanding, Phase I clinical trials confirmed the safe use of WGFE for the treatment of patients undergoing high doses of BEAM chemotherapy, which resulted in the reduction of the duration and severity of oral mucositis in patients (Dyer *et al.* 2006, Prince *et al.* 2005). Supporting animal studies demonstrated that WGFE treatment resulted in significant reduced mucosal ulceration (Clarke *et al.* 2002) with the protective effects on the cheek mucosa attributed to the anti-proliferative and anti-microbial effects of WGFE. WGFE has also been shown to inhibit the production of TNFα by cultured macrophages (Dyer *et al.* 2006), which together with the inhibition in IFNγ levels released in co-culture shown here, confirms that WGFE has strong anti-inflammatory potential. Animal based studies by Howarth et al (Howarth *et al.* 1996) showed that oral administration of this growth factor extract ameliorated methotrexate-induced damage in the small bowel of rats. This supports data by Tran et al and Porter et al, showing that WGFE improved the growth, adaptation, repair, and intestinal permeability of methotrexate induced gut damage in the rat and reduced colonic inflammation (Porter *et al.* 1998, Tran *et al.* 2003). In summary, this thesis proposes that WGFE may have beneficial properties based on its combination of anti-apoptotic and

anti-inflammatory effects, that are potentially provided by the growth factors in the mixture. Such evidence suggests that there is value in pursuing clinical applications using WGFE based treatments.

Based on the evidence presented in this thesis and the supporting literature, growth factor treatments could potentially be used as a preventive treatment for AE and provide relief to many sufferers of the disease. Such growth factor based treatments could be used as adjuvants, reducing the required doses and reliance on current treatments available, particularly in cases of AE where immunosuppressive therapy is not recommended or not well controlled by topical steroids. Furthermore, given that the growth factor treatments protect the basal keratinocytes, it would be interesting to determine if these agents could be used for treating other skin conditions where basal keratinocytes become the target of T-lymphocytes such as in toxic epidermal necrolysis (TEN). TEN is characterized by blistering and peeling of the epidermis as a result of FasL mediated basal keratinocyte apoptosis induced by inflammatory T-lymphocytes consequently leading the cells to become detached from the basement membrane (Abe *et al.* 2003, Viard *et al.* 1998).

Finally, this thesis provides support for further research into the development of growth factor based therapies for treating AE, with particular emphasis on alternative sources of growth factors such as those shown in this thesis that are derived from milk. These been readily available, safe for use on humans and more commercially viable than

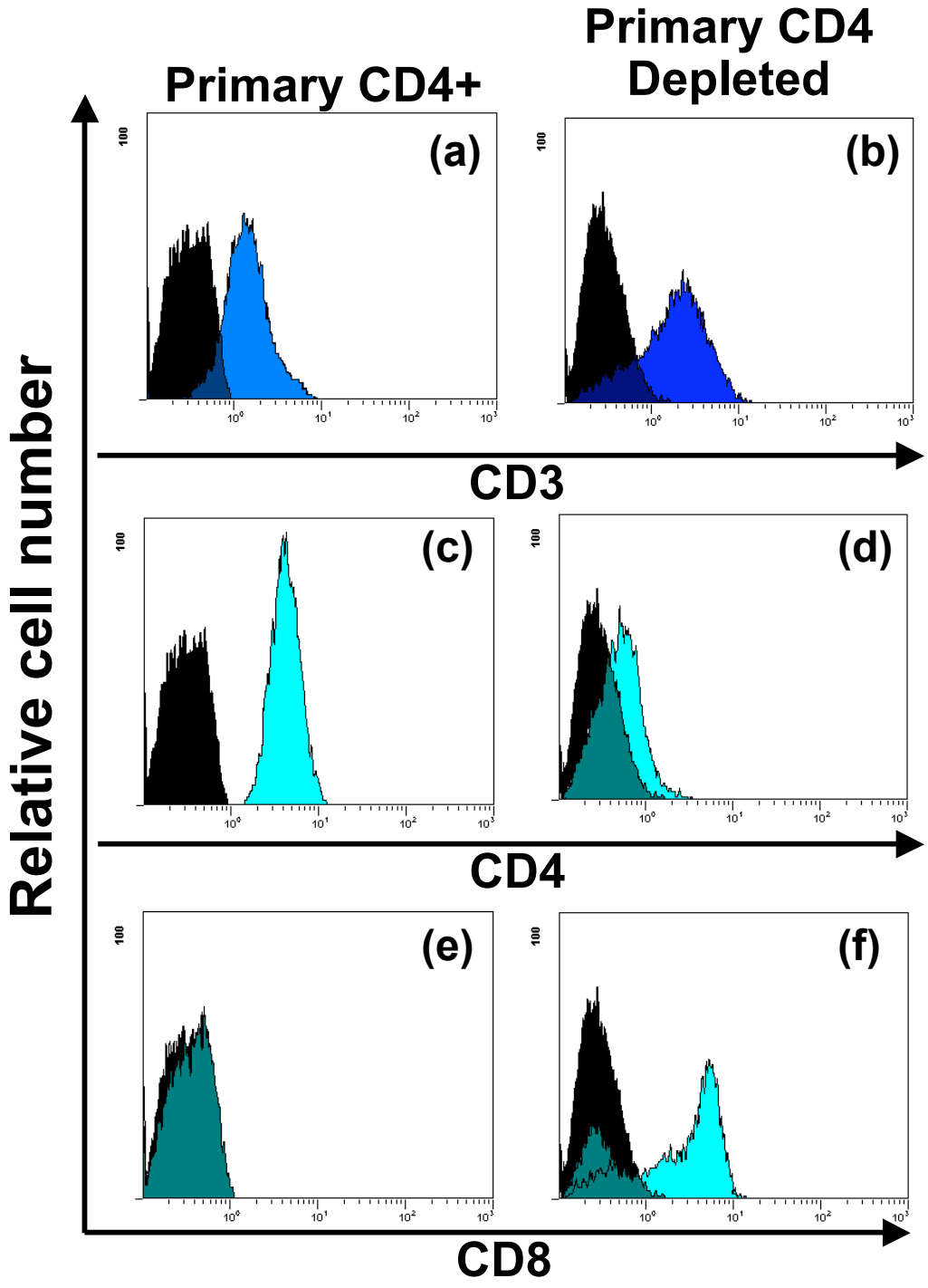
recombinant forms and ultimately offering the opportunity for a better quality of life to the 10-15% of children worldwide currently living with atopic eczema.

8.6 Conclusion

In conclusion, an *in vitro* T-lymphocyte and keratinocyte cell model was established, which was shown to mimic T-lymphocyte induced apoptosis of keratinocytes occurring in AE *in vivo*. The co-culture model established allowed the characterisation of the Fas associated mechanisms by which T-lymphocytes induce keratinocyte apoptosis and also provided a greater insight into the biology of keratinocyte cell death. T-lymphocytes were shown to induce early keratinocyte differentiation by loss of α6-integrin expression. This novel finding could point to a potential mechanism for death and survival, whereby decreased α6 expression may explain the loss of anti-apoptotic defences by keratinocytes. The work presented in this thesis supports the hypothesis that growth factors such as IGF-I and TGFβ_1 can protect keratinocytes from T-lymphocyte induced apoptosis. Studies performed in this thesis demonstrated that the decrease in T-lymphocyte induced keratinocyte apoptosis by the growth factor treatments was associated with maintenance of α6 expression by the keratinocytes. This finding suggested that these IGF-I and TGFβ_1 can reduce the susceptibility of keratinocytes to T-lymphocyte potentially by preventing them from entering terminal differentiation. The effects of IGF-I and TGFβ_1 in co-culture comprise of anti-apoptotic and potentially anti-inflammatory action, which suggests that specific growth factor combinations may ameliorate the reduced barrier function and inflammation associated with skin conditions where T-lymphocyte mediated apoptosis is involved in the pathology such as atopic eczema. Finally, this thesis provides evidence which may be used as a foundation for further investigation into the possible use of growth factor based therapies as an alternative approach for the treatment of atopic eczema.

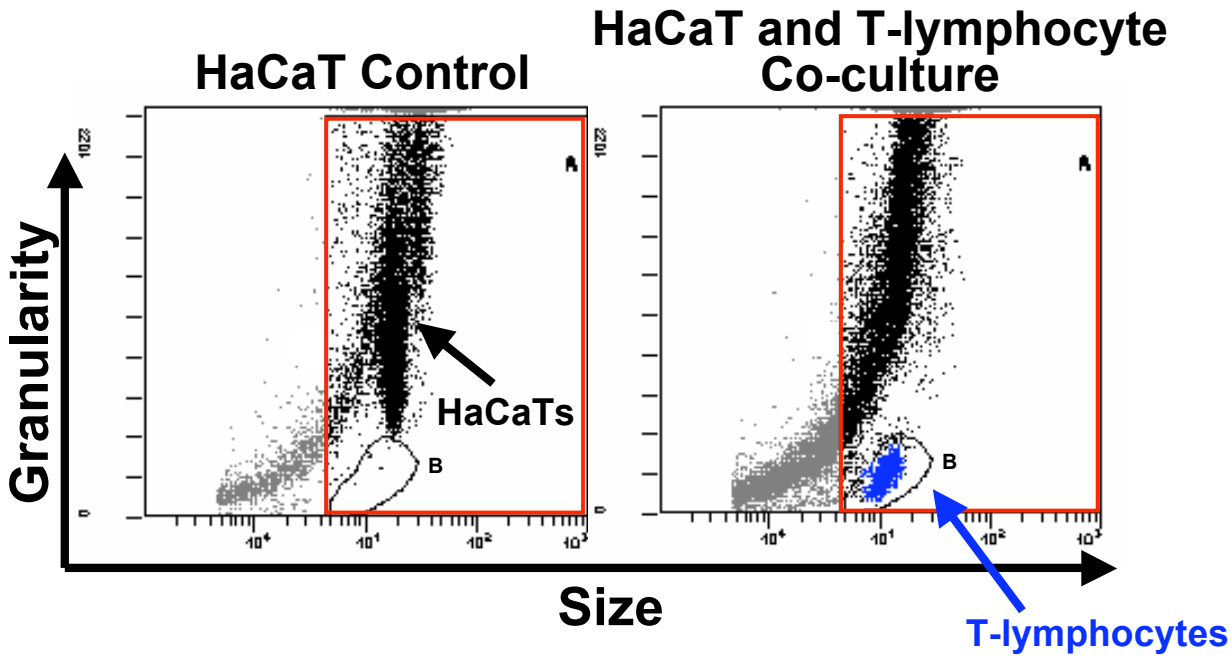
APPENDICES

Appendix 1



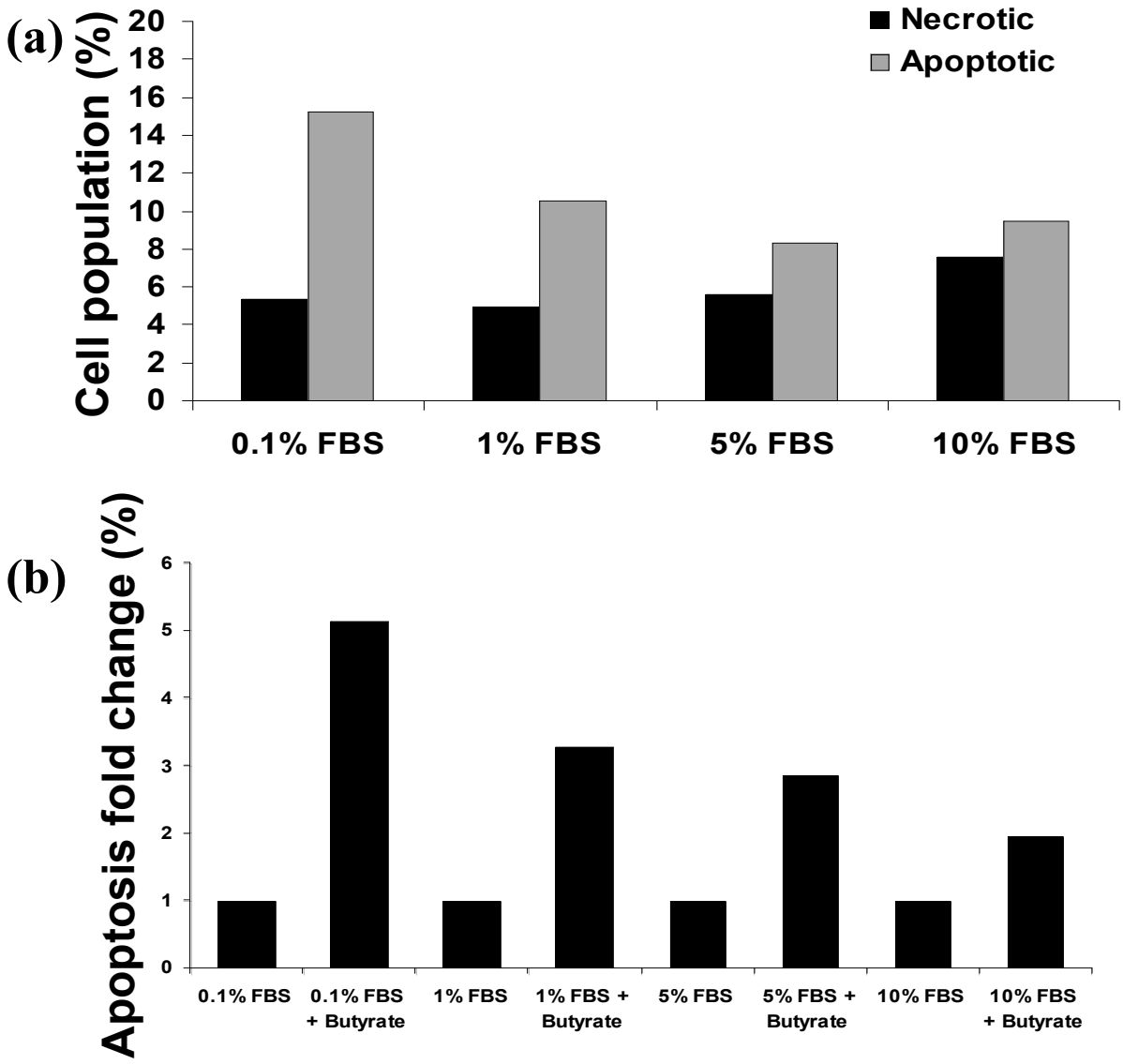
Appendix 1. Primary T-lymphocyte profile.
Surface marker expression of T-lymphocytes was analysed by flow cytometry. (a and b) blue histogram represents CD3 staining of CD4+ and CD4 depleted T-lymphocytes respectively. (c and d) aqua histogram represents CD4 staining of CD4+ and CD4 depleted T-lymphocytes respectively. (e and f) aqua histogram represents CD8 staining of CD4+ and CD4 depleted T-lymphocytes respectively. Histograms are a representation of 3 separate experiments. Black histogram represents staining of an isotype-matched control Ab

Appendix 2



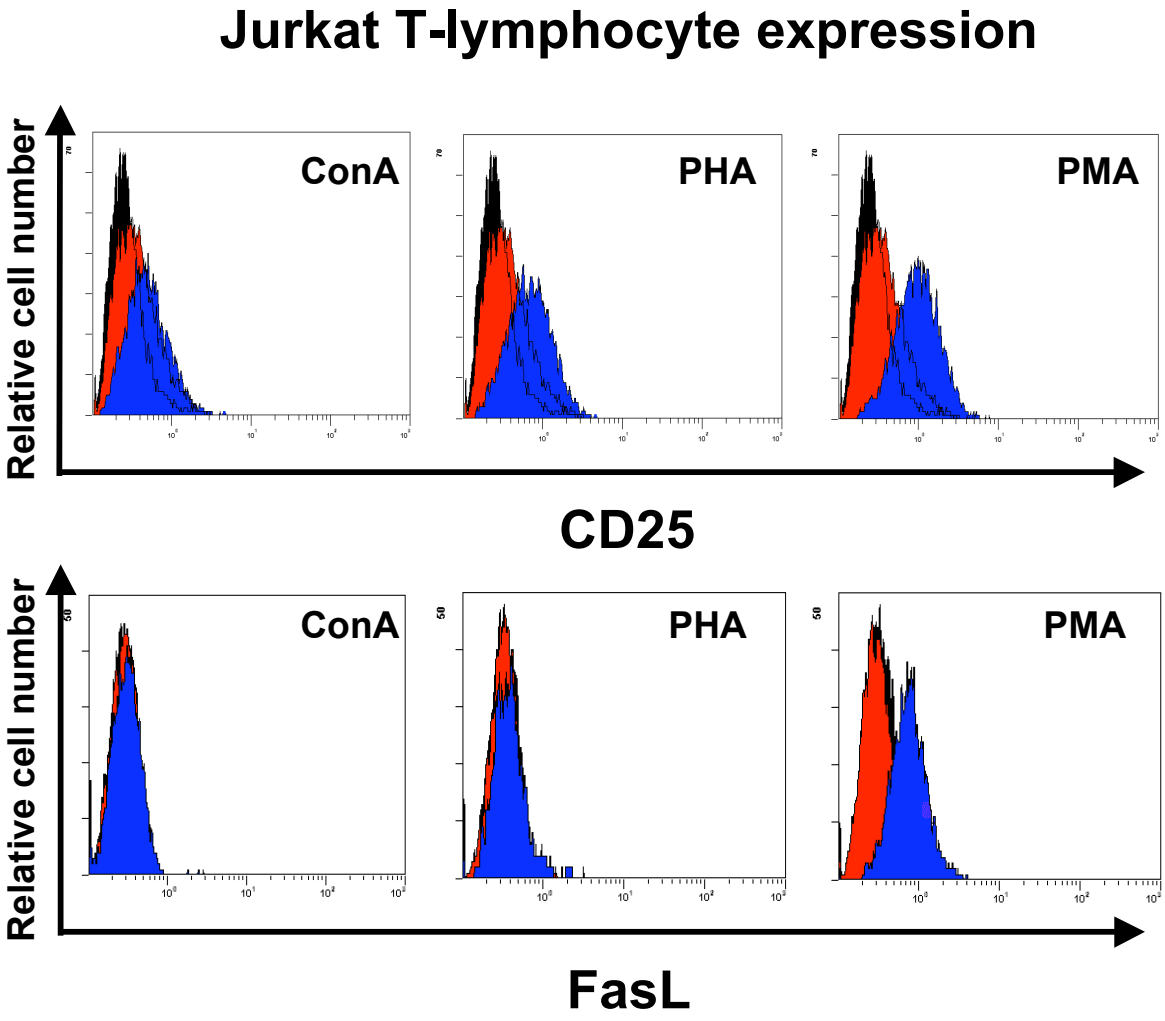
Appendix 2. T-lymphocyte size exclusion by FACS.
(a) HaCaT controls and (b) HaCaTs and T-lymphocytes in co-culture were gated based on size and light scatter properties by flow cytometry. HaCaT apoptosis was detected on cells in gate “A” excluding T-lymphocytes in gate “B”.

Appendix 3



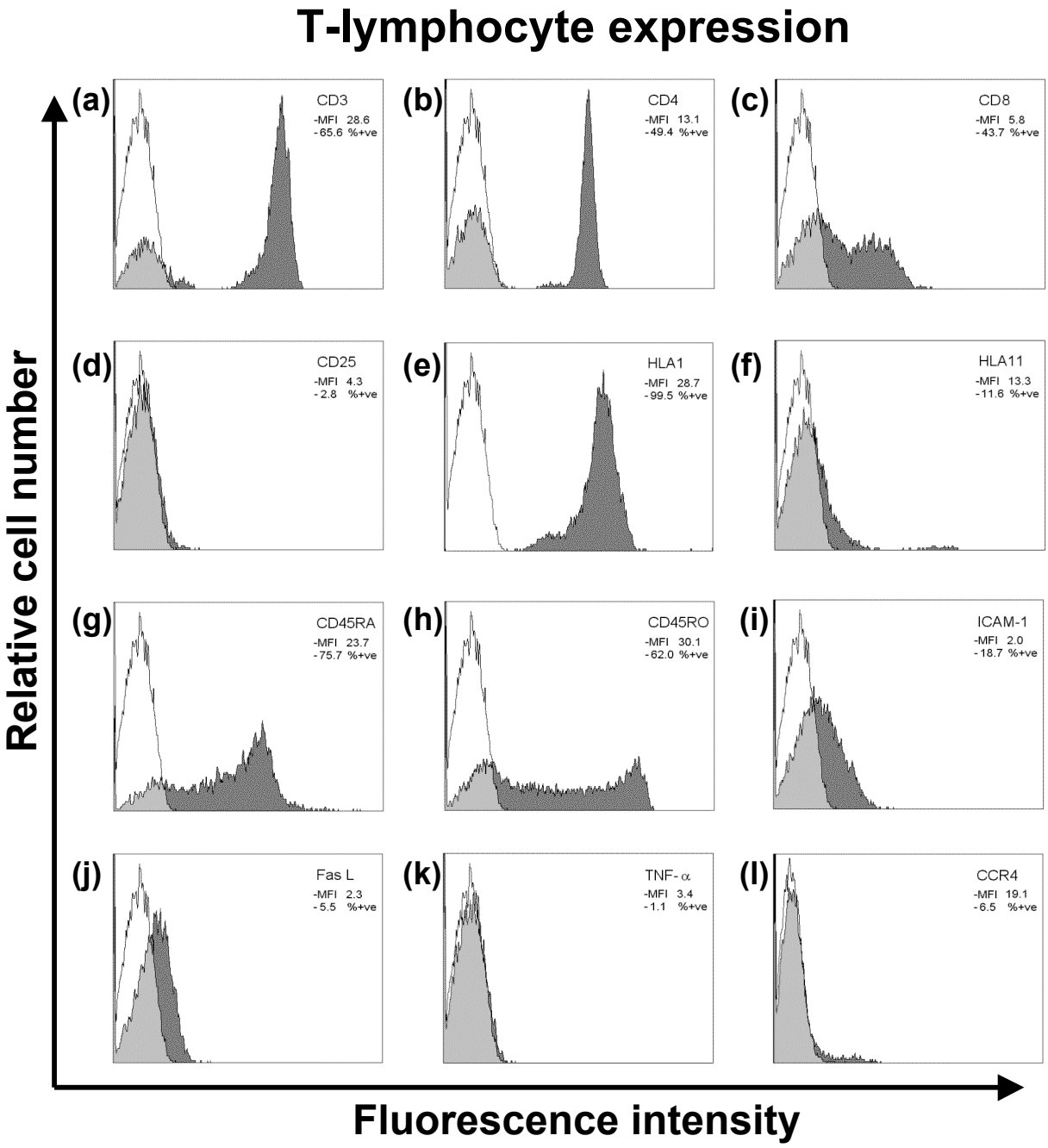
Appendix 3. Effect of FBS Titration on HaCaT apoptosis or necrosis and effect of sodium butyrate on HaCaT apoptosis. (a) Untreated HaCaTs or (b) sodium butyrate treated HaCaTs were incubated with 0.1 – 10% FBS for 24 hr. HaCaTs were stained with Annexin V and PI and analysed by flow cytometry. The results indicate that increasing concentration of FBS decreased apoptosis and necrosis of HaCaTs in 24 hr culture. Lowering the concentration of FBS, increased sensitivity of HaCaTs to sodium butyrate induced apoptosis.

Appendix 4



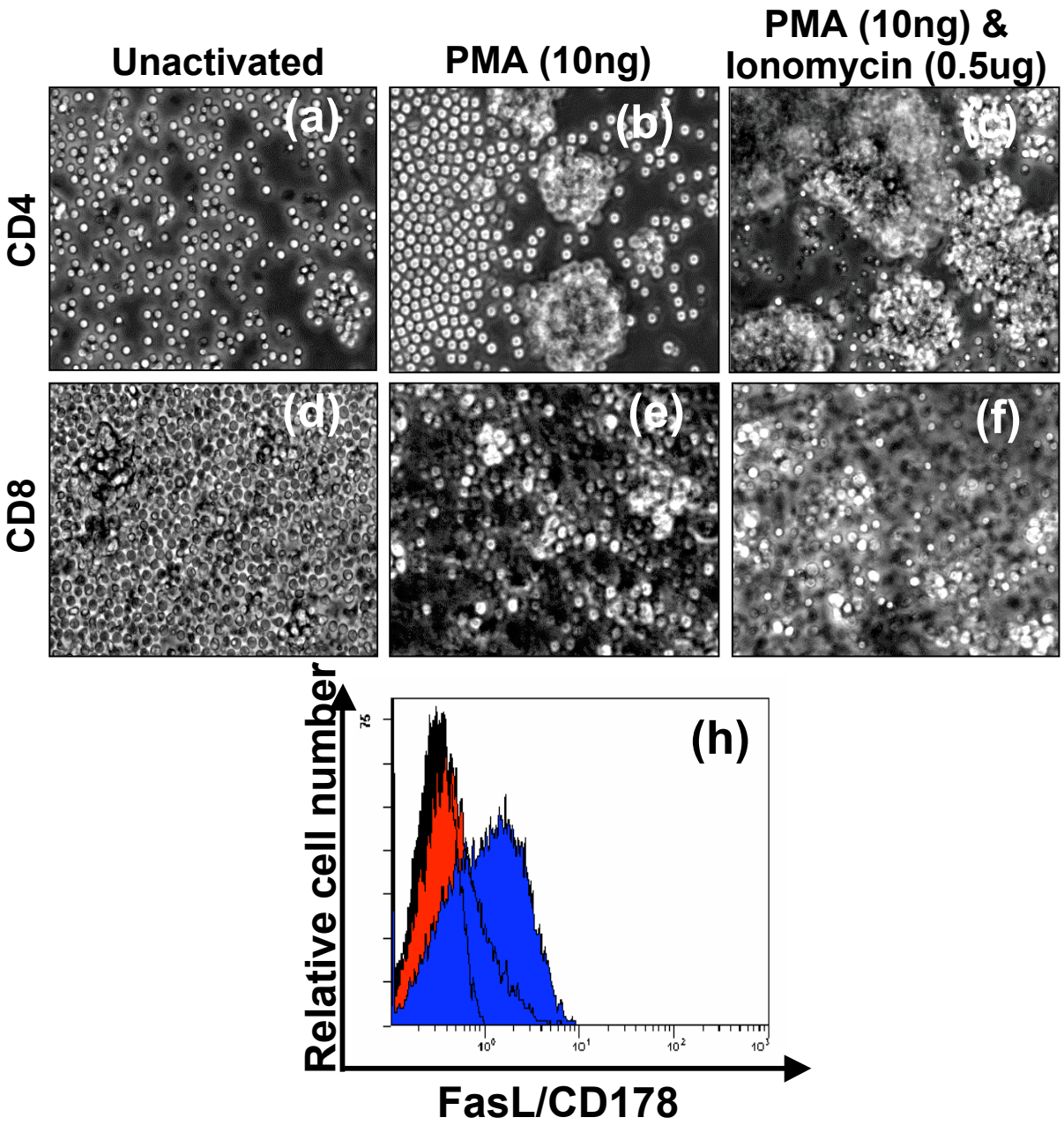
Appendix 4. Effect of mitogen activation on Jurkat T-lymphocyte activation and FasL expression. CD25 and FasL expression by Jurkat T-lymphocytes was analysed using flow cytometry. (a - c) CD25 expression of unactivated (red histogram) ConA (10ug/ml), or PHA (10ug/ml), or PMA (10ng/ml) activated Jurkat T-lymphocytes (blue histogram respectively). (d - e) FasL expression of unactivated (red histogram) ConA (10ug/ml), or PHA (10ug/ml), or PMA (10ng/ml) activated Jurkat T-lymphocytes (blue histogram respectively). Results indicate 10ng/ml PMA was the most efficient mitogen for inducing Jurkat T-lymphocyte activation and FasL expression.

Appendix 5



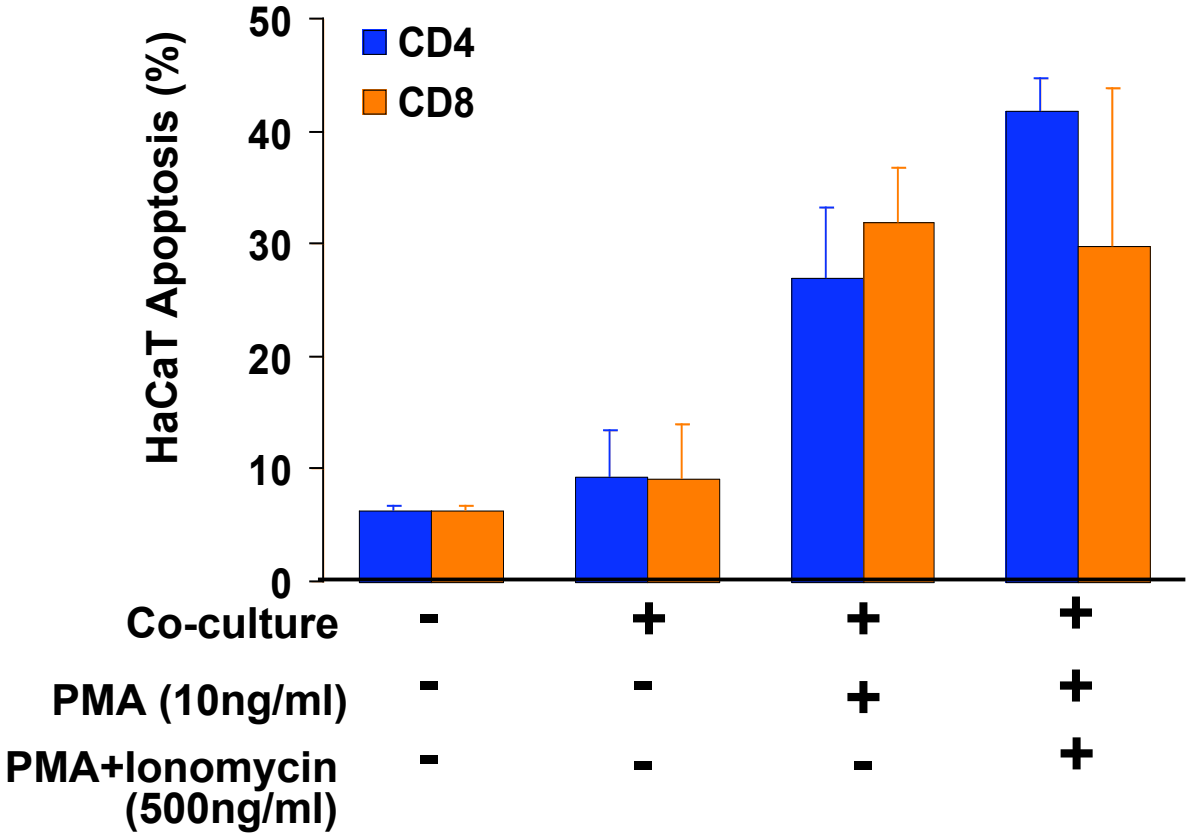
Appendix 5. PBMC resting profile.
Surface molecule expression of PBMCs was analysed by flow cytometry. PBMCs were isolated from flesh blood as described in Methods section 2.3.4 a and stained with markers as described in Methods section 2.5.2. The grey histogram represents surface marker expression by unstimulated PBMCs as indicated in each plot. The unfilled histogram represent staining with an isotype-matched control Ab.

Appendix 6 a



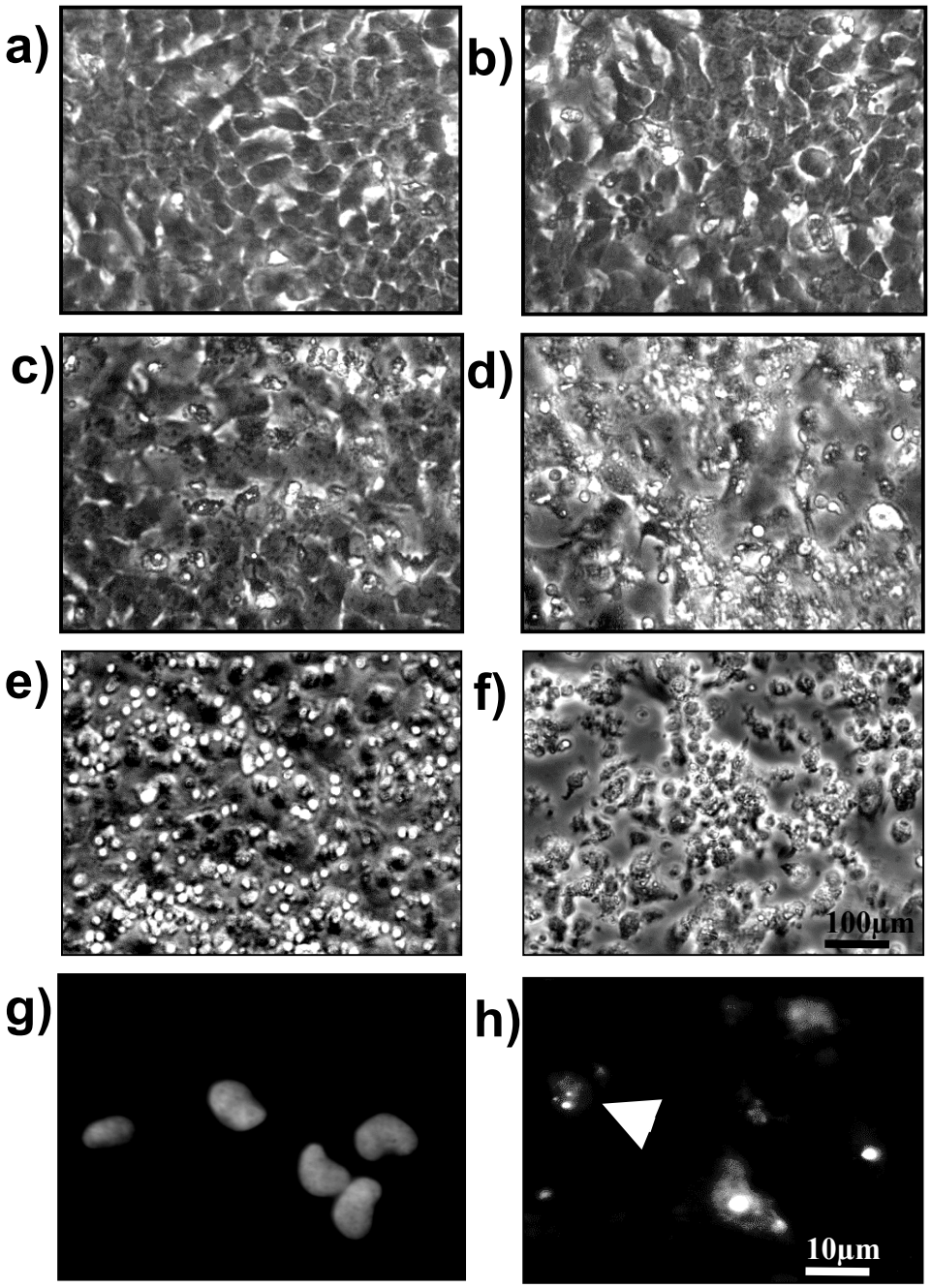
Appendix 6 a. PMA and ionomycin induced activation of CD4+ T-lymphocytes and CD4 depleted PBMCs . Phase contrast microscopy of (a, d) untreated CD4+ T-lymphocytes and CD4 depleted PBMCs respectively, (b, e) PMA (10ng/ml) treated CD4+ T-lymphocytes and CD4 depleted PBMCs respectively and (c, f) PMA (10ng) + ionomycin 0.5ug treated CD4+ T-lymphocytes and CD4 depleted PBMCs respectively for 48hr. The increase in aggregation by PMA and PMA + ionomycin are indicative of activation. (h) CD25 expression of untreated (red histogram) or PMA and ionomycin treated CD4 depleted PBMCs; blue histogram. The black histogram represents staining of an isotype-matched control Ab.

Appendix 6 b



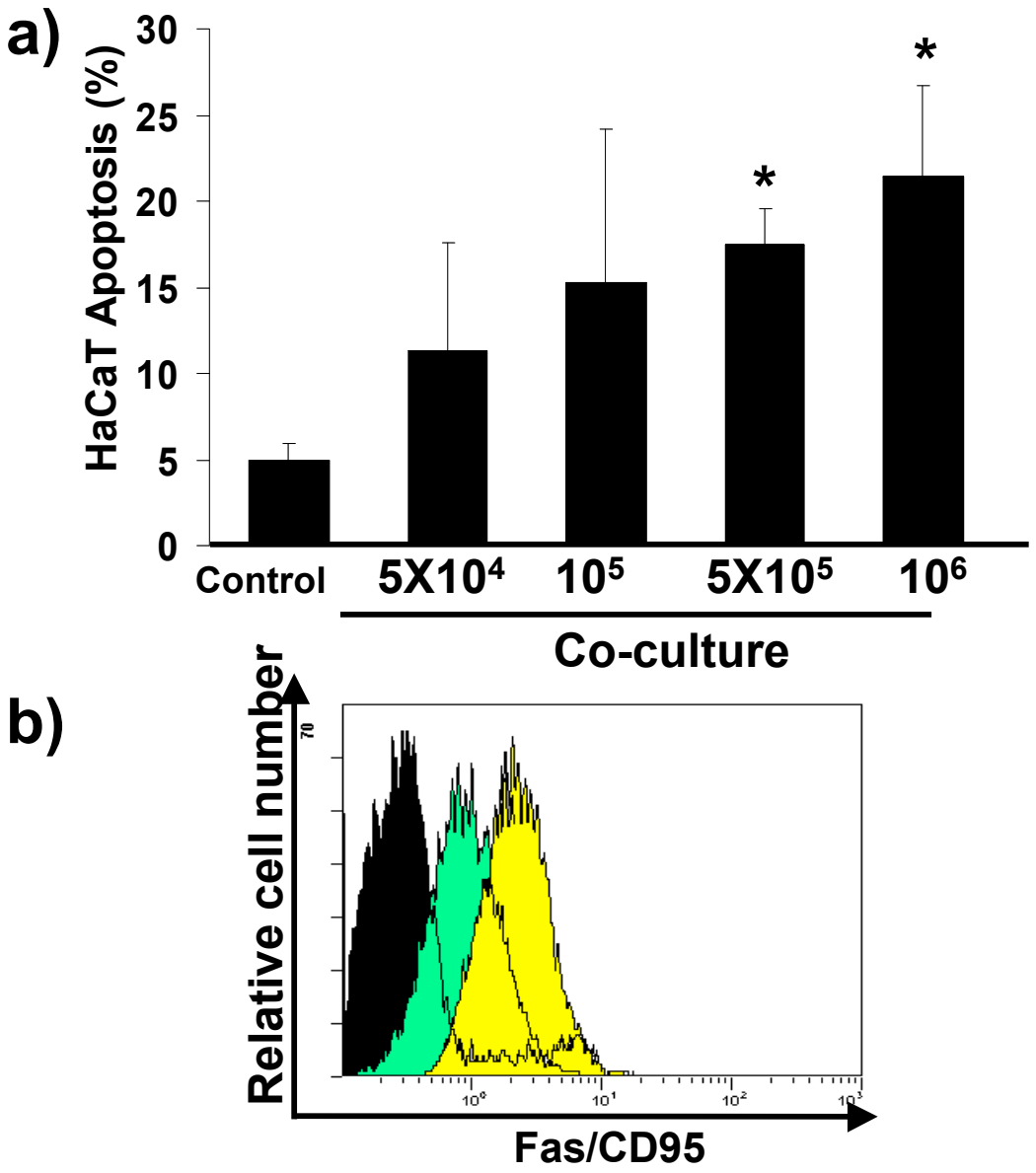
Appendix 6 b. PMA and ionomycin activation of CD4+ T-lymphocyte and CD4 depleted PBMCs induced HaCaT apoptosis in co-culture. HaCaT were co-cultured with T-lymphocytes and different mitogen combinations for 48hr. HaCaTs were stained with Annexin V and PI and analysed by flow cytometry. These studies showed that whist activation of 1 x 10^6 CD4+ T-lymphocytes or CD4 depleted PBMCs with PMA (10ng/ml) or combination of PMA and ionomycin (0.5ug/ml) both induced significant apoptosis, PMA and ionomycin induced a higher level of HaCaT apoptosis than PMA alone. Values represent the percentage of apoptotic cells mean ± SEM from 3 independent experiments. The data was analysed using one-way analysis of variance (ANOVA) with significance compared to controls shown (*$p<0.001$).

Appendix 7 a



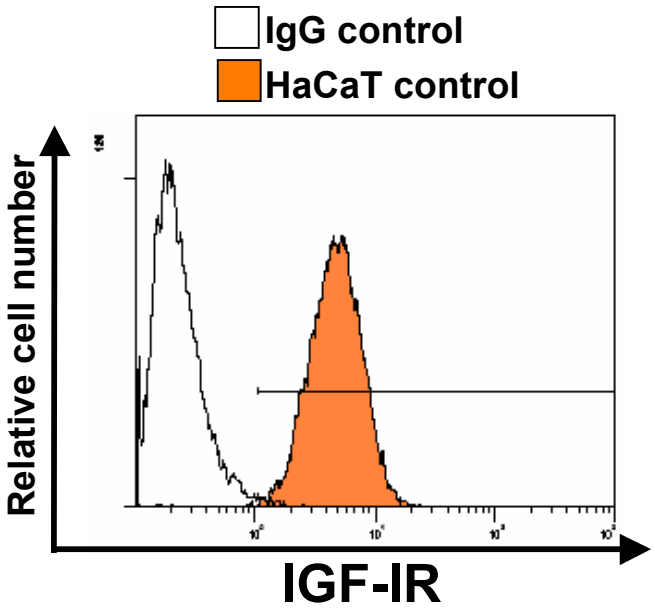
Appendix 7 a. Effect of PMA and ionomycin co-culture on HaCaT cell morphology and nuclear fragmentation. (a) Phase contrast microscopy of untreated HaCaTs, (b) HaCaT controls incubated with PMA and ionomycin, (c-f) HaCaTs co-cultured with $5x10^4$, $1x10^5$, $5x10^5$ and $1x10^6$ PMA and ionomycin activated CD4 depleted PBMCs for 48hrs respectively. Increasing the numbers of CD4 depleted PBMCs, resulted in a progressive increase in HaCaT cell shrinkage, membrane blebbing, cell separation indicative of loss of inter-cellular connections, the number of cell detached from culture plate. (g) HOESCHT 33342 fluorescent staining of control HaCaTs and (h) HaCaTs incubated with $5x10^5$ activated PMA and ionomycin for 48hrs, arrows point to fragmented nuclei. Scale bar represents 100µm for the phase contrast images and 10µm for the HOESCHT 33342 images.

Appendix 7 b



Appendix 7 b. Effect of CD4+ depleted PBMCs on HaCaT apoptosis and Fas expression. (a) Annexin V and propidium iodide stained HaCaT controls incubated with PMA and ionomycin and HaCaTs co-cultured with $5X10^4$, $1X10^5$, $5X10^5$ and $1X10^6$ PMA and ionomycin activated CD4 depleted PBMCs for 48hrs. Co-culture caused a PBMC number dependent increase in the number of HaCaTs induced to undergo apoptosis. Bar graphs represent the percentage of apoptotic cells mean ± SEM of 3 independent experiments. The data was analysed using one-way analysis of variance (ANOVA) with significance compared to control shown (*$p<0.05$).(b) Fas expression by control HaCaTs (green histogram) and HaCaTs co-cultured with activated T-lymphocytes (yellow histogram) was analysed using flow cytometry after 48hr co-culture. Compared to Fas expression by control HaCaTs (green histogram), CD4 depleted PBMCs caused a marked increased in HaCaT Fas expression. The black histogram represents staining of an isotype-matched control Ab. Histograms are a representation of 3 separate experiments.

Appendix 8



Appendix 8. HaCaT IGF-IR expression.
Surface IGF-IR expression of HaCaTs was analysed by flow cytometry. 100% of HaCaTs were shown to express IGF-RI (orange histogram). The unfilled histogram represent staining of an isotype-matched control Ab.

Appendix 9

Publications

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Sodium butyrate induced keratinocyte apoptosis

Ilse S. Daehn · Antiopi Varelias · Timothy E. Rayner

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Abstract Apoptosis of keratinocytes is a key mechanism required for epidermal homeostasis and the renewal of damaged cells. Its dysregulation has been implicated in many skin diseases including cancer and hyperproliferative disorders. In the present study, the effect of sodium butyrate, a histone deacetylase inhibitor, on keratinocyte apoptosis was investigated using the HaCaT human keratinocyte cell line. Sodium butyrate induced morphological changes associated with apoptosis and nuclear fragmentation of HaCaTs. Annexin V staining demonstrated that sodium butyrate induced apoptosis in a dose and time-dependent manner with 50% of HaCaTs apoptotic after exposure to 0.8 mg/ml sodium butyrate for 24 h. Apoptosis was associated with upregulation of cell surface expression of the death receptor Fas and activation of the extrinsic caspase pathway, with induction of caspase 8 activity peaking after 8 h. Caspase 3 activity peaked after 24 h and was associated with cleavage of the caspase 3 substrate, poly (ADP-ribose) polymerase (PARP). The intrinsic caspase pathway was not activated as caspase 9 activity was not detected, and there was no change in the expression of terminal differentiation markers keratin 10 and involucrin following sodium butyrate treatment. Together these results indicate that sodium butyrate is a potent inducer of Fas associated apoptosis via caspase activation in HaCaT keratinocytes, an effect that is independent of the induction of terminal differentiation.

Keywords Apoptosis · Caspase · Fas · Keratinocyte · Sodium butyrate

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Introduction

Skin barrier function is an important feature of normal epidermis and is dependent on stratification of the epidermal layer and proper development of the protective stratum corneum via terminal differentiation of keratinocytes [1]. In order to preserve this protective function in normal skin, keratinocyte proliferation, differentiation and cell death are tightly coordinated within the epidermis. However, a number of stimuli such as ultra-violet (UV) radiation and inflammation can disrupt the integrity of the epidermis by inducing apoptosis or programmed cell death [2–4]. It is generally considered that keratinocyte apoptosis has evolved to ensure the removal of damaged cells, particularly the removal of cells with unrepairable DNA damage caused by UV radiation that could ultimately result in skin cancers [5]. Although they result in the same end-point, terminal differentiation and apoptosis are morphologically distinct processes.

Terminal differentiation in keratinocytes can be considered a developmentally associated form of cell death that, like apoptosis, results in the loss of the nucleus, mitochondria and ribosomes but, unlike apoptosis, does not result in DNA fragmentation [6, 7]. In addition, terminally differentiating keratinocytes synthesize keratin proteins (keratinization) which is important for the maintenance of skin barrier function and clearly not a feature of cells undergoing apoptosis [8].

Given that apoptosis is necessary to remove damaged keratinocytes from the epidermis, it is not surprising that a number of cutaneous disease states are associated with a dysregulation of the apoptotic process. Most notably the development of non-melanoma skin cancers have been linked to inhibition of apoptosis, via the expression of the apoptosis inhibitor survivin [9] or by suppression of p53 levels [10]. Whilst reduced p53 activation and increased survivin have also been implicated in the epidermal hyperplasia seen in psoriasis [10, 11], premature cell death and accelerated keratinization also occur, making psoriatic plaque formation a complex, multi-factorial process [12]. Moreover the overexpression of differentially expressed apoptosis regulatory proteins such as Bcl-2 family members in psoriasis, non melanoma skin cancers and squamoproliferative lesions (*i.e.* Bowen's disease, keratoacanthomas) may contribute to the longevity of cells by blocking the normal apoptotic process of epidermal keratinocytes [13, 14]. Agents known to induce apoptosis via Fas, such as histone deacetylase inhibitors (HDI's) like sodium butyrate and trochostatin A, have been recognised as potent anti-tumor agents [15, 16] and potential topical treatments for epidermal malignancies in keratinocytes [17, 18].

Sodium butyrate is a short chain fatty acid that regulates proliferation, differentiation and apoptosis of colonic epithelial cells [19, 20]. HDI's such as sodium butyrate have been shown to potentiate Fas-mediated apoptosis in breast cancer and colon cancer cell lines, with antagonism of Fas found to inhibit butyrate induced caspase activation and apoptosis [21–23]. Whilst these studies demonstrate that Fas-dependent signalling and downstream activation of the caspase cascade is involved in butyrate induced apoptosis in a range of tumour derived cells, the effects of sodium butyrate on keratinocytes has not been defined.

In this study, we have investigated the effect of sodium butyrate on apoptosis and differentiation in the non-tumorigenic keratinocyte cell line HaCaT. This cell line demonstrates highly preserved epidermal characteristics and normal differentiation [24, 25].

Materials and methods

Cell culture

The transformed human keratinocyte cell line HaCaT derived from adult skin [24], was grown to 70–80% confluence in 6 well plates (unless otherwise indicated) before treatment with sodium butyrate for up to 72 h. HaCaTs were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 0.1 mg/ml streptomycin, 100 U/ml penicillin (both from GIBCO, Auckland, NZ) and supplemented with 10% fetal bovine serum (FBS; Thermotrace, Melbourne, AU). The cultures were maintained in 5% CO_2 in a 37°C incubator.

Flow cytometry

(a) Cell viability and apoptosis. Two-colour flow cytometry was used to assess keratinocyte viability, apoptosis and necrosis. A key characteristic of early apoptosis is the translocation of phosphatidylserine (PS) from the inner to outer membrane leaflet of the plasma membrane. Annexin V (BD Biosciences, San Jose, CA), is a PS binding protein and was used to detect apoptotic cells, while the nuclear stain, propidium iodide (PI) (Sigma Chemicals, St. Louis, MO), was used to identify late apoptotic or necrotic cells. HaCaT cells were treated with sodium butyrate (BDH, Poole, England) at doses ranging from 0.08–0.8 mg/ml. After incubation for up to 72 h, the adherent cells were collected by incubating with trypsin (0.25%)-EDTA (GIBCO, Auckland, NZ) and pooled with the non-adherent cells. The cells were washed twice with phosphate-buffered saline pH 7.4 (PBS) and 1×10^5 cells/ml were incubated with Annexin V-FITC (1 μg/ml) and PI (5 μg/ml) in binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) for 15 min at room temperature in the dark. Samples were analysed within 1 h of staining using an Epic Elite ESP Beckman Coulter flow cytometer. Quadrant markers were set on dotplots of unstained cells (viable cell population) and then subsequently applied to other samples.

(b) Fas expression. HaCaTs were treated with 0.8 mg/ml sodium butyrate for 24 h and cells collected using trypsin (0.25%)-EDTA. 5×10^5 cells were washed with pre-chilled buffer (PBS with 2% fetal bovine serum and 0.09% sodium azide) centrifuged at $100 \times g$ for 5 min at 4°C and after blocking with 10% fetal bovine serum for 15 min, cells were stained with anti-Fas monoclonal antibody (R&D Systems, Minneapolis, US) for 30 min at 4°C. Duplicate samples were stained with IgG isotype matched control antibody (BD Pharmingen, San Diego, CA). Cells were subsequently incubated with FITC-conjugated antimouse antibody (Sigma Chemical) for 30 min followed by fixation with 1xFACS Lysing Solution (BD Biosciences, San Jose, CA) at RT. Cells were resuspended in sterile saline solution (0.9% NaCl) followed by analysis using an Epic Elite ESP Beckman Coulter flow cytometer.

(c) Differentiation marker expression. HaCaTs were treated and harvested as described in Section (b) above. Cells were permeabilised using FACS-Perm (BD Biosciences San Jose, CA) for 15 min at RT before blocking with 10% fetal bovine serum. Cells were stained with either anti-K14, or anti-K10 monoclonal antibodies (Neomarkers, Fremont, CA), or with

anti-Involucrin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4°C. Duplicate samples were stained with IgG isotype matched control antibody (BD Pharmingen, San Diego, CA). Cells were subsequently incubated with the appropriate biotin-conjugated antibody (Vector Laboratories, Burlinghame, USA) for 30 min followed by incubation with phycoerythrin (PE)-conjugated streptavidin (BD Biosciences, San Jose, CA) for a further 30 min in the dark. Cells were fixed with 1xFACS Lysing Solution (BD Biosciences, San Jose, CA), resuspended in sterile saline solution (0.9% NaCl) and analysed using an Epic Elite ESP Beckman Coulter flow cytometer.

DNA fragmentation and cell morphology

The effect of sodium butyrate on cell morphology was assessed by phase contrast microscopy in all experiments prior to the preparation of the HaCaT cells for the measurement of apoptosis by flow cytometry. Phase contrast images were collected using a Nikon Eclipse TE2000-U microscope. To assess the effect of sodium butyrate on nuclear condensation, cells from parallel cultures were stained with the dye HOECHST 33342. HaCaT cells were plated into 24 well tissue culture plates for these experiments and after treatment for the required time the cells were washed with PBS, fixed with 4% buffered formaldehyde for 15 min at room temperature and stained with 1 μg/ml HOECHST 33342 dye (Sigma Chemicals) for 15 min in the dark. Stained cells were evaluated using a Nikon inverted fluorescent microscope fitted with a DAPI filter.

Caspase activity assay

Caspase 3, 8 and 9 activity was measured in protein extracts by measuring the capacity of the sample to cleave a substrate specific for each caspase resulting in the release of the fluorogen AFC. The peptides include caspase 3 substrate IV (DEVD-AFC), caspase 8 substrate II (IETD-AFC) and caspase 9 substrate I (LEHD-AFC), all from CalBiochem, La Jolla, CA. Protein was extracted from control and sodium butyrate treated HaCaTs by lysing the cells with NP40 lysis buffer (5 mM Tris-HCL pH 7.4, 5 mM EDTA pH 8.0, 0.5% NP40; Sigma Chemical) for 15 min. The wells were scraped and the insoluble material pelleted at 13,400 $\times$ g for 15 min at 4°C. A 50 μl aliquot of the cell lysate was tested for caspase activity by incubation with 1 ml of protease buffer (50 mM HEPES, 10% Sucrose, 0.1% CHAPS pH 7.4), supplemented with 10 mM DTT and containing 8 μM of the relevant fluorogenic substrate, for 24 h at room temperature in the dark. The release of AFC was quantified by measuring the fluorescent emission at 490 nm (excitation 400 nm) for caspases 3 and 8 and at 460 nm (excitation 400 nm) for caspase 9 using a Perkin-Elmer LS50 spectrofluorimeter. A fluorescence unit of 1 was determined as the equivalent to the amount of caspase required to produce 1 pmol of AFC/min at 25°C. In some experiments the caspase 3 inhibitor DMQD-CHO (CalBiochem, La Jolla, CA) was used. HaCaTs were pretreated with DMQD-CHO for 24 h, with fresh DMQD-CHO added to the media at the same time the cells were treated with sodium butyrate.

Western blotting

Cells were incubated in lysis buffer (10% sodium dodecyl sulfate, 170 mM TrisHCl, 22% glycerol) containing protease inhibitor cocktail set 1 (CalBiochem, La Jolla, CA) for 15 min. The wells were scraped and insoluble material was pelleted at 13,400 $\times$ g for 5 min at 4°C. Protein concentration was calculated using the Bradford method as per manufacturer's instructions (Bio-Rad Hercules, USA). Equal amounts of protein were loaded per well and electrophoresed through a 15% agarose gel (Bio-Rad Hercules, US) at 60 mA for 90 min. The proteins were transferred onto a Hybond nitrocellulose membrane (Amersham Life Sciences, Castle Hill, AU), which was blocked with 2% powdered skim milk in PBS containing 0.05% Tween 20 (PBST) for 30 min at room temperature and then incubated with mouse anti-caspase 3 monoclonal antibody (R&D Systems, Minneapolis, US) overnight at 4°C. Membranes were incubated with anti-mouse-HRP IgG (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Poly (ADP-ribose) polymerase (PARP) cleavage was assessed by probing with rabbit anti-PARP IgG (Santa Cruz, California, US) overnight at 4°C followed by anti-rabbit HRP IgG (DAKO A/S, Denmark) for 1 h at room temperature. Fas expression of cell lysates, was assessed using an anti-Fas monoclonal antibody (R&D Systems, Minneapolis, US) overnight at 4°C, followed by incubation with anti-mouse-HRP IgG (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Membranes were then soaked for 2 min in ECL Western Blot reagent (Amersham Life Sciences, Sydney, AU) and bands detected by autoradiography. Rainbow molecular weight markers (Amersham Life Sciences, Sydney, AU) were used for the determination of protein size.

Statistics

Each experiment was performed at least three times. Results are expressed as mean $\pm$ SEM. Apoptosis and caspase activity data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc t-test using Dunnett's method (multiple comparisons versus the untreated control) or a Bonferroni modification (all-pairwise comparison). When data was not normally distributed, a Kruskal-Wallis

one-way ANOVA on ranks was performed. Data was considered to be statistically significant when $p < 0.05$.

Results

Sodium butyrate induces HaCaT apoptosis

To investigate the effect of sodium butyrate on human skin cells, we cultured the HaCaT keratinocyte cell line with increasing concentrations of sodium butyrate and assessed apoptosis by flow cytometry. Early apoptotic cells were identified by positive Annexin V staining which binds with high affinity to phosphatidylserine exposed on the cell surface. Cells double stained with Annexin V and the nuclear stain propidium iodide (PI) signified late apoptosis. Cells stained with PI only represent necrotic cells. As shown in Fig. 1, most HaCaTs incubated under control conditions (10% FBS) were negative for Annexin or PI staining and were considered to be viable cells (Fig. 1(a)). However, when the cells were treated for 24 h with increasing concentrations of sodium butyrate (0.08, 0.4 and 0.8 mg/ml), there was a concomitant increase in cells induced to undergo apoptosis. This is shown by the dose dependent increase in cells found in the early apoptotic (EA; Annexin V positive/PI negative) and late apoptotic (LA; Annexin V positive/PI positive) quadrants (Fig. 1(b–d)). Despite the effect of butyrate on apoptosis, there was only a slight increase in the number of necrotic cells (N; Annexin V negative/PI positive).

The percentage of apoptotic cells (EA and LA) increased significantly when HaCaTs were treated with 0.4 and 0.8 mg/ml butyrate for 24 h ($p \leq 0.05$ Fig. 1(e)). Compared to 8% of cells in control cultures, 46% of cells treated with 0.8 mg/ml butyrate were apoptotic with a concomitant decrease in the percentage of viable cells also found to be significant (Fig. 1(e)). The temporal induction of apoptosis was investigated using the most effective concentration of sodium butyrate (0.8 mg/ml). Apoptosis was significantly increased by 18 h, with 66% of the total cell population found to be apoptotic after 72 h (Fig. 1(f)). Although necrosis did not change in the first 48 h, 15% of cells were necrotic after 72 h however this was not significantly different to untreated controls (Fig. 1(f)).

Analysis of HaCaT cell morphology demonstrated that sodium butyrate treated cells appeared shrunken, displayed fewer intercellular connections, and a greater number of cells were detached from the culture plate after 24 h (Fig. 2(c) and (e)) compared to control cultures (Fig. 2(a)). Apoptotic bodies were also evident (Fig. 2(e) arrow), with the effect of sodium butyrate on nuclear fragmentation assessed in parallel cultures using the HOESCHT 33342 nuclear stain. Figure 2(b) shows minimal nuclear staining in controls, whereas HaCaTs treated with 0.8 mg/ml sodium butyrate showed increased nuclear condensation and fragmentation (Fig. 2(d) and (f)).

Sodium butyrate activates the caspase cascade

Activation of the caspase cascade is important for mediating the induction of apoptosis via death receptors [26]. The activity of initiator caspases 8 and 9 as well as the downstream effector caspase 3 was measured in HaCaTs treated with sodium butyrate. Given that the temporal profile of activity is different for each caspase, we examined the response to butyrate at time points up to 72 h. Sodium butyrate (0.8 mg/ml) induced caspase 8 activity at 3 h, reaching a significant 2-fold increase after 8 h (Fig. 3(a)). Caspase 8 activity gradually declined after 8 h, returning to basal levels by 48 h. Caspase 3 activity on the other hand reached a significant 1.5–2 fold increase after 6 h with a peak of more than 4-fold at 24 h (Fig. 3(a)). Caspase 3 activity declined after 24 h and was undetectable by 72 h. This may be due to increased protease levels within the cultures as the total protein content of extracts from butyrate treated cells was also reduced between 24 and 72 h (data not shown). Caspase 9 was not activated by sodium butyrate (Fig. 3(a)).

Having established the time of maximal activity for caspases 8 and 3, we sought to confirm the dose effect of sodium butyrate on caspase activity at those times. Consistent with our apoptosis data, sodium butyrate was shown to dose-dependently increase caspase 8 activity in HaCaTs after 8 h and caspase 3 after 24 h with this significant at the 0.8 mg/ml concentration ($p < 0.05$; Fig. 3(b)).

Caspase 3 proteolytic activity is dependent on the cleavage of the 35 kDa pro-caspase 3 precursor to a 17 kDa active caspase 3 fragment. Western blot analysis showed that sodium butyrate dose-dependently decreased pro-caspase 3 in HaCaTs after 24 h although the active fragment was not detected (Fig. 3(c)). The generation of active 17 kDa caspase 3 fragment from pro-caspase 3 was confirmed however by the cleavage of PARP, a known protein substrate of caspase 3 [27]. Figure 3(d) demonstrates that sodium butyrate dose-dependently converted the 113 kDa pro-form of PARP to the 89 kDa fragment concomitant with pro-caspase 3 cleavage.

Sodium butyrate induces Fas expression by HaCaTs

Given that the caspase cascade is often activated by the death receptor Fas, we next investigated whether butyrate modulated Fas expression by HaCaTs. As shown in Fig. 4(a), HaCaTs treated with sodium butyrate for 24 h showed an increase in the level of Fas surface expression with this represented by the mean fluorescence intensity (MFI) of Fas

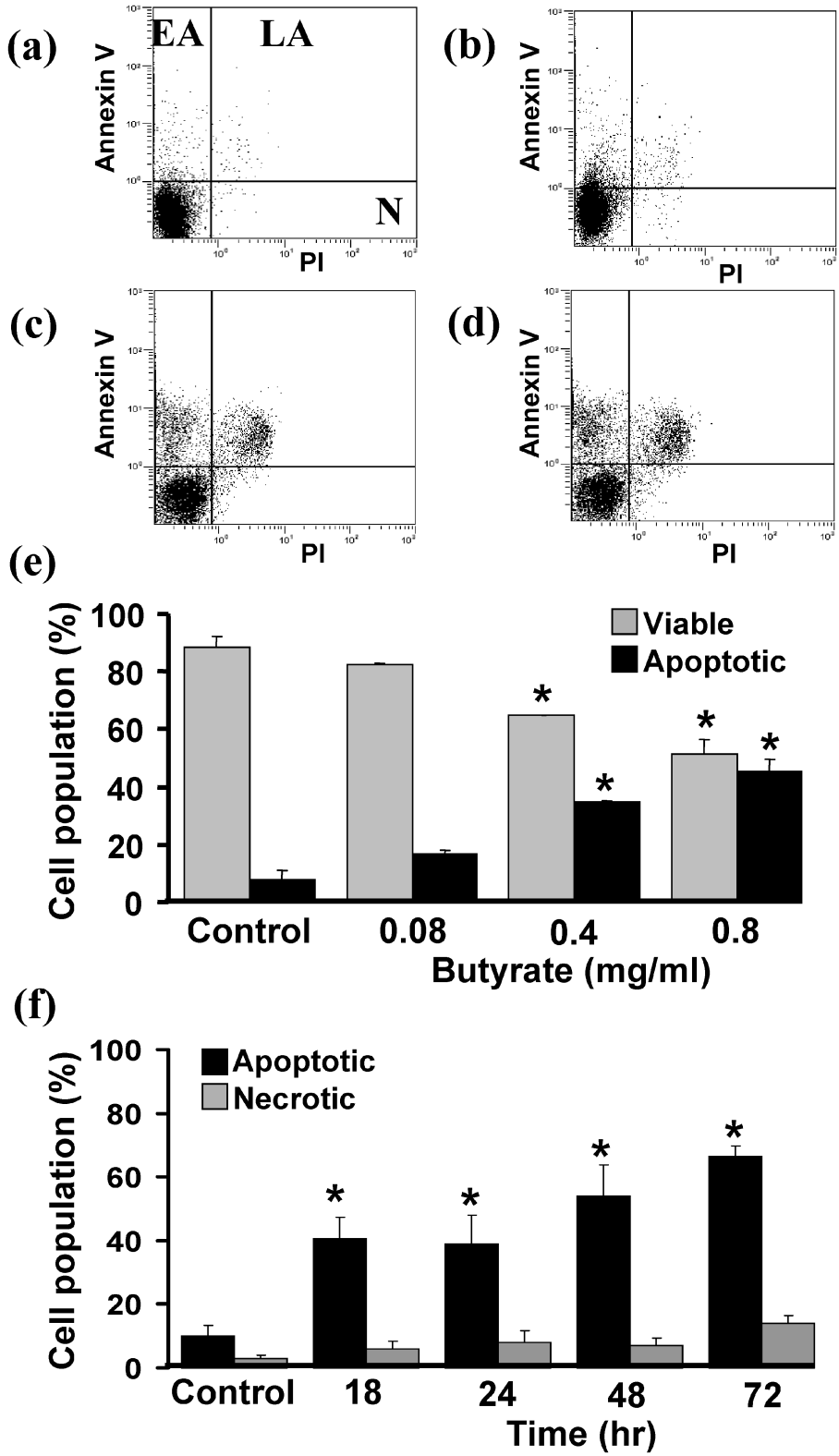

Fig. 1 Effect of sodium butyrate on HaCaT apoptosis. (a) Representative dot plot of Annexin V and propidium iodide (PI) stained control HaCaTs after 24 h. (b–d) HaCaTs treated with 0.08, 0.4 and 0.8 mg/ml sodium butyrate respectively for 24 h. Unstained cells (Annexin V negative/PI negative) were considered viable, Annexin V positive/PI negative cells were considered early apoptotic (EA), Annexin V positive/PI positive cells were considered late apoptotic (LA) and Annexin V negative/PI positive cells represented the necrotic population (N). (e) Collated data from 3 separate experiments ($n = 3$) showing the effect of sodium butyrate (0.08–0.8 mg/ml) on HaCaT viability (grey bar) and apoptosis ([EA + LA]; black bar). (f) HaCaTs were treated with 0.8 mg/ml sodium butyrate for up to 72 h and the combined data from 3 separate experiments ($n = 3$) presented showing the effect on apoptosis (black bar) and necrosis (grey bar). Control cells were incubated in the absence of sodium butyrate for 72 h. Bar graphs show the parameters measured as a percentage of the total cell population with the data presented as mean ± SEM. Data was analysed using one-way analysis of variance (ANOVA) and post-hoc *t*-test with significant differences compared to controls shown ($^*p < 0.05$)

staining increasing from 0.7 to 1.3. We also investigated the expression of the heterogenous forms of Fas by western blot which showed the higher molecular weight Fas fragment (50 kDa) was elevated in sodium butyrate treated HaCaTs (Fig. 4(b)), consistent with the flow cytometry data shown in Fig. 4(a) and other studies associating this form of Fas with increased susceptibility to Fas-mediated death [28].

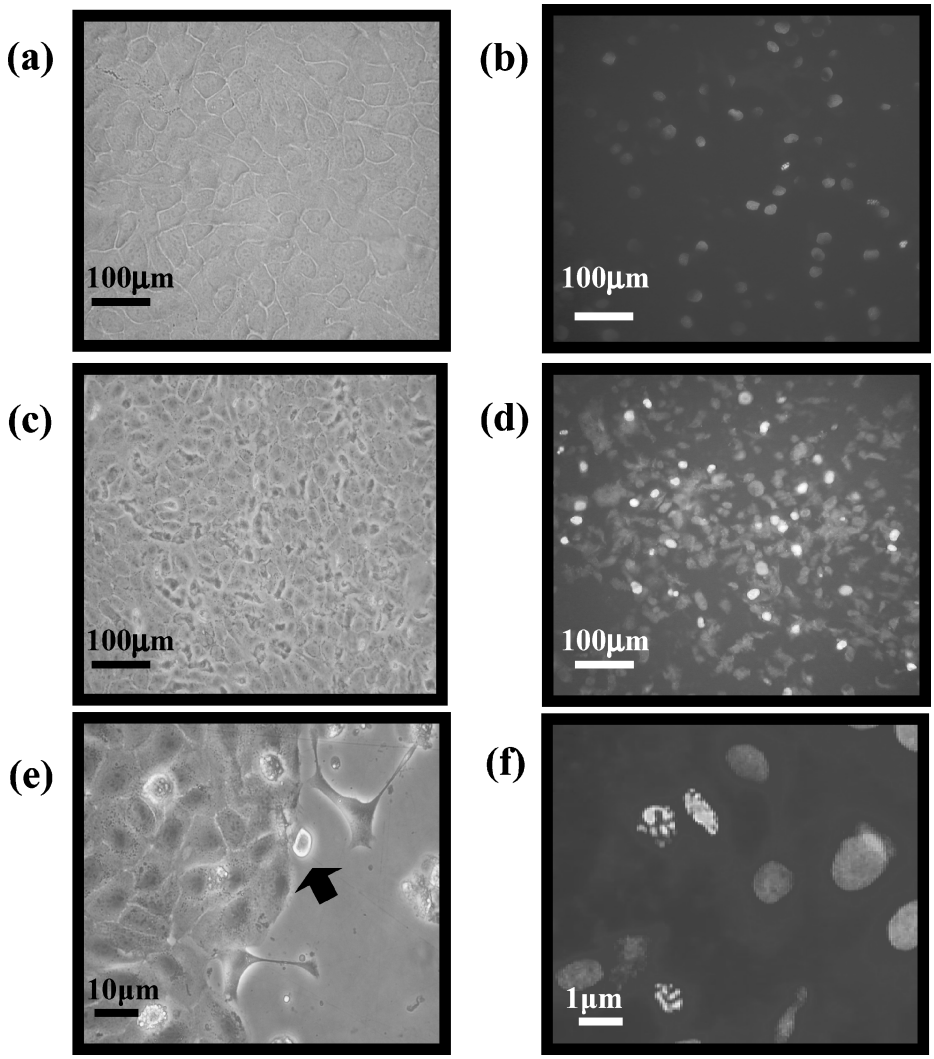

Fig. 2 Effect of sodium butyrate on HaCaT morphology and DNA fragmentation. HaCaT morphology was assessed on cultures prior to flow cytometric analysis of apoptosis while chromatin condensation and DNA fragmentation was assessed in parallel cultures using the HOESCHT 33342 fluorescent stain. (a, b) Control HaCaTs imaged by phase contrast microscopy or by HOESCHT 33342 stain, respectively (low magnification). (c, d) HaCaTs treated with 0.8 mg/ml sodium butyrate for 24 h imaged by phase contrast microscopy or by HOESCHT 33342 stain, respectively (low magnification). (e, f) HaCaTs treated with 0.8 mg/ml sodium butyrate for 24 h imaged by phase contrast microscopy or by HOESCHT 33342 stain, respectively (high magnification). The arrow in (e) points to an apoptotic body and scale bars are shown

Caspase 3 inhibition suppresses apoptosis but not Annexin V staining

A membrane permeable caspase 3 inhibitor DMQD-CHO [29, 30] was used to determine if preventing activation of caspase 3 could protect cells from sodium butyrate induced apoptosis. Pre-treating HaCaTs for 24 h with the DMQD-CHO (10 and 100 μg/ml) significantly decreased sodium butyrate induced caspase 3 activity (Fig. 5(a)). The reduction in sodium butyrate induced caspase 3 activity was confirmed by the ability of DMQD-CHO to also dose-dependently prevent sodium butyrate mediated PARP cleavage (Fig. 5(b)).

Although pre-treatment of HaCaTs with DMQD-CHO blocked sodium butyrate induced caspase 3 activity and PARP cleavage, this inhibitor did not prevent the increase in Annexin V staining (Fig. 6(a)). HaCaT morphology was assessed before cells were prepared for Annexin V and PI staining and showed that the apoptosis related morphological changes induced by butyrate, such as cell shrinkage and detachment from the culture plate, were reduced by DMQD-CHO (Fig. 6(d) and (f)). Staining of parallel experiments with HOESCHT 33342 demonstrated that DMQD-CHO also decreased nuclear fragmentation induced by butyrate (Fig. 6(e) and (g)).

Sodium butyrate does not induce HaCaT differentiation

Given that sodium butyrate has been reported to induce terminal differentiation in primary keratinocytes [31, 32], we sought to determine if differentiation was altered in HaCaTs. We investigated the intracellular expression of keratin 14 (K14; a basal cell marker), keratin 10 (K10; identifies cells committed to differentiate) and involucrin (a marker for differentiated cells) by flow cytometry. As shown by Fig. 7(a–c), butyrate (0.8 mg/ml) had no effect on the relative expression of any of the keratinocyte markers after 24 h, suggesting that butyrate did not modulate the normal

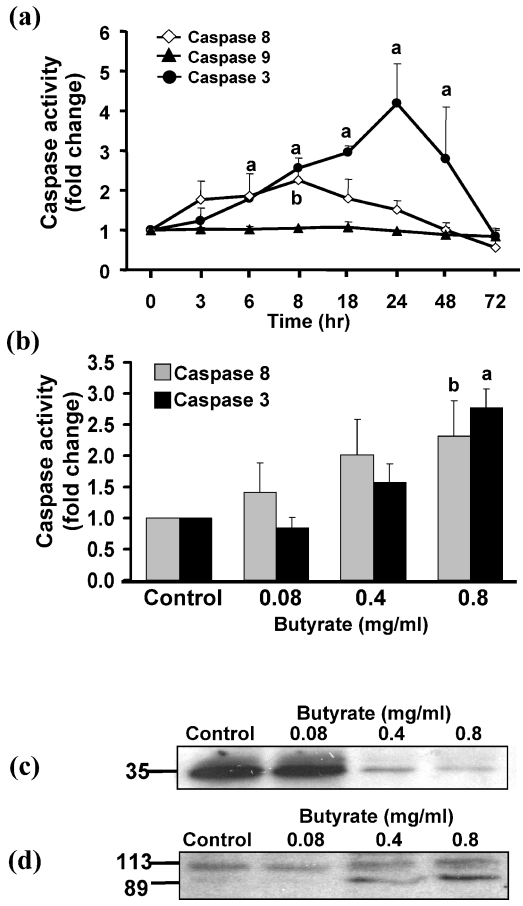

Fig. 3 Effect of sodium butyrate on caspase activity and PARP cleavage. (a) The activity of caspases 8, 9, and 3 were measured in HaCaTs treated with 0.8 mg/ml sodium butyrate using caspase-specific fluorogenic substrates. Cells were harvested at times up to 72 h after the addition of sodium butyrate and results normalised (fold change) to the activity levels in untreated controls. (b) The dose effect of sodium butyrate (0.08–0.8 mg/ml) on the peak activity of caspase 8 at 8 h (grey bar) and caspase 3 at 24 h (black bar) was determined and normalised to untreated controls. Data is presented as the mean ± SEM of 3 independent experiments and was analysed using one-way analysis of variance (ANOVA) and post-hoc *t*-test with significant differences compared to controls shown (caspase 3, a; caspase 8, b; $p < 0.05$). (c) Western blot showing pro-caspase 3 (35 kDa) protein expression in HaCaTs cultured with or without sodium butyrate for 24 h. (d) Western blot showing the effect of sodium butyrate on PARP cleavage after 24 h. The 113 kDa band is the pro-form of PARP and the 89 kDa band is the product of caspase 3 cleavage

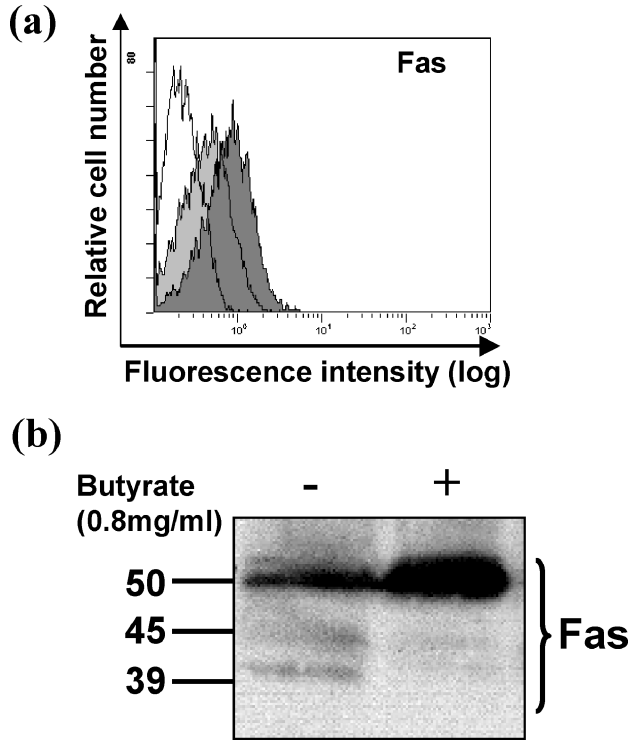

Fig. 4 Effect of sodium butyrate on HaCaT Fas expression. (a) Compiled dot blots showing the change in surface Fas expression by HaCaTs treated with sodium butyrate (0.8 mg/ml) for 24 h. The light grey histogram shows the degree of Fas expression by control HaCaTs and the dark grey histogram the Fas levels by HaCaTs treated with sodium butyrate. The unfilled histogram is the negative control (isotype-matched Ab). (b) Western blot showing Fas protein levels in whole cell lysates from control HaCaTs (−) and cells treated with 0.8 mg/ml sodium butyrate for 24 h (+). Fas fragments were seen at 50, 45 and 39 kDa

progression of the HaCaTs through the phases of differentiation.

Discussion

Histone deacetylase inhibitors such as sodium butyrate have been recognised as potent anti-tumour agents due to their capacity to induce growth arrest, cell differentiation and apoptosis [33, 34]. In the present study, we demonstrate that sodium butyrate effectively induces programmed cell death of HaCaT keratinocytes by inducing apoptosis rather than by increasing keratinocyte terminal differentiation.

Consistent with studies in other epithelial cell lines, sodium butyrate reduced HaCaT cell viability and induced changes in cell morphology characteristic of apoptotic cell death. Other studies have demonstrated using the WST-1 cell proliferation assay that a similar concentration range of sodium butyrate as that used here, significantly reduced viability of the intestinal cell line $CaCo_2$ by approximately 55% after 72 h [35]. Both the magnitude and time dependent nature of the response seen in HaCaTs was similar to that reported in $CaCo_2$ cells, with a 40% decrease in HaCaT viability after 72 h. Our study shows that the decrease in HaCaT cell viability was accompanied by a significant increase in apoptotic cells as identified by Annexin V staining and characteristic morphological changes. Similarly, studies by Litvak et al. [16] in gastric cancer cell lines reported sodium butyrate increased the number of condensed and fragmented nuclei detected using the HOECHST 33342 stain by 24 h. Collectively, our data is consistent with these studies

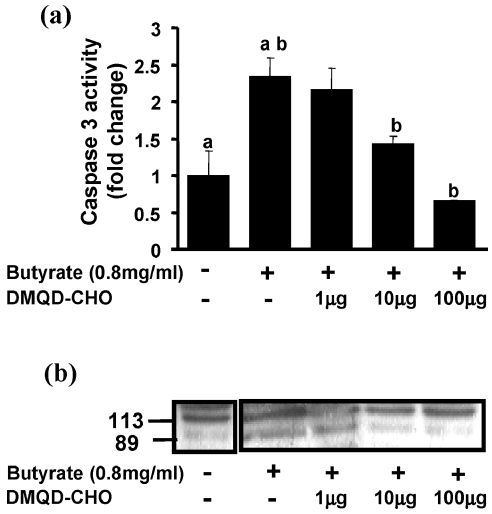

Fig. 5 Effect of the caspase 3 inhibitor DMQD-CHO on sodium butyrate induced caspase 3 activity and PARP cleavage. (a) Caspase 3 activity was measured in HaCaTs pre-treated for 24 h with DMQD-CHO (1–100 μg/ml) before incubation with sodium butyrate (0.8 mg/ml) for a further 24 h (+). Values were normalised to the caspase 3 activity of control HaCaTs incubated for the same period of time without sodium butyrate or DMQD-CHO (−) and represent the mean ± SEM of 5 independent experiments. The data was analysed using one-way analysis of variance (ANOVA) and post-hoc *t*-test with significance ($p < 0.05$) between treatments shown by the matching symbols ($^{a, b}$). (b) Representative western blot showing the effect of DMQD-CHO (1–100 μg/ml) on sodium butyrate (0.8 mg/ml) induced PARP cleavage. Cells were treated as described in (a) and the 113 kDa band is the pro-form of PARP and the 89 kDa band the product of caspase 3 cleavage

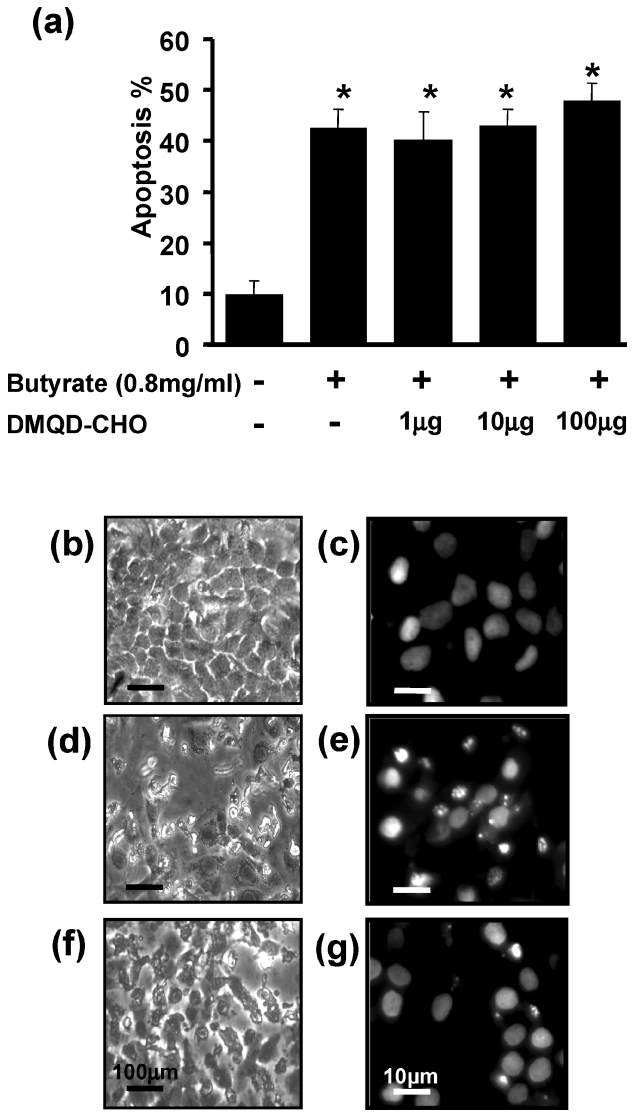

Fig. 6 Effect of the caspase 3 inhibitor DMQD-CHO on sodium butyrate induced HaCaT apoptosis. (a) HaCaTs were pre-treated for 24 h with DMQD-CHO (1–100 μg/ml) before incubation with 0.8 mg/ml sodium butyrate for a further 24 h (+). Cells were stained with Annexin V and PI and analysed by flow cytometry. Values represent the percentage of Annexin V positive cells (mean ± SEM) of 5 independent experiments. The data was analysed by one-way analysis of variance (ANOVA) and post-hoc *t*-test with significant differences compared to the untreated controls (−) shown (*; $p < 0.05$). Panels (b–g) HaCaT morphology was assessed on cultures prior to flow cytometry while chromatin condensation and DNA fragmentation was assessed in parallel cultures using the HOESCHT 33342 fluorescent stain. (b, c) Control HaCaTs imaged by phase contrast microscopy or by HOESCHT 33342 stain, respectively. (d, e) HaCaTs treated with 0.8 mg/ml sodium butyrate for 24 h imaged by phase contrast microscopy or by HOESCHT 33342 stain, respectively. (f, e) HaCaTs pre-treated for 24 h with 100 μg DMQD-CHO before incubation with 0.8 mg/ml sodium butyrate for 24 h imaged by phase contrast microscopy or by HOESCHT 33342 stain, respectively. Scale bar represents 100 μm for the phase contrast images and 10 μm for the HOESCHT 33342 images

conducted in intestinal epithelial cell lines and identifies the induction of apoptosis as the primary mode of HaCaT cell death induced by sodium butyrate.

A number of studies suggest that the death receptor pathways such as tumour necrosis related apoptosis-inducing ligand (TRAIL) and Fas are involved in butyrate induced tumour cell apoptosis [21, 22, 36, 37]. The results reported here also suggest a central role for Fas as sodium butyrate increased surface expression of Fas on HaCaTs. However, the involvement of FasL in sodium butyrate induced HaCaT apoptosis needs to be confirmed, as Fas-mediated apoptosis in keratinocytes appears able to proceed via a FasL independent or dependent mechanism. UVB radiation has been reported to induce apoptosis by upregulating and directly activating Fas on both HaCaTs and transformed human keratinocytes without the requirement of FasL [38, 39]. Normal, resting keratinocytes possess a functional Fas death pathway as *in vivo* and *in vitro* studies report the presence of both Fas and FasL [40, 41]. Spontaneous apoptosis is prevented however by the intracellular localisation of FasL within the cytosol and intermediate filaments [42]. It has been proposed

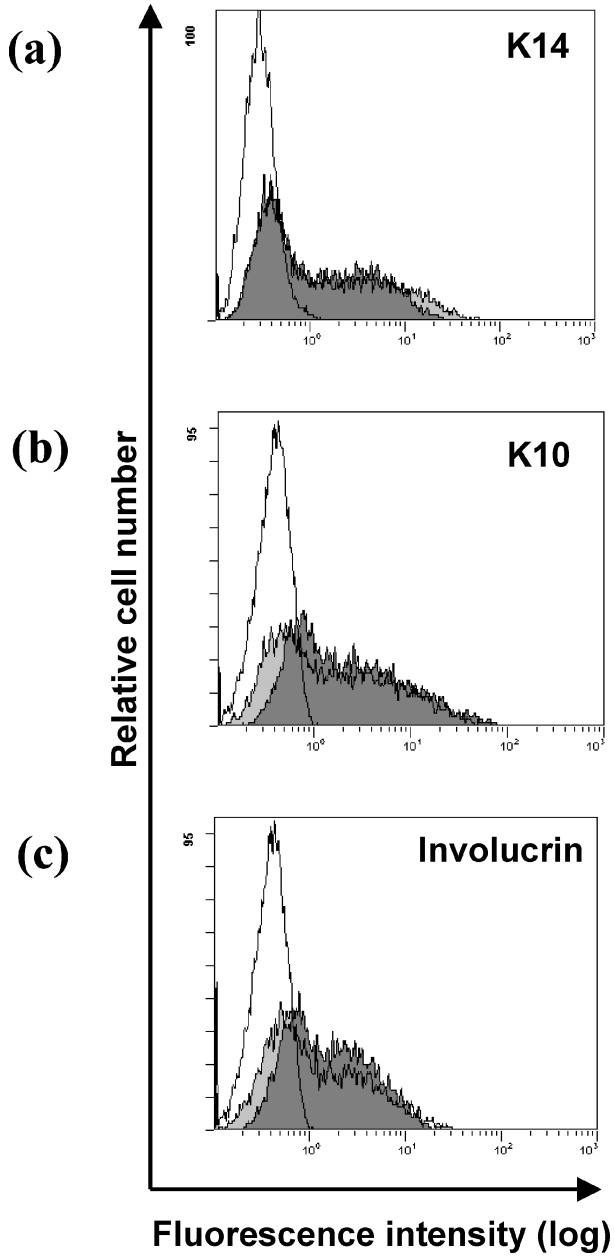

Fig. 7 Effect of sodium butyrate on keratinocyte differentiation. The intracellular expression of keratinocyte differentiation markers was assessed by flow cytometry. (a) representative histogram of keratin 14 expression, (b) keratin 10; (c) involucrin. In each panel the light grey histogram shows staining in control HaCaTs and the overlaying dark grey histogram staining in HaCaTs treated for 24 h with sodium butyrate (0.8 mg/ml). The unfilled histogram is the negative control (isotype-matched Ab)

that when keratinocytes are exposed to a cytotoxic stimulus such as UV radiation, FasL is translocated to the cell membrane where it can interact with Fas, induce apoptosis and promote cell death [42]. In the light of these opposing mechanisms, additional studies are required to determine if sodium butyrate is promoting keratinocyte apoptosis in a FasL dependent or independent fashion.

The increase in caspase 8 activity we observed supports the view that Fas mediates sodium butyrate induced keratinoctye apoptosis. Activation of Fas results in the recruitment of adapter protein Mort1 to the Fas death domain (FADD). A protein complex known as the death-inducing signalling complex (DISC) is formed when inactive caspase 8 is recruited to FADD, resulting in activation of caspase 8 and initiation of caspase-mediated apoptosis [26, 43]. We sought to further characterise the events downstream of caspase 8 and examined caspase 9 and caspase 3 activity as well as the cleavage of PARP, a specific nuclear substrate of caspase 3 [27]. Previous studies report that the HDI azelaic bishydroxamic acid caused the cleavage of PARP in keratinocytes [44], however, the direct connection between Fas, caspase activation and PARP cleavage following HDI treatment in keratinocytes has not been defined.

It has been shown that sodium butyrate induces both the intrinsic and extrinsic caspase pathways in carcinoma cells of different origin [37, 45]. Our results indicate that sodium butyrate induced HaCaT apoptosis, however, occurred exclusively via the extrinsic pathway as we observed increased caspase 3 activity but no concomitant caspase 9 activation. Although we were unable to detect an increase in the active caspase 3 fragment by western blot, activation of caspase 3 was confirmed by a reduction in the levels of pro-caspase 3 and cleavage of PARP. Proteolysis of pro-caspase 3 has been directly associated with increased caspase activity in keratinocytes induced to undergo apoptosis by chemotherapeutic agents and UV radiation [46, 47]. Moreover, the reduced levels of pro-caspase 3 following UV exposure corresponded to increased PARP cleavage which was reduced by a specific caspase 3 inhibitor [47], consistent with the results shown here (Fig. 5).

The finding that sodium butyrate induced apoptosis of HaCaTs independently of the intrinsic pathway is surprising given the ability of sodium butyrate to directly activate this pathway in a variety of cells of epithelial origin and that activation of the intrinsic pathway is central for UV-induced keratinocyte apoptosis [14, 48, 49]. As the primary role of apoptosis in keratinocytes is the removal of cells irreparably damaged by UV exposure, it would be of considerable interest and potential benefit to determine if sodium butyrate can act synergistically with UV radiation to potentiate cell death, particularly when failure to remove damaged cells can lead to the development of skin cancers. Sodium butyrate and HDIs in general appear to have the capacity to act synergistically with other apoptotic stimuli to augment cell death [50, 51]. Both lymphoid and colorectal cancer cells primed with a low concentration of butyrate (by itself unable to induce activation of caspase 3 or apoptosis), became highly susceptible to apoptosis when the release of cytochrome *c* from the mitochondria was stimulated by staurosporine [52]. These reports suggest that investigating the interaction of bu-

tyrate with UV radiation has merit, but the ability of butyrate to stimulate keratinocyte apoptosis via the extrinsic pathway indicates this agent alone might be useful for the treatment of skin disorders such as skin cancers and psoriasis.

Increased Fas expression is generally associated with keratinocyte apopotosis stimulated by UV radiation [4, 53, 54] however a decrease in Fas expression has been linked to malignant cell survival and the progression of actinic keratoses to squamous cell carcinoma [55, 56]. Moreover, regulatory points upstream of the mitochondria such as the Bax/Bcl-2 ratio are altered to favour survival in both melanoma and non-melanoma skin cancers [14, 49]. Similarly, keratinocytes within psoriatic plaques are more resistant to apoptosis directed by the intrinsic pathway than uninvolved keratinocytes due to increased expression of the anti-apoptotic protein Bcl-xL [57]. By upregulating Fas and activating the extrinsic pathway in keratinocytes as shown here, sodium butyrate may bypass the interference of anti-apoptotic proteins from the intrinsic pathway and overcome the capacity of malignant cells and hyperproliferative psoriatic cells to resist apoptosis. Recent work showing butyrate downregulates Bcl-xL in pancreatic cancer cells gives additional support to the contention it may be of benefit in conditions where keratinocyte apoptosis is dysregulated [58].

The specific caspase 3 inhibitor DMQD-CHO was able to diminish sodium butyrate induced caspase 3 activity, inhibit PARP cleavage and decrease the number of HaCaT cells with fragmented nuclei and those lifting off the culture plate. These results show from a biological viewpoint that DMQD-CHO appeared to suppress apoptosis in HaCaTs, consistent with studies in a Pre-B leukaemia cell line ALL-697 where DMQD-CHO also decreased chromatin condensation and inhibited PARP cleavage [59]. In our studies however, DMQD-CHO did not prevent the translocation of PS to the outer membrane leaflet as measured by Annexin V staining.

PS translocation is an early event in apoptosis and, somewhat controversially, is generally considered to be linked to activation of the initiator caspase 8 and the cleavage of plectin, a major cross-linking protein involved in apoptosis-induced reorganization of the actin cytoskeleton [26, 60]. Others have reported that Annexin V staining induced by death receptors can be decreased by inhibiting effector caspases using z-VAD-fmk [61, 62], however this is a relatively non specific inhibitor that can inhibit initiator caspases as well as effector caspases [63]. Our finding that an increase in caspase 8 activity preceded the activation of caspase 3 together with the inability of DMQD-CHO to prevent PS exposure lends support to the view, at least in keratinocytes, that the initiator caspases are indeed responsible for this event and that inhibition of effector caspases does not decrease Annexin V staining [64, 65]. Thus it appears in keratinocytes that the early apoptotic related changes in cell membrane symmetry are controlled by upstream initiator caspases [30], with caspase 3 mediating chromatin condensation and DNA fragmentation [30, 66] and blockade of caspase 3 being capable of limiting the progression of apoptosis but not preventing its initiation.

Sodium butyrate has also been linked to the induction of terminal differentiation, a process that has been shown to also activate caspase 3 [67]. Sodium butyrate has been reported to promote differentiation in colon cells [68] and has also been shown to induce terminal differentiation and formation of cornified envelopes in primary keratinocytes [31, 32]. Our study shows that in HaCaTs, sodium butyrate did not alter the expression of keratinocyte markers associated with the cells committing to differentiation (K10) and becoming terminally differentiated (involucrin), suggesting that apoptosis can be induced in keratinocytes in a manner distinct from terminal differentiation. This is in agreement with other studies identifying apoptosis and differentiation as different processes [6, 69, 70].

Conclusion

These studies show that in the HaCaT keratinocyte cell line, apoptosis was induced by sodium butyrate in a mechanism likely to be Fas-associated rather than coupled to terminal differentiation. Sodium butyrate induced apoptosis was mediated by activation of caspase 8 and 3, leading to PARP cleavage. Moreover, the effects of sodium butyrate appear to act at a point that bypasses the apoptotic protection provided by anti-apoptotic proteins known to be important in malignant cell survival. These findings give support to the view that HDI's like sodium butyrate may be useful treatments for epidermal disorders such as keratinocyte hyperproliferation in psoriasis or in the treatment of cutaneous malignancies where the normal mechanisms activating keratinocyte cell death are downregulated.

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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 to direct cell-cell

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

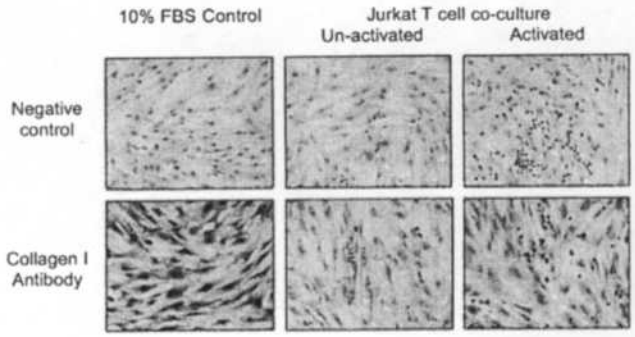
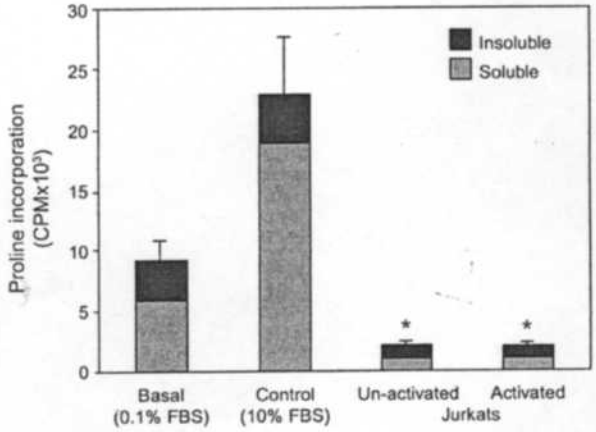


Figure 2. Fibroblast/T cell co-culture: fibroblast collagen synthesis.



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.

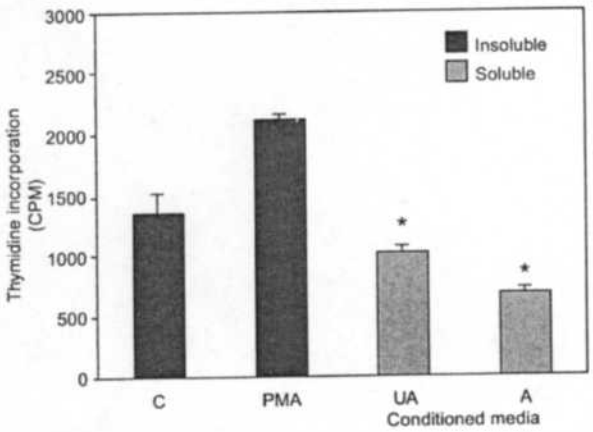
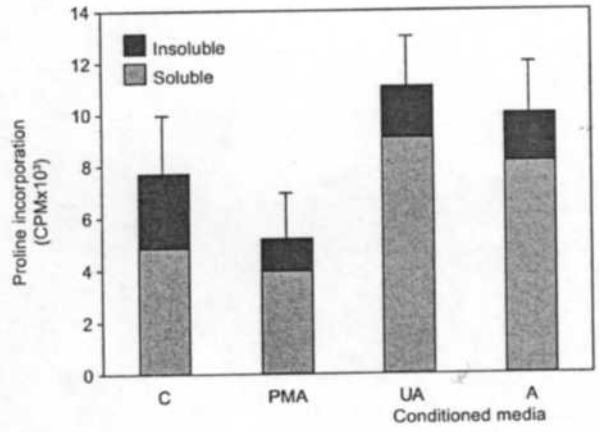


Figure 4. Fibroblast/T cell conditioned media: fibroblast collagen synthesis.



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.

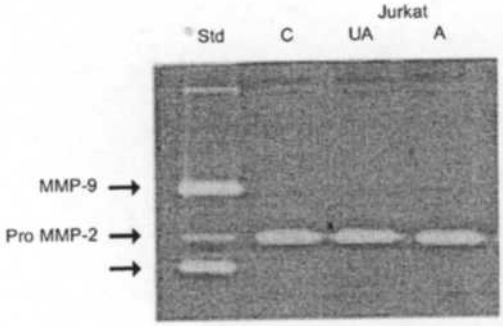


Figure 6. Fibroblast/T cell conditioned media: MMP activity – casein.

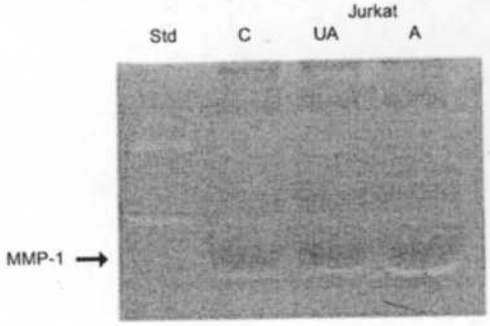
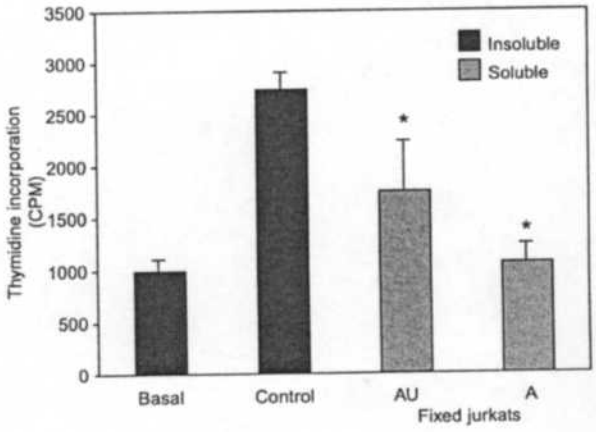
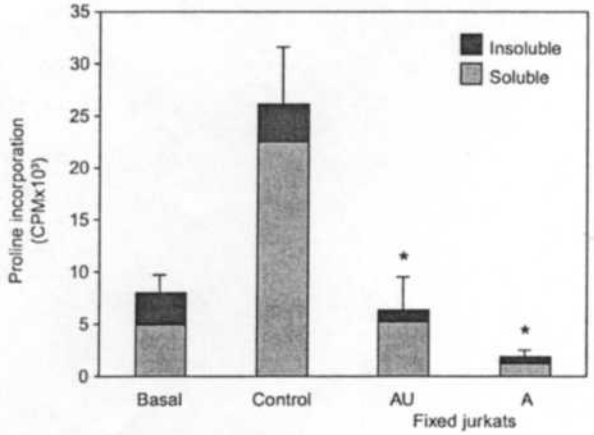


Figure 7. Fibroblast/Fixed T cell co-culture: fibroblast proliferation.



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.

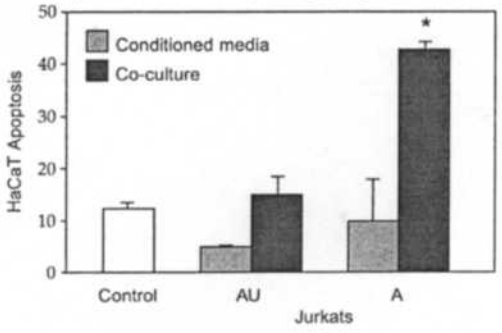


Figure 10. FasL expression by PMA activated Jurkats: measured by flow cytometry.

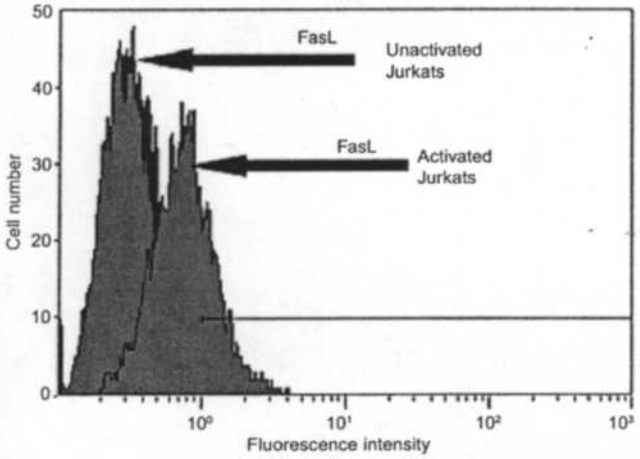
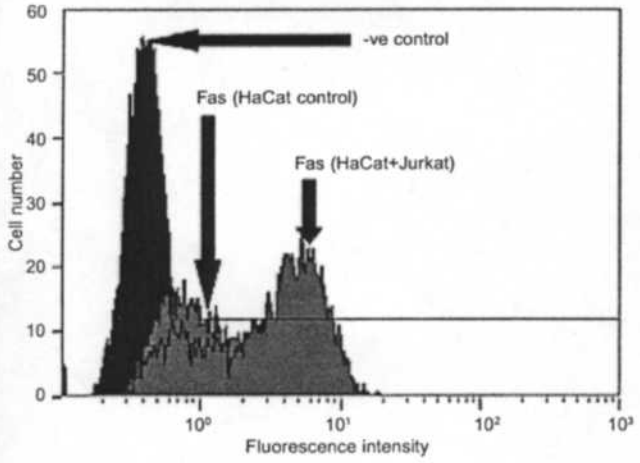


Figure 11. Jurkat induced Fas expression by HaCats: measured by flow cytometry.



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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