CHAPTER 6

6 TGFβ AND IGF-I EFFECTS ON T-LYMPHOCYTE INDUCED KERATINOCYTE APOPTOSIS

6.1 Introduction

The primary hypothesis of this thesis is that insulin-like growth factor-1 (IGF-1) and transforming growth factor β_1 (TGFβ_1) protect keratinocytes from T-lymphocyte induced apoptosis. Both IGF-I and TGFβ promote keratinocyte survival via the Akt signalling pathway (Decraene *et al.* 2002, Kuhn *et al.* 1999, Shin *et al.* 2001), however their effect on keratinocyte apoptosis mediated by T-lymphocytes has not been previously investigated. The following specific aims were investigated:

1. Determine the effect of TGFβ_1 and IGF-I on keratinocyte apoptosis induced by T-lymphocytes.

A combination of IGF-1, epidermal growth factor and basic fibroblast growth factor was required to inhibit IFNγ induced apoptosis of granulosa cells (Quirk *et al.* 2000). The effect of TGFβ_1 and IGF-I individually or as a combination on T-lymphocyte induced keratinocyte apoptosis was determined using the T-lymphocyte and keratinocyte co-culture model characterised in Chapters 4 and 5.

The effects of IGF-I on keratinocytes are mediated via IGF-IR and modulated by a family of high affinity binding proteins (IGFBPs) that sequester free IGFs, limiting the availability of IGF-I to bind to the receptor (Butt *et al.* 1999, Clemmons 1998, Edmondson *et al.* 1999b, Edmondson *et al.* 2001). LONG™R3 IGF-I (LR3-IGF) is an IGF-I analog which has reduced affinity for IGFBPs compared to native IGF-I, making it an efficient activator of IGF-1R that is about 10-fold more potent than IGF-I at stimulating hypertrophy and proliferation of cultured cells (Ballard *et al.* 1996, Francis *et al.* 1992, Francis *et al.* 1993). Given that keratinocytes produce IGFBP3, which may interfere with the availability of IGF-I in co-culture, the effect of LR3-IGF on T-lymphocyte induced keratinocyte apoptosis was also examined.

2. Examine the effect of growth factors on keratinocyte Fas expression.

Consistent with other reports (Trautmann *et al.* 2000a), it was shown in Chapter 4 and Chapter 5 that T-lymphocytes induce keratinocyte apoptosis via a Fas dependent pathway, which is potentially driven by IFNγ due to its ability to upregulate Fas. The capacity of TGFβ_1 and IGF-I to modulated IFNγ and T-lymphocyte induced Fas expression by keratinocytes was assessed.

3. Assess the effect of growth factors on T-lymphocyte induced keratinocyte differentiation.

It was demonstrated in Chapter 5, that T-lymphocyte co-culture induced early keratinocyte differentiation (Section 5.3.9). Studies extended these findings and

investigated the effect of TGFβ_1 and IGF-I on keratinocyte differentiation associated with T-lymphocyte co-culture by measuring changes in α6 integrin expression.

6.2 Methods

6.2.1 Co-culture treatment with IGF-1, TGFβ_1 or LR3-IGF

HaCaTs and NHEKs were grown to 70-80% confluence in 12 well plates and were co-treated with activated CD4+ T-lymphocytes (as described in Sections 5.2.1-5.2.2) and incubated for 48hs in DMEM or KBM. Keratinocytes were co-cultured with T-lymphocytes and TGFβ_1, IGF-1, LR3-IGF or combinations of TGFβ_1 and IGF-I or TGFβ_1 and LR3-IGF. The growth factors were added at the initiation of the co-culture or 6 hr after the T-lymphocytes were added to the keratinocytes. The concentrations used in these experiments ranged from 0.1-100 ng/ml and analysis was performed after 48 hr.

Keratinocyte apoptosis was measured by Annexin V staining (Methods Section 2.5.1) and cell morphology was imaged by phase contrast microscopy. Wells stained with HOECHST 33342 were first washed twice with PBS to remove T-lymphocytes (Methods Section 2.6.1). The cell-free conditioned media from these experiments was collected and stored at -20°C for IFNγ measurements as described in Methods Chapter 2 (Section 2.11).

In this chapter keratinocytes from co-cultures treated with growth factors were compared with keratinocytes from untreated T-lymphocyte co-cultures.

6.3 Results

6.3.1 Effect of growth factors on T-lymphocyte induced HaCaT apoptosis

6.3.1.1 Morphology and nuclear condensation

Images in Figure 6.1 demonstrate the effect of TGFβ_1 on the morphological changes induced in HaCaTs by T-lymphocyte co-culture. Compared to co-culture controls shown in Figure 6.1 b, TGFβ_1 (1 and 10ng/ml) treated HaCaTs displayed reduced cell shrinkage and membrane blebbing with the monolayer maintained due to fewer cells found detached from the culture plate (Figure 6.1 c and d). Parallel wells were used to identify nuclear condensation and fragmentation of HaCaTs by HOESCHT 33342 staining. TGFβ_1 (1 and 10ng/ml) shown in Figures 6.1 g and 6.1 h respectively, demonstrated a reduced number of HOESCHT 33342 positive condensed and fragmented nuclei induced by T- lymphocytes controls (Figure 6.1 f).

IGF-1, like TGFβ_1 also reduced the T-lymphocyte related changes in HaCaT morphology. Figure 6.2 c and d respectively, show that 10 and 100ng/ml IGF-I reduced HaCaT cell shrinkage and membrane blebbing compared to co-culture controls (Figures 6.2 b). Both 10 and 100ng/ml IGF-I reduced the number of T-lymphocyte induced HOESCHT 33342 positively stained nuclei and the incidence of fragmentation (Figure 6.2 f - h).

Figure 6.3 shows the effects of the IGF-I analogue, LR3-IGF on HaCaT morphology and nuclear staining. Both 10 and 100ng/ml LR3-IGF decreased T-lymphocyte induced changes in HaCaT morphology (Figure 6.3 c and d) and T-lymphocyte induced nuclear fragmentation of the HaCaTs (Figure 6.3 g and h) compared to controls (Figure 6.3 b and f respectively). Moreover, the 10ng/ml dose of IGF-LR3 (Figure 6.3 c and g) seemed to be as effective as the 100ng/ml dose of IGF-I in reducing apoptosis related changes in morphology (Figure 6.2 d and h).

6.3.1.2 Annexin V and PI staining

HaCaTs treated with TGFβ_1 (10ng/ml), IGF-I (100ng/ml) and LR3-IGF (10ng/ml), the concentrations shown to be most effective at reducing apoptosis related changes in HaCaT morphology (Figures 6.1, 6.2 and 6.3 respectively), were assessed by Annexin V/PI staining. Figure 6.4 shows the combined results from four independent experiments and consistent with results in Chapter 5 (Section 5.3.1), demonstrates that the total number of apoptotic cells was significantly increased from 7% in HaCaT controls to 26% by co-culture ($p<0.05$ Figure 6.4). Despite an overall trend suggesting that TGFβ_1 (10ng/ml), IGF-I (100ng/ml) and LR3-IGF (10ng/ml) decreased Annexin V staining consistent with the decreased apoptosis related morphological changes in HaCaTs (Figure 6.1 – 6.3), these results were not significantly different to untreated co-culture controls ($p<0.05$ Figure 6.4).

Figure 6.1

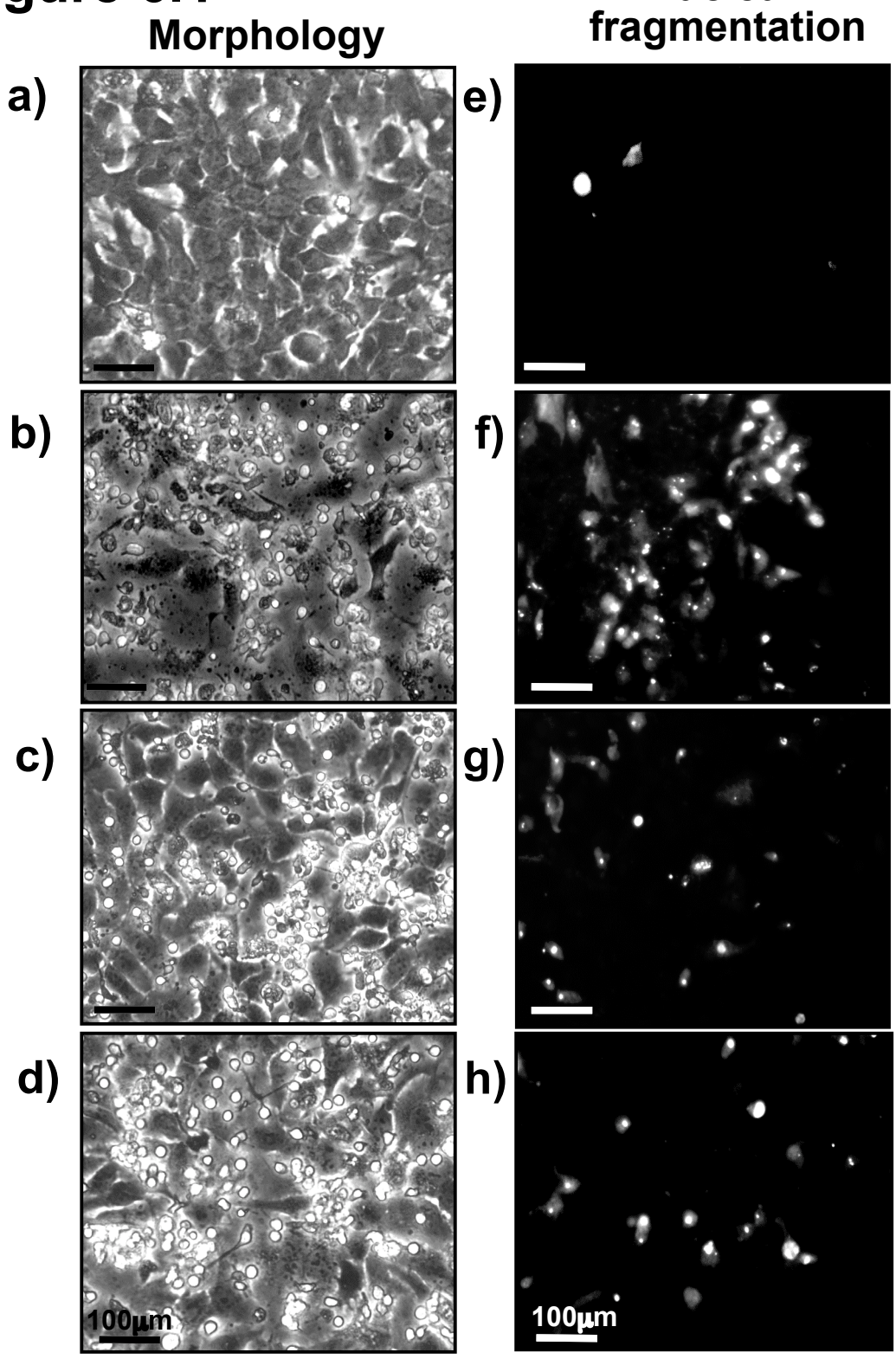

Figure 6.1 Effect of TGFβ on T-lymphocyte induced HaCaT apoptosis. (a) Phase contrast microscopy was used to assess morphology of HaCaT controls, (b) HaCaTs co-cultured with $5X10^5$ T-lymphocytes, and (c) HaCaTs T-lymphocyte co-cultures with 1ng/ml TGFβ, or (d) 10ng/ml TGFβ for 48hrs. (e) HOESCHT fluorescent stain was used to detect condensed and fragmented nuclei of HaCaT controls, (f) HaCaTs co-cultured with T-lymphocytes, (g) HaCaTs T-lymphocyte co-cultures with 1ng/ml TGFβ, or (h) 10ng/ml TGFβ for 48hrs.

Figure 6.2

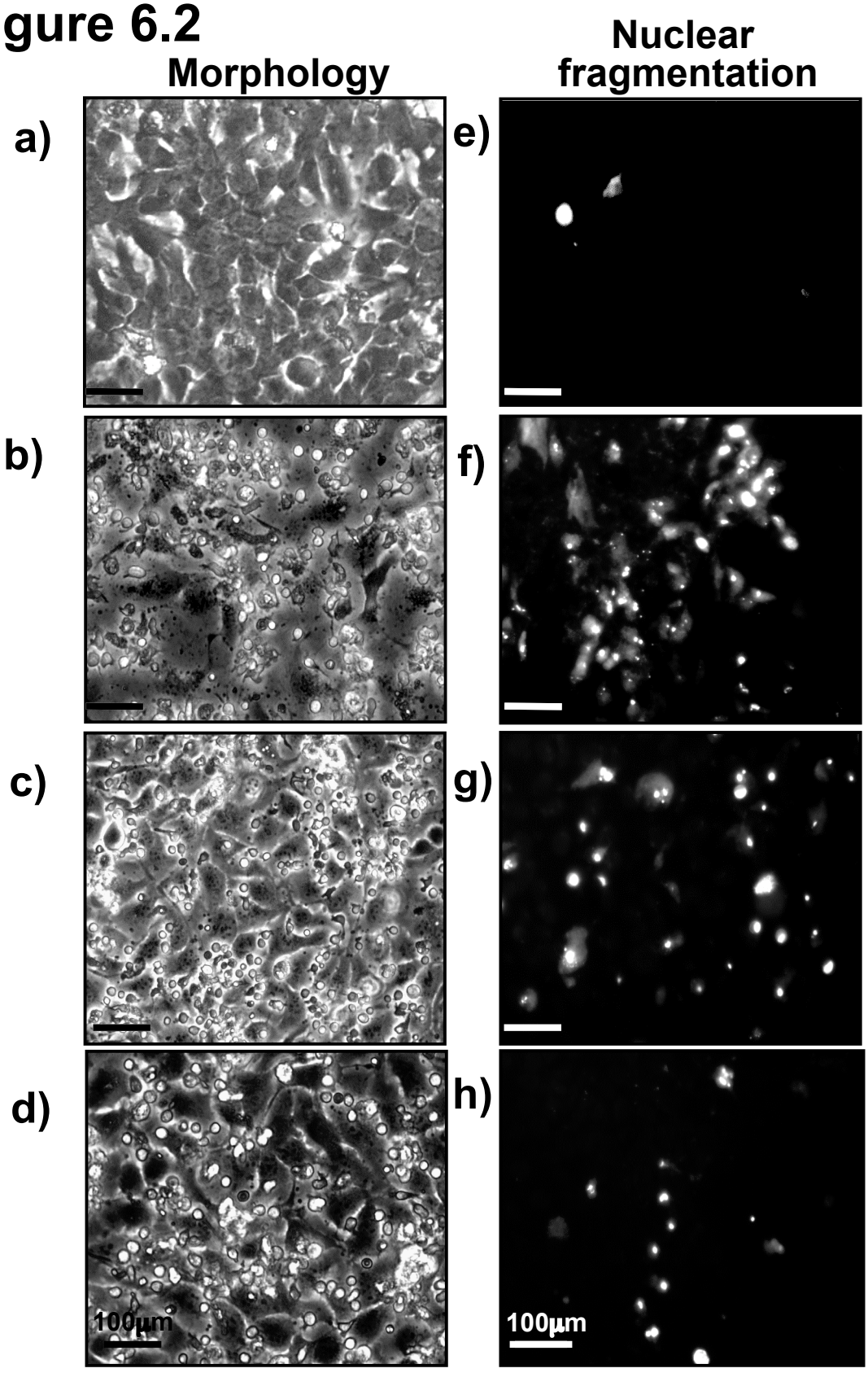

Figure 6.2 Effect of IGF-I on T-lymphocyte induced HaCaT apoptosis. (a) Phase contrast microscopy of HaCaT controls, (b) HaCaTs co-cultured with $5X10^5$ T-lymphocytes, (c) co-cultured with 10ng/ml IGF-1, and (d) 100ng/ml IGF-1for 48hrs. (e) HOESCHT fluorescent staining of HaCaT controls, (f) HaCaTs co-cultured with T-lymphocytes, (g) co-cultured with 10ng/ml IGF-1, and (h) 100ng/ml IGF-I for 48hrs.

Figure 6.3

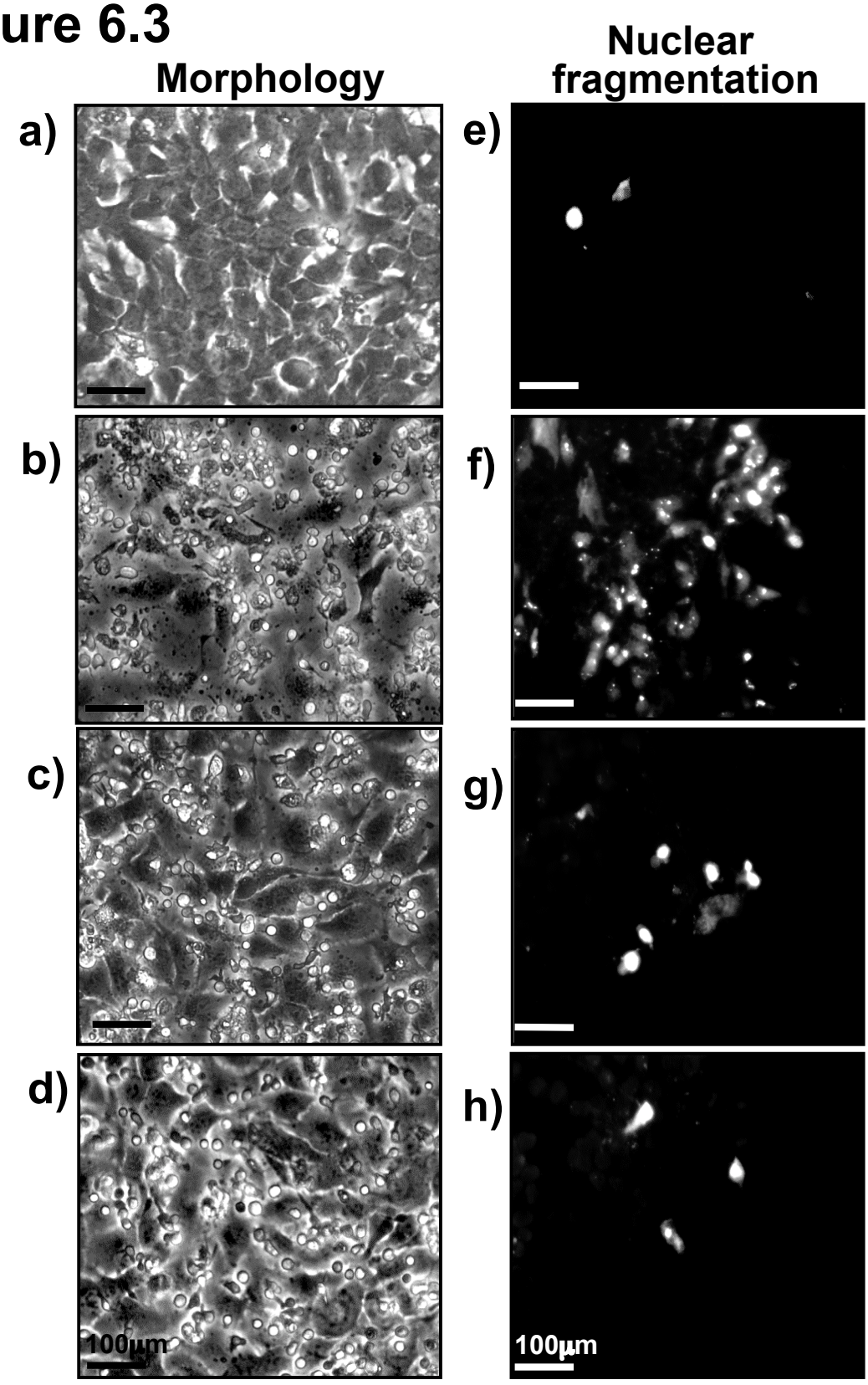

Figure 6.3 Effect of LR3-IGF on T-lymphocyte induced HaCaT apoptosis. (a) Phase contrast microscopy of HaCaT controls, (b) HaCaTs co-cultured with $5X10^5$ T-lymphocytes, (e) co-cultured with 10ng/ml LR3-IGF, and (d) 100ng/ml LR3-IGF for 48hrs. (e) HOESCHT fluorescent staining of HaCaT controls, (f) HaCaTs co-cultured with T-lymphocytes, (g) co-cultured with 10ng/ml LR3-IGF, and (h) 100ng/ml LR3-IGF for 48hrs.

Figure 6.4

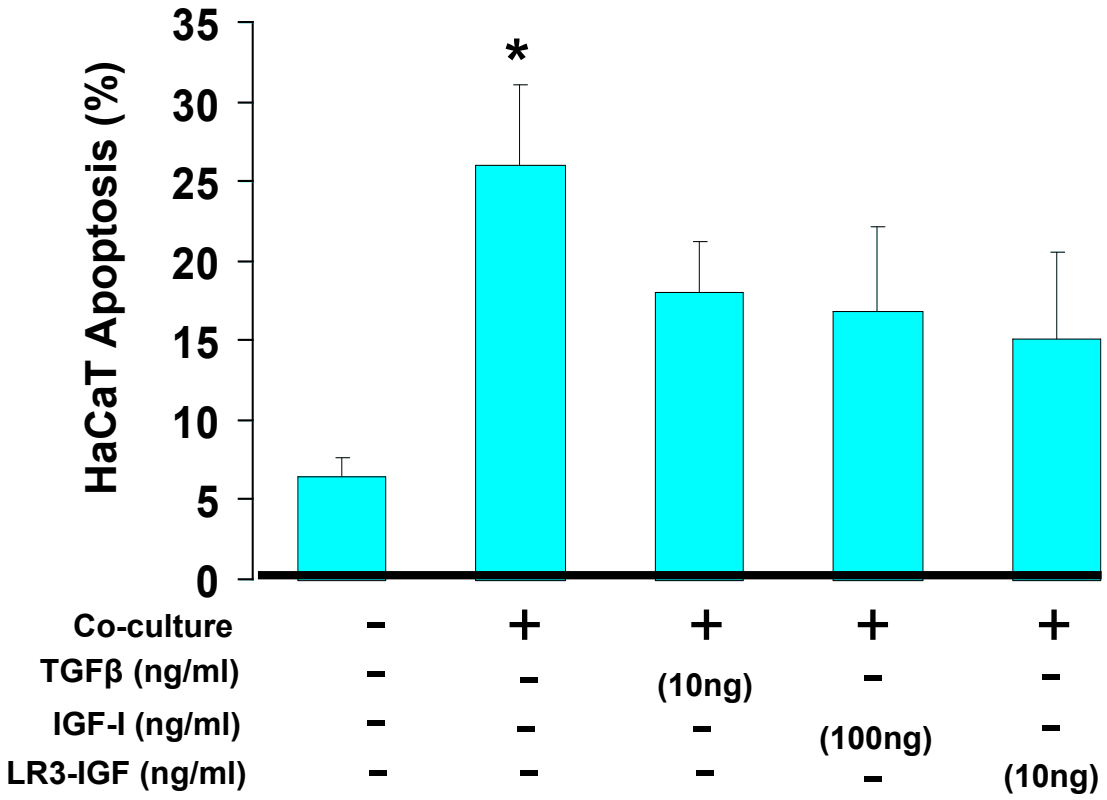

Figure 6.4 Effect of TGFβ, IGF-I and LR3-IGF on T-lymphocyte induced HaCaT apoptosis. Apoptosis was quantified by Annexin V and propidium iodide (PI) staining. Apoptotic population was determined in HaCaT controls, HaCaTs co-cultured with activated T-lymphocytes for 48hr and HaCaTs co-cultured with T-lymphocytes together with either TGFβ, IGF-I or LR3-IGF. Bar graphs represent the mean ± SEM percentage from cells in 3 separate experiments. The data was analysed using one-way analysis of variance (ANOVA) and considered significant at *p<0.05 to co-culture control.

6.3.2 A combination of TGFβ_1 and IGF-I decreased T-lymphocyte induced HaCaT apoptosis

The effect of a combination of TGFβ_1 and IGF-I was investigated. Figures 6.5 c and d demonstrate that HaCaT co-cultures treated with TGFβ_1 and IGF-I at concentrations of 1 and 10ng/ml or 10 and 10ng/ml respectively, had decreased morphological features of apoptosis induced by T- lymphocytes compared to co-culture controls (Figure 6.5 b). Moreover, HaCaTs treated with TGFβ_1 (10ng/ml) and IGF-I (100ng/ml) demonstrated almost no cell shrinkage or membrane blebbing and there was no evidence of cell separation or detachment from the culture plate with these cells looking like control HaCaTs (Figure 6.5 e). Treatment of HaCaTs with each TGFβ_1 and IGF-I combination also decreased T-lymphocyte induced nuclear condensation and fragmentation (Figures 6.5 g, h, i and j)

The effect of TGFβ_1 and LR3-IGF combination on T-lymphocyte induced HaCaT apoptosis is shown in Figure 6.6. Treatment of HaCaTs with TGFβ_1 (1ng/ml) and LR3-IGF (10ng/ml) decreased apoptosis related morphological features induced by T-lymphocytes (Figure 6.6 c) compared to controls (Figure 6.6 b). Combinations of TGFβ_1 and LR3-IGF at concentrations of 10 and 10ng/ml or 10 and 100ng/ml respectively, prevented T-lymphocyte induced HaCaT cell shrinkage and cell separation (Figure 6.6 d and 6.6 e respectively). The TGFβ_1 and LR3-IGF combinations also decreased the number of HOESCHT 33342 positive nuclei (Figure 6.6 h, i and j respectively) compared to untreated T-lymphocyte co-cultures (Figure 6.6 g).

Figure 6.7 demonstrates the total number of apoptotic cells from four independent experiments identical to those shown in Figures 6.5 and 6.6. Consistent with the morphological observations and nuclear staining, a combination of TGFβ_1 (10ng/ml) and IGF-I (100ng/ml) significantly decreased T-lymphocyte induced HaCaT apoptosis from 29.3% to 14.5% ($p<0.05$; Figure 6.7 a). A significant decrease in apoptosis from 29.3% to 17.1%, was also observed in HaCaTs treated with TGFβ_1 (10ng/ml) and LR3-IGF (10ng/ml) ($p<0.05$; Figure 6.7 a).

It has been previously demonstrated that TGFβ induces T-lymphocyte apoptosis (Chung *et al.* 2000, Lomo *et al.* 1995). To ensure the effect of growth factors on the T-lymphocyte induced HaCaT apoptosis was not attributed to T-lymphocyte apoptosis during co-culture, T-lymphocytes were collected from co-cultures treated with TGFβ_1 (10ng/ml) and IGF-I (100ng/ml) or TGFβ_1 (10ng/ml) and LR3-IGF (10ng/ml) and stained with Annexin V and PI and the analysis was performed in all cells present and then T-lymphocytes were gated based on size (see Appendix 2), in order to discriminate all T-lymphocytes from keratinocytes. Figure 6.7 b demonstrates that T-lymphocyte viability was not affected by either growth factor combinations, with cell viability found to be 100%.

Figure 6.5

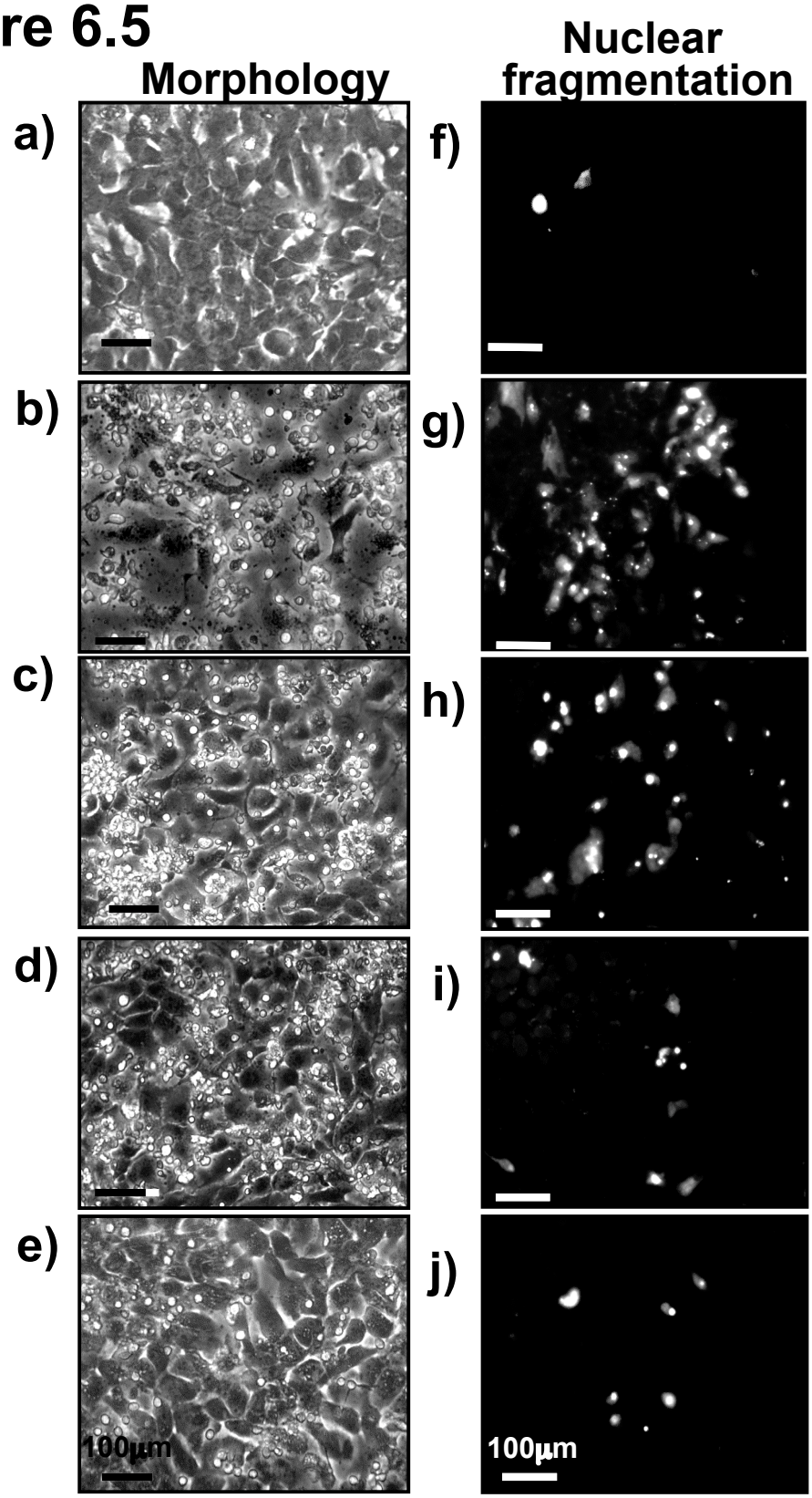

Figure 6.5 Effect of a combination of TGFβ and IGF combination on T-lymphocyte induced HaCaT apoptosis. (a) Phase contrast microscopy of HaCaT controls, (b) HaCaTs T-lymphocytes co-culture, and HaCaTs co-cultured with TGFβ and IGF-I at (c) 1:10, (d) 10:10 and (e) 10:100ng/ml concentration for 48hrs. (f) HOESCHT staining of HaCaT controls, (g) HaCaTs co-cultured with T-lymphocytes, and HaCaTs co-cultured with TGFβ and IGF-I at (h) 1:10, (i) 10:10 and (j) 10:100ng/ml 48hrs.

Figure 6.6

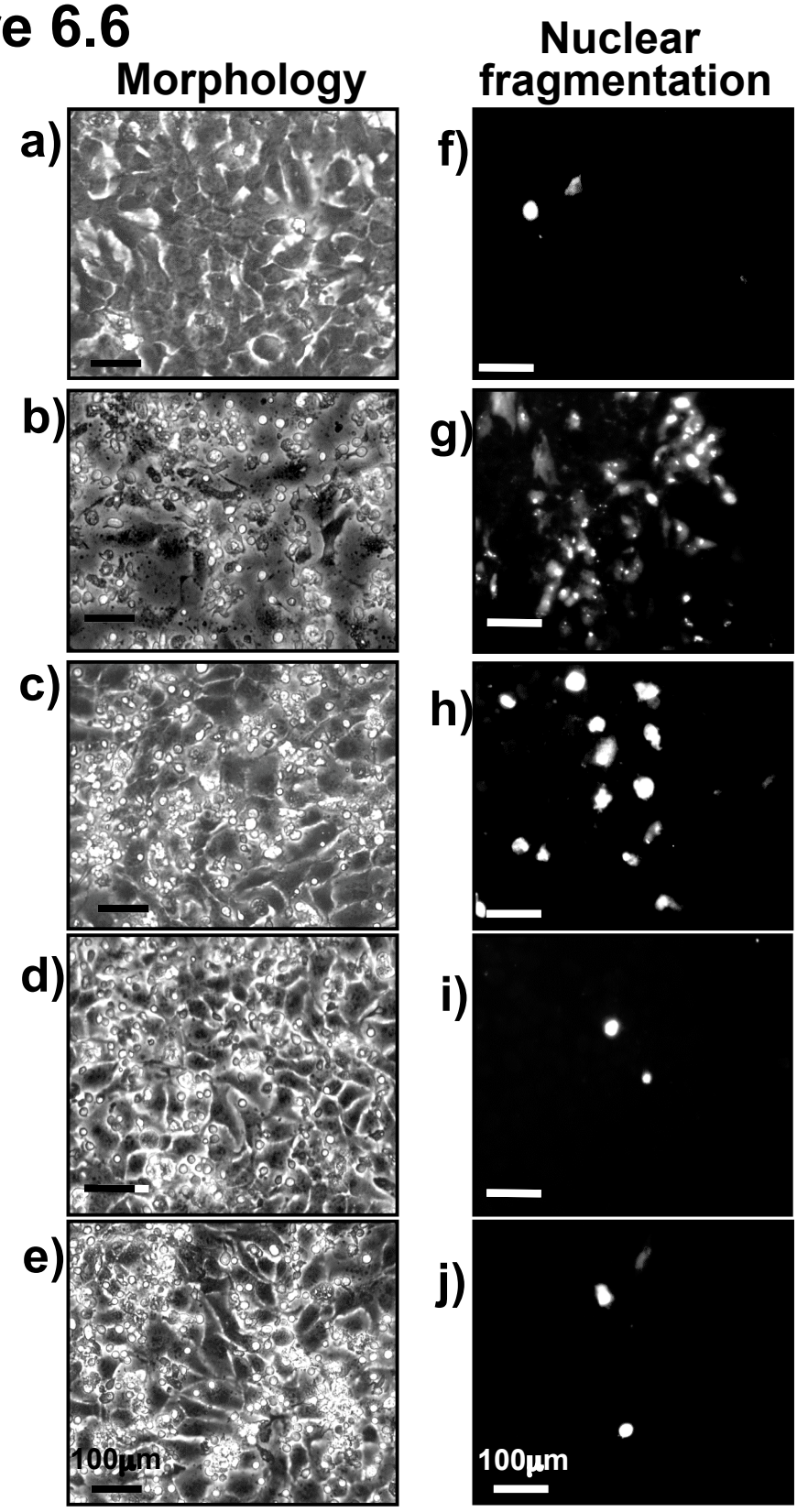

Figure 6.6 Effect of a combination of TGFβ and LR3-IGF combination on T-lymphocyte induced HaCaT apoptosis. (a) Phase contrast microscopy of HaCaT controls, (b) HaCaTs T-lymphocytes co-culture, and HaCaTs co-cultured with TGFβ and LR3-IGF at (c) 1:10, (d) 10:10 and (e) 10:100ng/ml concentration for 48hrs. (f) HOESCHT staining of HaCaT controls, (g) HaCaTs co-cultured with T-lymphocytes, and HaCaTs co-cultured with TGFβ and LR3-IGF at (h) 1:10, (i) 10:10 and (j) 10:100ng/ml 48hrs.

Figure 6.7

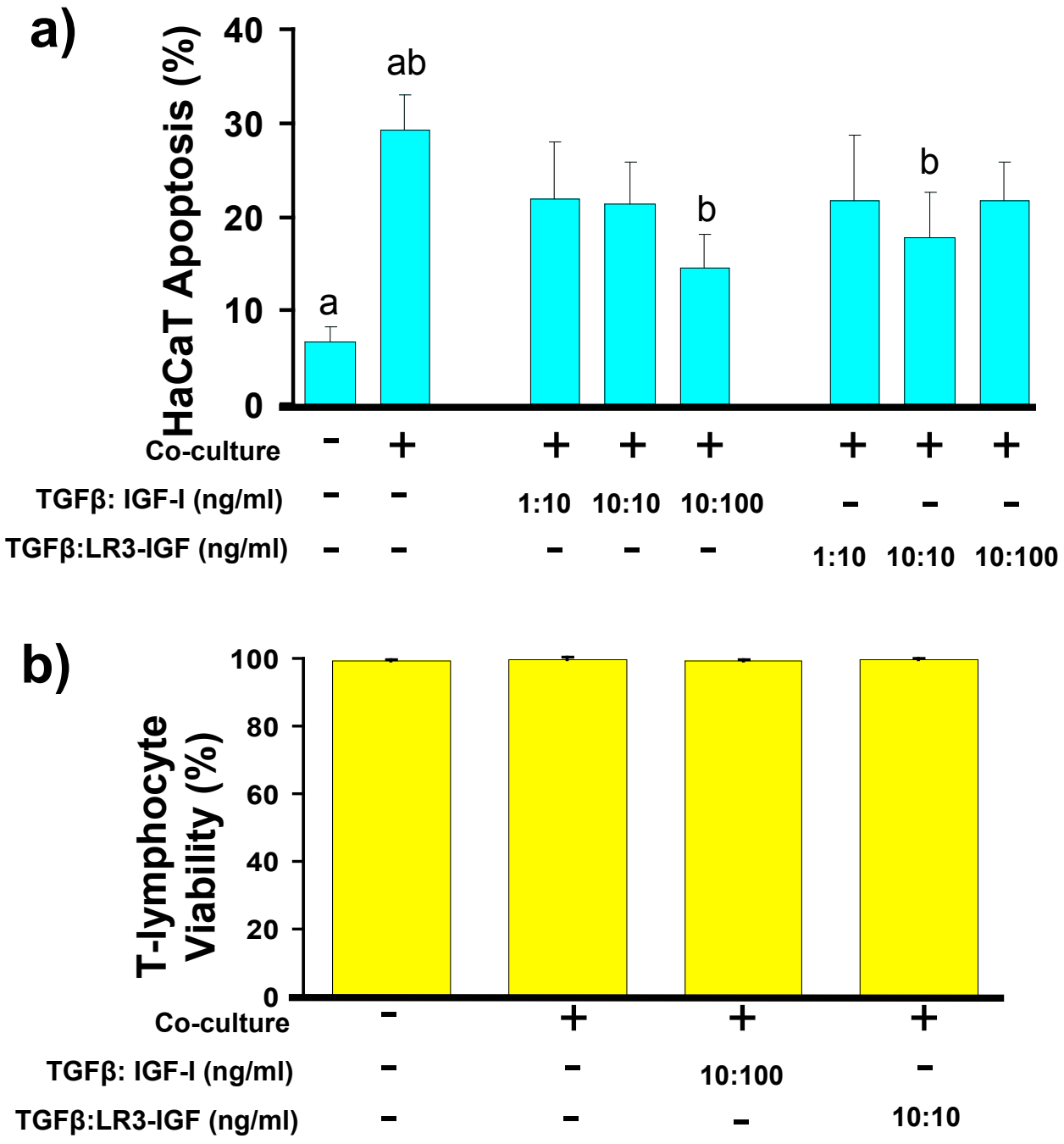

Figure 6.7 Effect of TGFβ and IGF-I or TGFβ and LR3-IGF on T-lymphocyte induced HaCaT apoptosis and T-lymphocyte viability. Apoptosis was quantified by Annexin V and propidium iodide (PI) staining. (a) Apoptotic population was determined on HaCaT controls and co-cultures with activated T-lymphocytes together with increasing concentrations of either TGFβ and IGF-I or TGFβ and LR3-IGF for 48hr. (b) T-lymphocyte viable population [Annexin –ve and PI -ve] was determined after co-culture with HaCaTs together with either TGFβ and IGF-I (10:100ng/ml) or TGFβ andLR3-IGF (10:10ng/ml). Bar graphs represent the mean ± SEM percentage from cells in 3 separate 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).

6.3.3 Post-treatment with TGFβ_1 and IGF-I did not rescue T-lymphocyte induced HaCaT apoptosis

Section 6.3.2 demonstrated that HaCaTs treated at the induction of co-culture with TGFβ_1 and IGF-I or TGFβ_1 and LR3-IGF was able to prevent T-lymphocyte induced apoptosis. Given that some key apoptotic events occur in the first few hours after activation of Fas (Hirata *et al.* 1998, Zhang *et al.* 1999), studies in this chapter investigated whether post-treatment with growth factor combinations could reverse the apoptosis related changes in morphology and Annexin V staining induced by T-lymphocytes.

For these studies, TGFβ_1 (10ng/ml) and IGF-I (100ng/ml), or TGFβ_1 (10ng/ml) and LR3-IGF (10ng/ml) were added into the co-culture 6 hr after induction. Post-treatment with either TGFβ_1 and IGF-I or TGFβ_1 and LR3-IGF (Figures 6.8 c and d respectively) had no effect on the apoptosis associated changes in HaCaT morphology induced by T-lymphocytes shown in Figure 6.8 b. Consistent with these observations, apoptosis measured by Annexin V and PI staining showed that T-lymphocyte induced HaCaT apoptosis was not prevented when these growth factor combinations were added to the co-cultures 6 hr after induction (Figure 6.8 e).

6.3.4 TGFβ$_1$ and IGF-I inhibited the release of IFNγ in co-culture

Results in Chapter 5 showed that IFNγ released into the conditioned media of co-cultures may be involved in T-lymphocyte induced keratinocyte apoptosis by upregulating keratinocyte Fas expression. IFNγ released after treatment of co-cultures with growth factors was examined in the following experiments.

Consistent with Chapter 5 (Section 5.3.6), Figure 6.9 a demonstrated increase in IFNγ levels were significantly increased in HaCaT – T-lymphocyte co-culture conditioned media compared to control. However, the IFNγ released into the co-culture media was significantly decreased from 1.27ng/ml to less than 0.1ng/ml by 10ng/ml TGFβ$_1$, 100ng/ml IGF-I and 10ng/ml LR3-IGF ($p<0.001$). Furthermore, when of IFNγ was measured in the conditioned media from co-cultures treated with combinations of TGFβ$_1$ and IGF-I or TGFβ$_1$ and LR3-IGF, it was significantly decreased from 1.1 ng/ml to less than 0.1ng/ml by all the concentrations tested ($p<0.001$; Figure 6.9 b).

6.3.5 Effect of TGFβ and IGF-I on HaCaT Fas expression

T-lymphocyte induced keratinocyte apoptosis has been demonstrated to proceed via a Fas dependent mechanism potentially driven by IFNγ (Chapter 5 Section 5.3.5 to 5.3.8). Given that growth factor combinations prevented T-lymphocyte induced apoptosis, their effect on HaCaT Fas expression was determined.

Figure 6.8

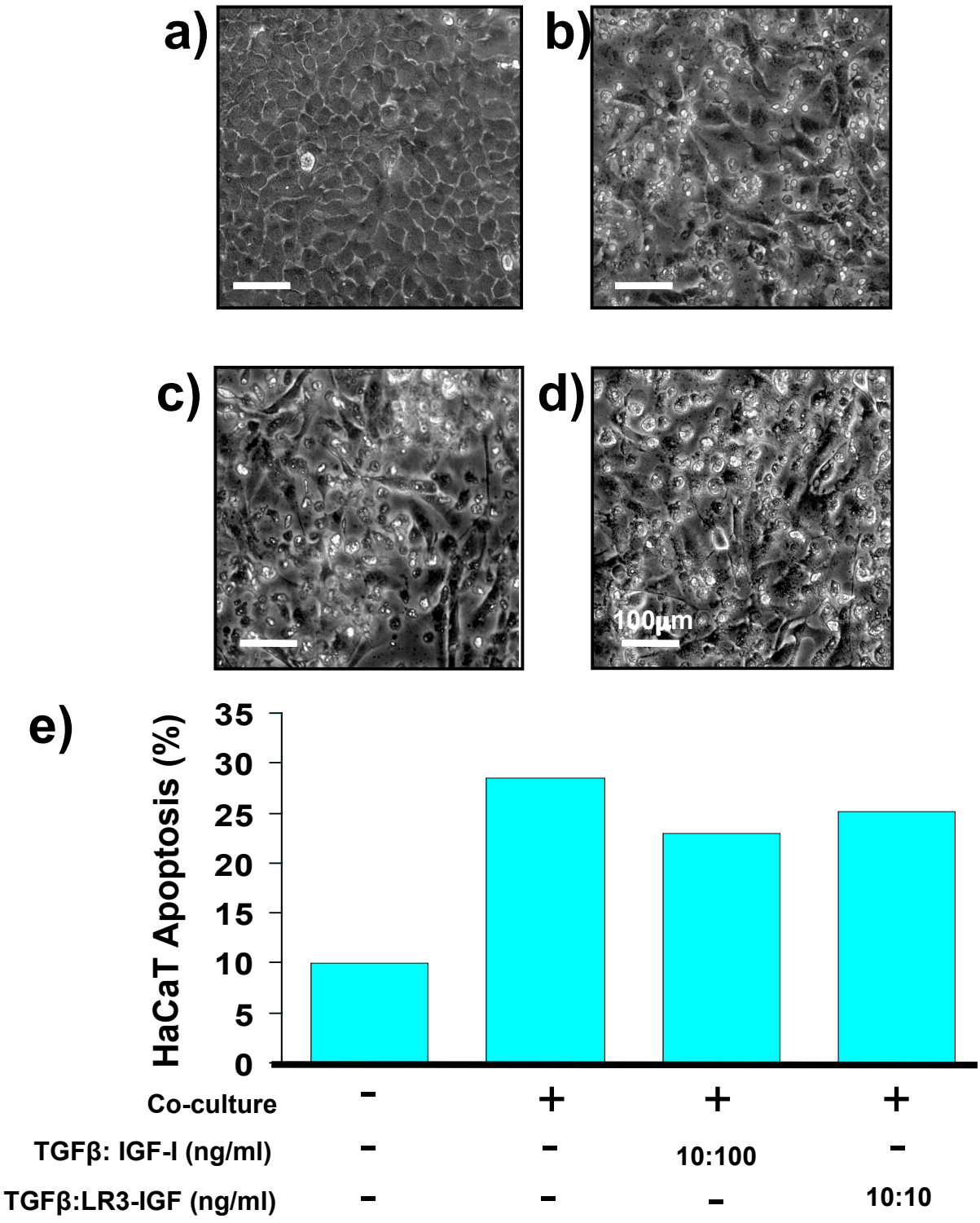

Figure 6.8 Effect of post-treatment with a combination of TGFβ and IGF-I or TGFβ and LR3-IGF on T-lymphocyte induced HaCaT apoptosis. (a) Phase contrast microscopy of HaCaT controls, (b) HaCaTs T-lymphocytes co-culture, and HaCaT co-cultures treated with (c) TGFβ and IGF-I (10:100ng/ml) or (d) TGFβ and LR3-IGF (10:10ng/ml) after 6hr co-culture. (e) Apoptosis of samples described in (a-d) was measured by Annexin staining. Bar graphs represent 1 experiment.

Figure 6.9

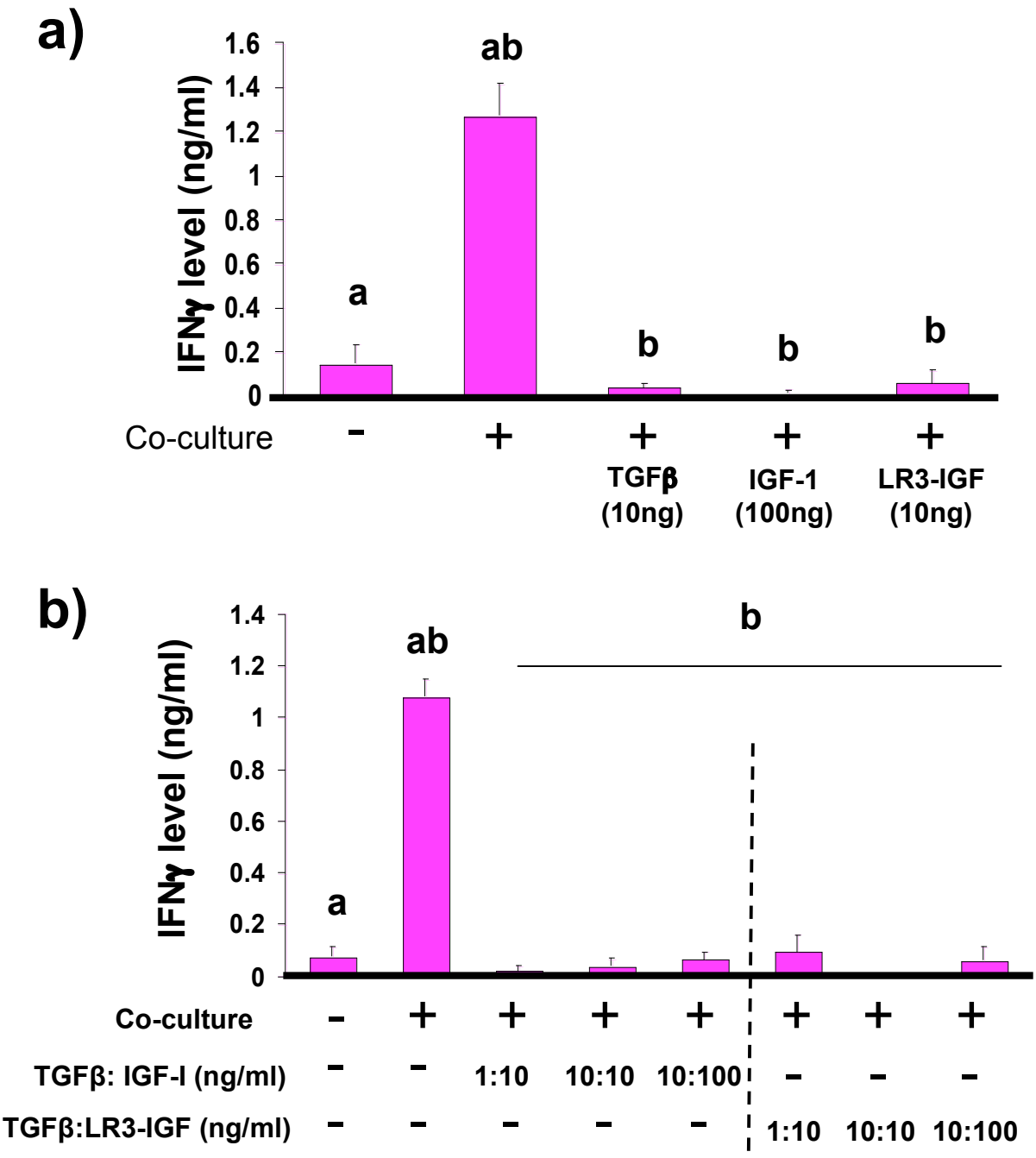

Figure 6.9 Effect of TGFβ,IGF-1, LR3-IGF on co-culture IFNγ levels. (a) IFNγ in the conditioned media from HaCaT controls, HaCaTs co-cultured with activated T-lymphocytes and HaCaTs co-cultured in the presence of TGFβ, IGF-I and LR3-IGF. (b) Levels of IFNγ in conditioned media from HaCaTs co-cultured in the presence of increasing concentrations of TGFβ and IGF-1, or TGFβ and LR3-IGF. Values represent the mean release of cytokine ± SEM from 4 independent experiments the data was analysed using one-way analysis of variance (ANOVA) and post-hoc t-test with significance ($p < 0.001$) between treatments shown by the matching symbols (a, b).

HaCaT basal Fas expression (green histogram) and IFNγ (100ng/ml; pink histogram) induced Fas expression are shown in Figures 6.10 a and d respectively. Figures 6.10 b and c show that HaCaT basal Fas expression was reduced by TGFβ_1 and IGF-I (blue histogram; MFI: from 1.2 to 0.9) and by TGFβ_1 and LR3-IGF (orange histogram; MFI: from 1.2 to 0.7). Similarly, IFNγ induced Fas expression was also reduced by TGFβ_1 and IGF-I or TGFβ_1 and LR3-IGF (blue histogram; MFI: from 1.4 to 0.5) or (orange histogram; MFI: from 1.4 to 1.05) (Figures 6.10 e and f respectively). In contrast, the TGFβ_1 and IGF-I (blue histogram; Figure 6.11 b) or TGFβ_1 and LR3-IGF (orange histogram; Figure 6.11 c) were found to have no effect on T-lymphocyte induced Fas expression (yellow histogram; Figure 6.11 a).

6.3.6 TGFβ_1 and IGF-I prevented T-lymphocyte induced keratinocyte differentiation

Given that T-lymphocytes were shown to initiate terminal differentiation of keratinocytes (Chapter 5 Section 5.3.9), the following series of studies examined the effect of growth factor combinations on T-lymphocyte induced HaCaT differentiation by assessing changes in α6 expression.

Figure 6.12 demonstrates that compared to control HaCaTs (Figure 6.12 a), T-lymphocytes decreased the number of α6-bright expressing HaCaTs and increased the number of α6-dim expressing HaCaTs (blue histogram; Figure 6.12 b). In contrast, the

Figure 6.10

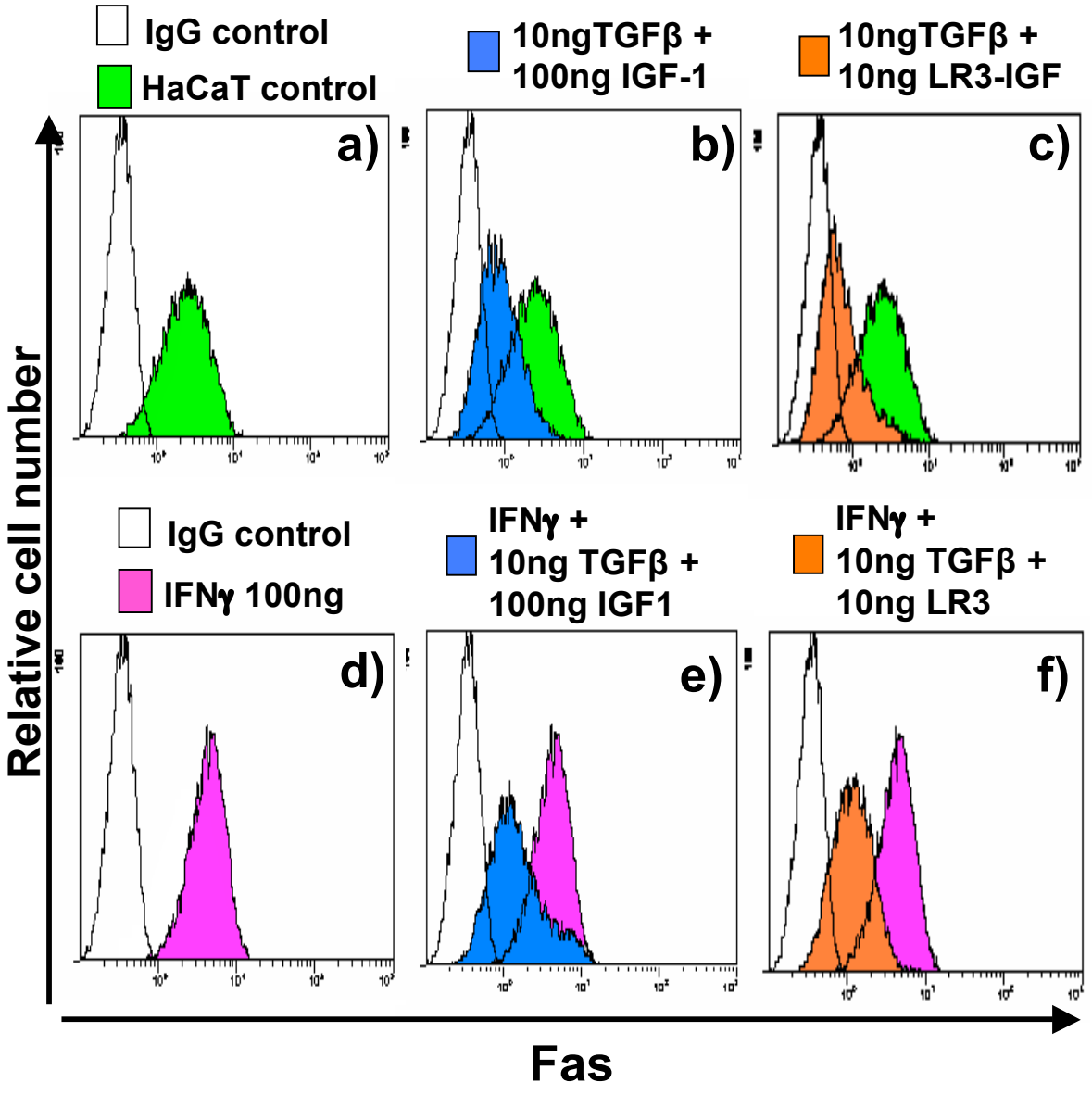

Figure 6.10. Effect of a combination of TGFβ and IGF-I or TGFβ and LR3-IGF on HaCaT Fas. Surface Fas expression of HaCaTs was analysed by flow cytometry. (a) Green histogram demonstrates Fas staining by HaCaT controls, (b) Fas of control HaCaTs incubated with TGFβ and IGF-I (10:100ng/ml) blue histogram and (c) control HaCaTs incubated with TGFβ and LR3-IGF (10:10ng/ml) orange histogram. (d) The pink histogram represents 100ng IFNγ induced Fas staining, (e) blue histogram demonstrate Fas staining of HaCaT + IFNγ incubated with TGFβ and IGF-I (10:100ng/ml) and (c) HaCaTs + IFNγ incubated with TGFβ and LR3-IGF (10:10ng/ml) orange histogram. Unfilled histogram represent staining with an isotype-matched control Ab.

Figure 6.11

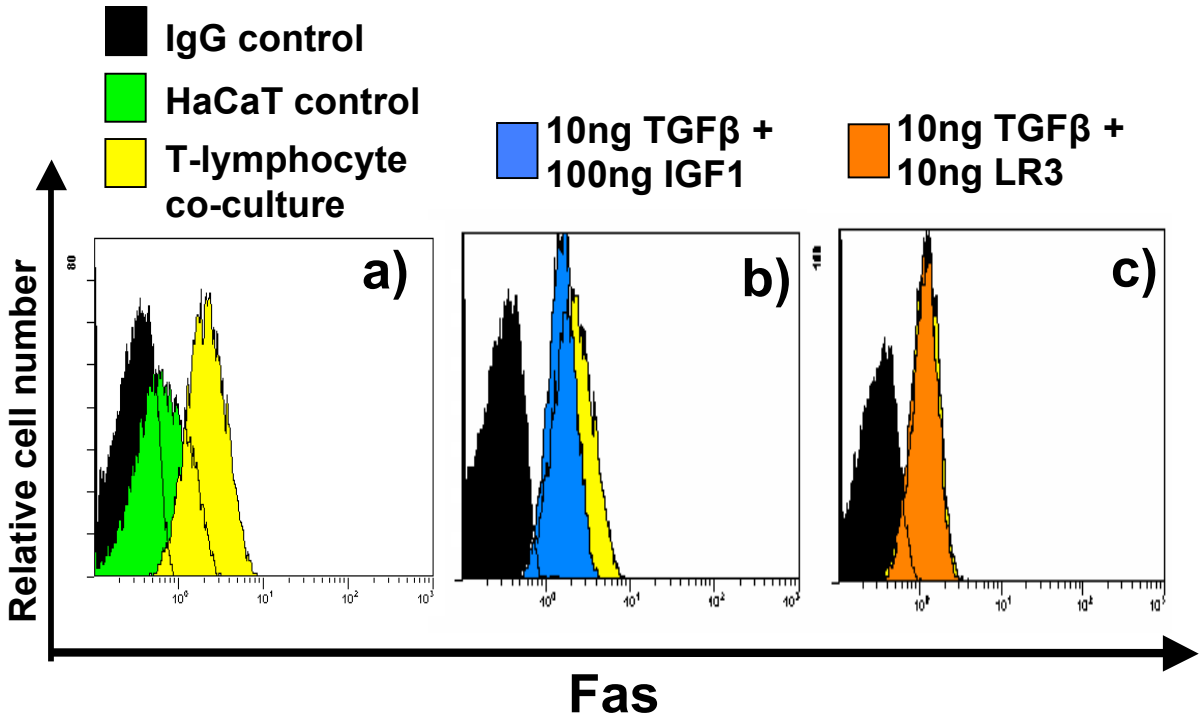

Figure 6.11. Effect of a combination of TGFβ and IGF-I or TGFβ and LR3-IGF on T-lymphocyte induced HaCaT Fas. Surface Fas expression of HaCaTs was analysed by flow cytometry. (a) Green histogram demonstrates Fas staining by HaCaT control and yellow histogram demonstrates T-lymphocyte co-culture induced Fas staining. (b) Fas expression of HaCaT co-cultures treated with TGFβ and IGF-I (10:100ng/ml) blue histogram and (c) HaCaT co-cultures treated with TGFβ and LR3-IGF (10:10ng/ml) orange histogram. Black histogram represent staining with an isotype-matched control Ab.

Figure 6.12

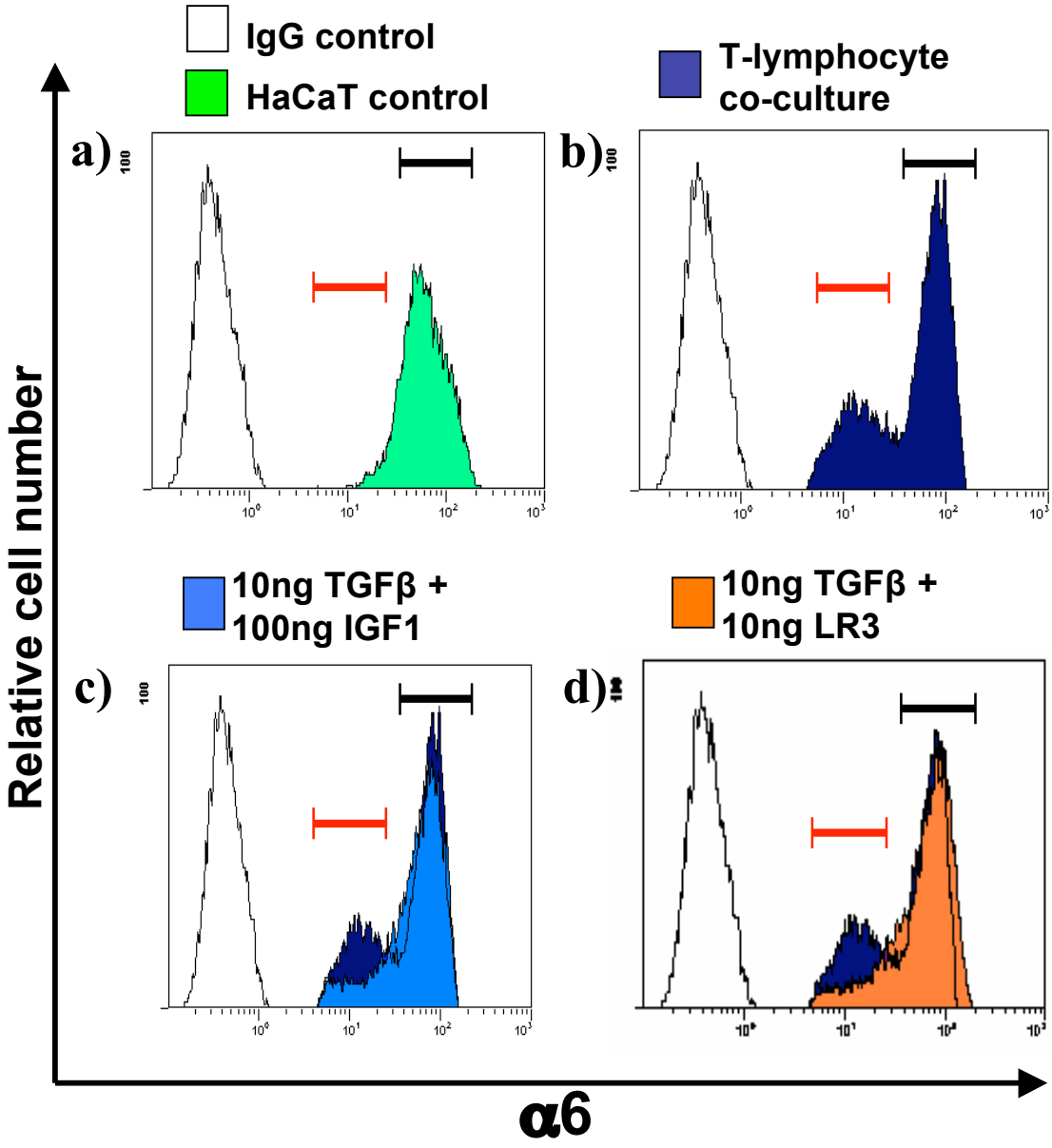

Figure 6.12. Effect of TGFβ and IGF-I or TGFβ and LR3-IGF on HaCaT α6 expression. Expression of α6 integrin was assessed by flow cytometry on (a) HaCaT controls (green histogram), (b) HaCaTs after T-lymphocyte co-culture (dark blue histogram) and (c) T-lymphocyte co-cultures treated with TGFβ and IGF-I (10:100ng/ml; light blue histogram) and (d) HaCaTs after T-lymphocyte co-cultures treated with TGFβ and LR3-IGF (10:10ng/ml; orange histogram). Unfilled histogram represent staining with an isotype-matched control Ab.

combination of TGFβ_1 (10ng/ml) and IGF-I (100ng/ml) maintained the number of α6-bright expressing HaCaTs, preventing the T-lymphocyte induced shift towards a α6-dim population (light blue histogram; Figure 6.12 c). TGFβ_1 (10ng/ml) and LR3-IGF-I (10ng/ml) had a similar effect with the number of α6-bright T-lymphocytes found to be similar to control HaCaTs. Only a small number of cells appeared to become α6-dim expressing cells after T-lymphocyte co-culture (orange histogram; Figure 6.12 d). Figure 6.13 confirms these observations and shows that the percentage of HaCaTs found to be α6-dim expressors following T-lymphocyte co-culture was significantly decreased by a combination of TGFβ_1 and IGF-I as well as a combination of TGFβ_1 and LR3-IGF ($p<0.05$; Figure 6.13).

In Chapter 5 (Section 5.3.10), T-lymphocyte induced HaCaT apoptosis was correlated with a loss of α6 staining by increasing the number of α6-dim HaCaTs. This indicated a direct association between cells induced to undergo differentiation and their susceptibility to apoptosis. To determine if the ability of TGFβ_1 and IGF-I to decrease apoptosis was associated with them preventing loss of α6 expression, staining of HaCaTs with Annexin V and α6 was performed after co-culture. Results in Figure 6.14 show that compared to control HaCaTs (Figure 6.14 a), T-lymphocytes increased the number of α6-dim expressing HaCaTs, as well as the number of Annexin V positive HaCaTs (Figure 6.14 b). A combination of TGFβ_1 and IGF-I however, prevented the shift of HaCaTs towards α6-dim and Annexin V positive staining (Figure 6.14 c).

Figure 6.13

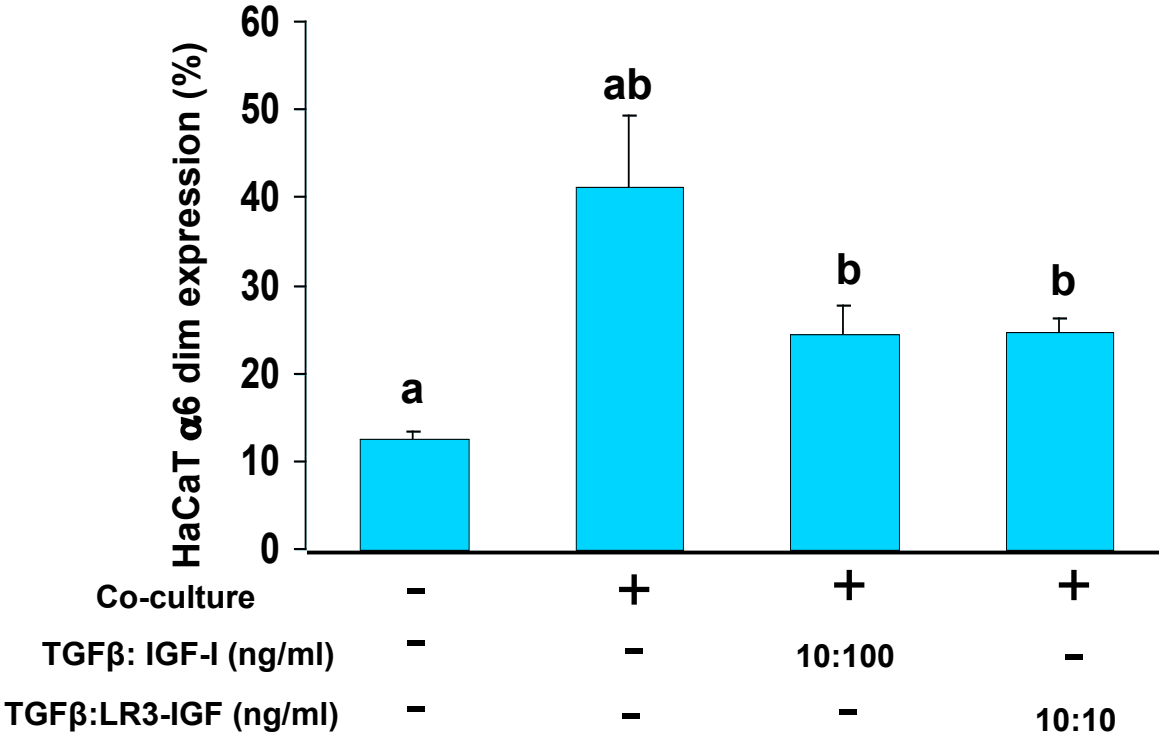

Figure 6.13. Effect of a combination of TGFβ and IGF-I or TGFβ and LR3-IGF on T-lymphocyte induced HaCaT α6 dim expression. Percentage α6 dim HaCaTs in controls, HaCaTs after T-lymphocyte co-culture and co-cultures treated with TGFβ and IGF-I (10:100ng/ml) or TGFβ and LR3-IGF (10:10ng/ml). Bar graphs represent the mean ± SEM percentage from cells in 3 separate 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 ($^{a, b}$).

Figure 6.14

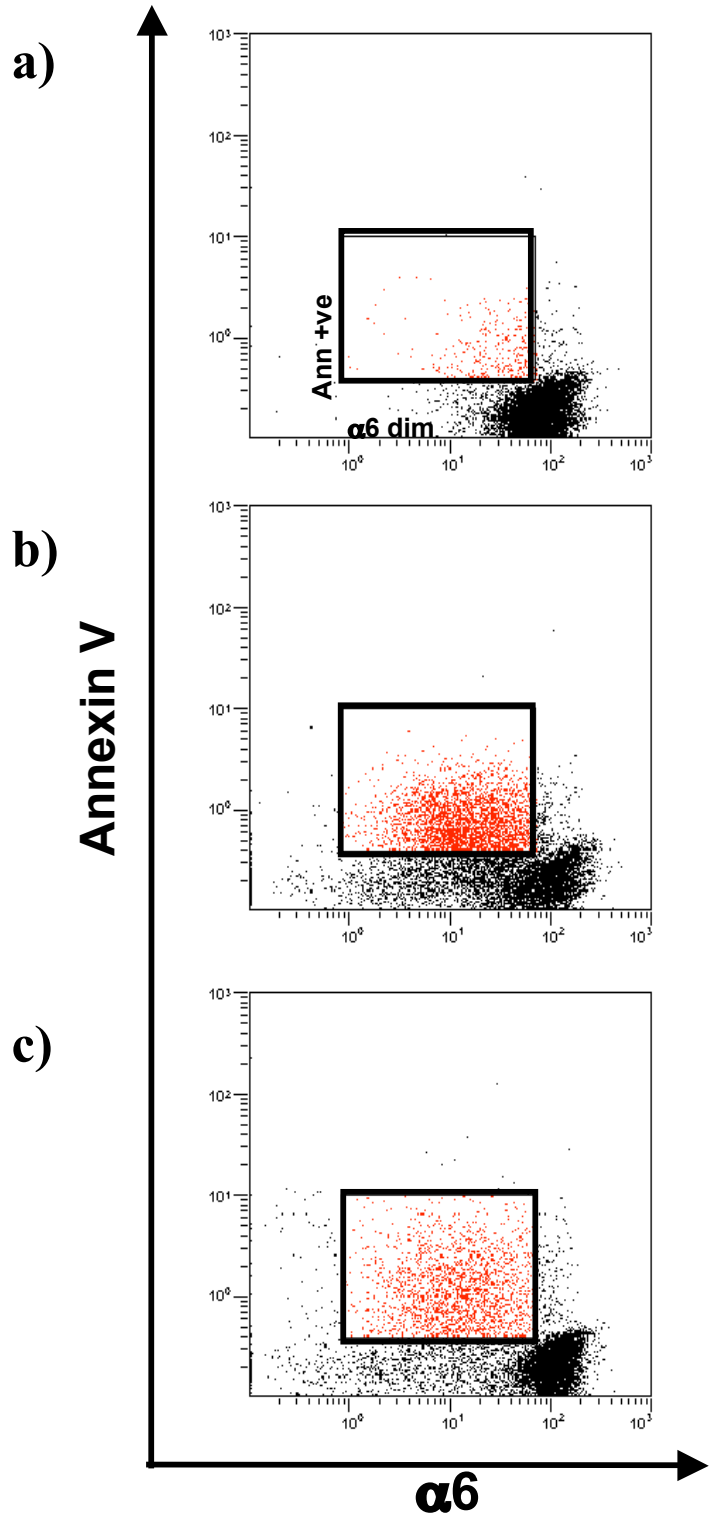

Figure 6.14. Effect of a combination of TGFβ and IGF-I on T-lymphocyte induced HaCaT apoptosis and differentiation. HaCaTs co-staining with Annexin V and α6 were analysed by flow cytometry. (a) HaCaTs controls, (b) HaCaTs after T-lymphocyte co-culture and (c) HaCaTs after T-lymphocyte co-culture treated with TGFβ and IGF-I (10:100ng/ml). Gated region indicated the population of HaCaTs staining positive for α6 (dim) and Annexin V. Dot-plots are a representation of 2 separate experiments.

6.3.7 IGF-I prevents T-lymphocyte induced apoptosis of NHEKs

The effect of TGFβ_1, IGF-I and LR3-IGF on primary T-lymphocyte and NHEK co-culture was examined. Apoptosis of NHEK co-cultures treated with TGFβ_1 (10ng/ml) or IGF-I (100ng/ml) was quantified by Annexin V and PI staining. Figure 6.15 a demonstrates that TGFβ_1 had no effect on T-lymphocyte induced NHEK apoptosis, however, this was significantly decreased by IGF-I (100ng/ml) and LR3-IGF (10ng/ml) ($p<0.001$; Figure 6.15 a).

The HaCaT data presented in Sections 6.3.2, indicated a combination of TGFβ_1 and IGF-I was required to significantly decrease T-lymphocyte induced apoptosis. Experiments were performed to determine the effect of combinations of TGFβ_1 and IGF-I as well as TGFβ_1 and LR3-IGF at a range of concentrations on T-lymphocyte induced NHEK apoptosis. Figure 6.15 b demonstrates that only NHEKs treated with he lowest concentration of TGFβ_1 and IGF-I (10ng/ml) showed a significant decreased in T-lymphocyte induced apoptosis ($P<0.05$). No decrease in T-lymphocyte induced NHEK apoptosis was observed when NHEKs were treated with TGFβ_1 and LR3-IGF (Figure 6.15 b).

Given these results, the effect of IGF-I and LR3-IGF on apoptosis associated morphology of NHEK was investigated. Figure 6.16 demonstrates that IGF-I (100ng/ml) and LR3-IGF (10ng/ml) prevented the membrane blebbing and granular appearance of

Figure 6.15

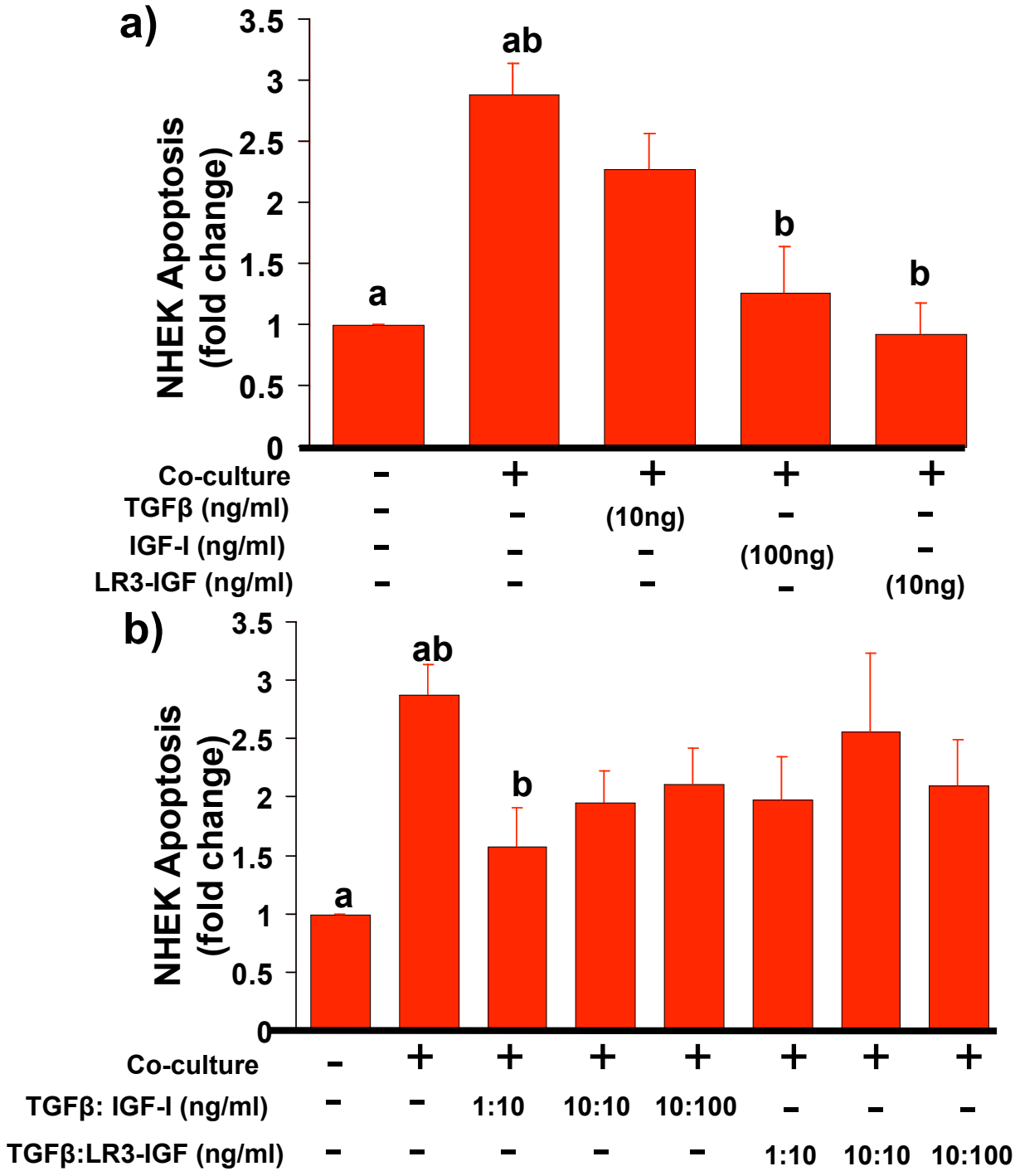

Figure 6.15 Effect of TGFβ, IGF-I and LR3-IGF on T-lymphocyte induced NHEK apoptosis. Apoptosis was quantified by Annexin V and propidium iodide (PI) staining. (a) NHEKs co-cultured with T-lymphocytes plus TGFβ (10ng/ml), IGF-I (100ng/ml) or LR3-IGF (10ng/ml). (b) NHEKs co-cultured with T-lymphocytes plus increasing concentrations of either TGFβ and IGF-I or TGFβ and LR3-IGF for 48hr (concentration ratio ng/ml). Bar graphs represent the mean ± SEM percentage from cells in 3 separate 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 ($^{a, b}$).

Figure 6.16

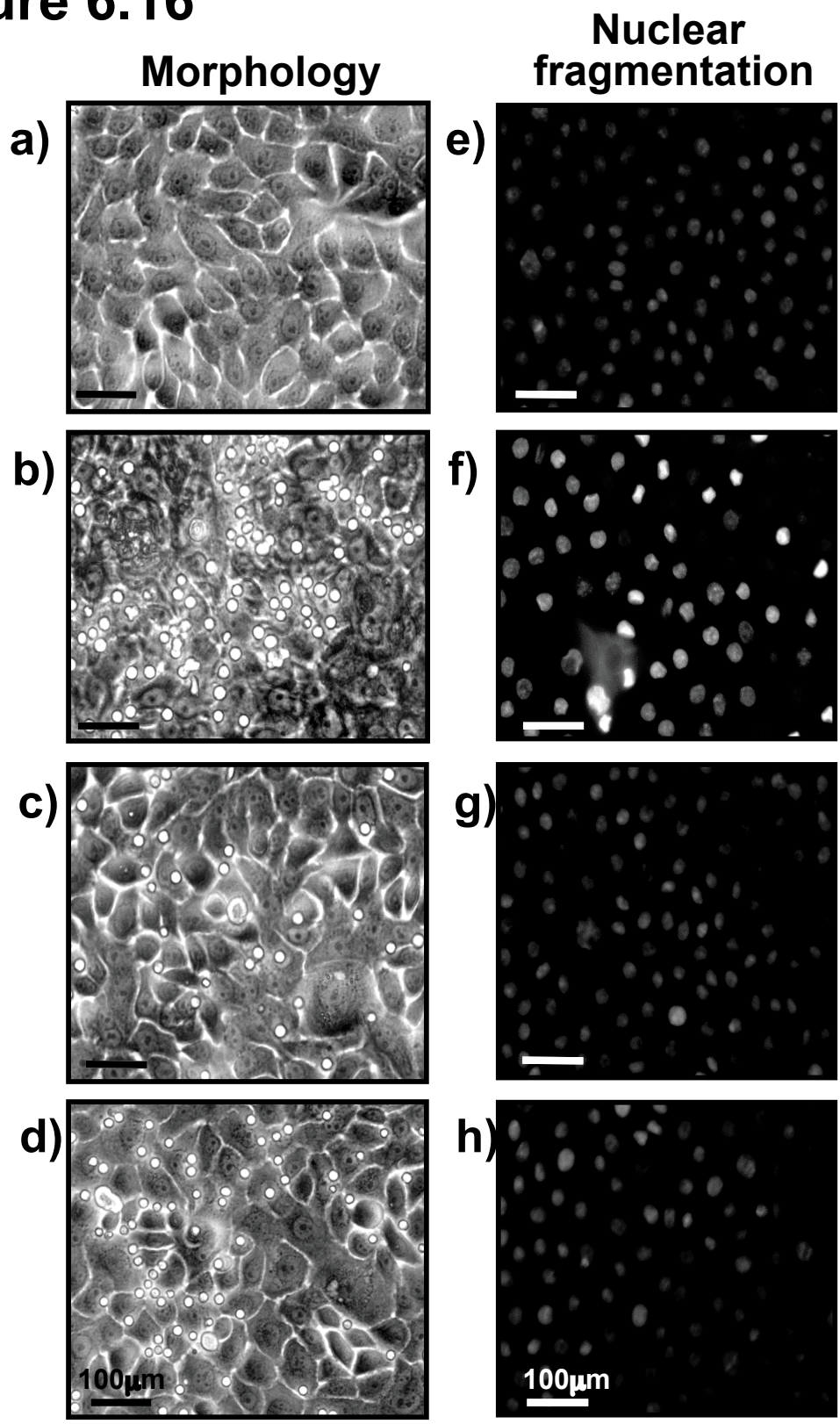

Figure 6.16 Effect of IGF-I and LR3-IGF on T-lymphocyte induced NHEK apoptosis. (a) Phase contrast microscopy of NHEK controls, (b) NHEK and T-lymphocytes co-culture, and NHEKs co-cultured with (c) IGF-I (100ng/ml) and (d) LR3-IGF (10ng/ml) for 48hrs. (e) HOESCHT staining of NHEK controls, (f) NHEKs co-cultured with T-lymphocytes, and (g) NHEKs co-cultures + IGF-I (100ng/ml) and (h) NHEKs co-cultures + LR3-IGF (10ng/ml) for 48hrs.

NHEKs induce by T-lymphocyte co-culture (Figure 6.16 b, c and d). IGF-I and LR3-IGF also resulted in a decreased number of HOESCHT 33342 positive cells (Figure 6.16 g and Figure 6.16 h respectively) compared to control co-cultures (Figure 6.16 f).

6.3.8 Effect of IGF-I on NHEK Fas expression and early differentiation

As T-lymphocyte induced NHEK apoptosis was shown to be mediated by Fas (Chapter 5 Section 5.3.3), the effect of IGF-I and LR3-IGF on T-lymphocyte induced Fas expression was investigated. Results in Figure 6.17 show that expression was not altered by IGF-I and LR3-IGF (pink and blue histogram; Figure 6.17 b and c respectively). This was consistent with the results obtained from HaCaT experiments (Section 6.3.5).

In Section 6.3.6 it was demonstrated that the ability of the growth factors to decrease T-lymphocyte induced HaCaT apoptosis was correlated with an apparent ability to prevent early keratinocyte differentiation. The effect of IGF-I (100ng/ml) and LR3-IGF (10ng/ml) on T-lymphocyte induced differentiation of NHEKs was assessed by measuring changes in α6 expression. Compared to NHEKs co-cultured with T-lymphocytes alone, which demonstrate a marked shift from α6-bright to α6-dim expression (green histogram; Figure 6.18 a), IGF-I and LR3-IGF maintained α6 expression (pink and orange histogram; Figure 6.18 b and c respectively). Keratinocytes showed that same level of α6 expression as untreated NHEK controls (Figure 6.18 a (MFI: 30)).

Figure 6.17

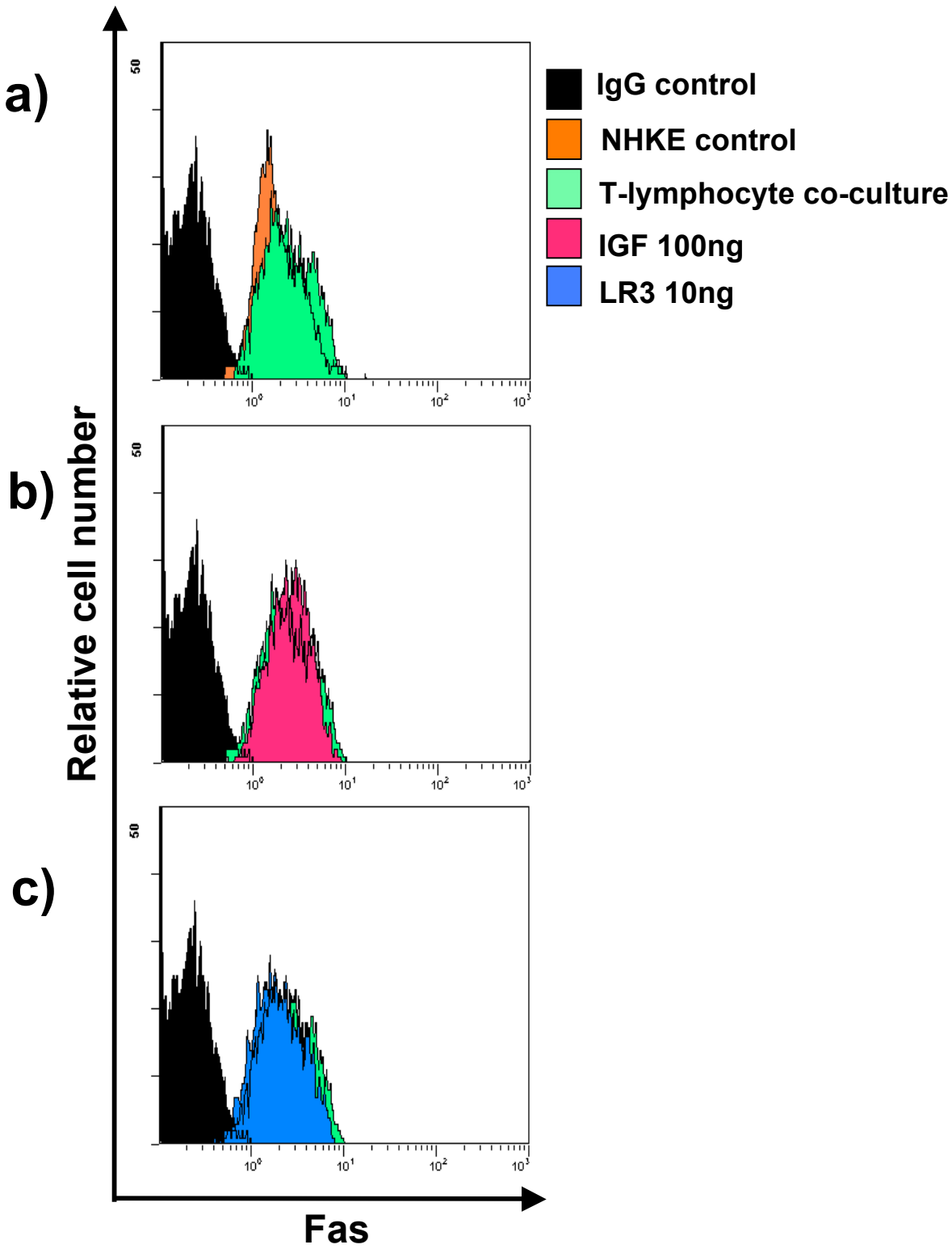

Figure 6.17. Effect of IGF-I and LR3-IGF on T-lymphocyte induced NHEK Fas. Fas expression of NHEKs was analysed by flow cytometry. (a) Orange histogram demonstrates NHEK control Fas staining and green histogram demonstrates T-lymphocyte induced Fas staining. (b) Fas expression of NHEK co-cultures treated with IGF-I (100ng/ml) pink histogram and (c) NHEK co-cultures treated with LR3-IGF (10ng/ml) blue histogram. Black histogram represent staining with an isotype-matched control Ab.

Figure 6.18

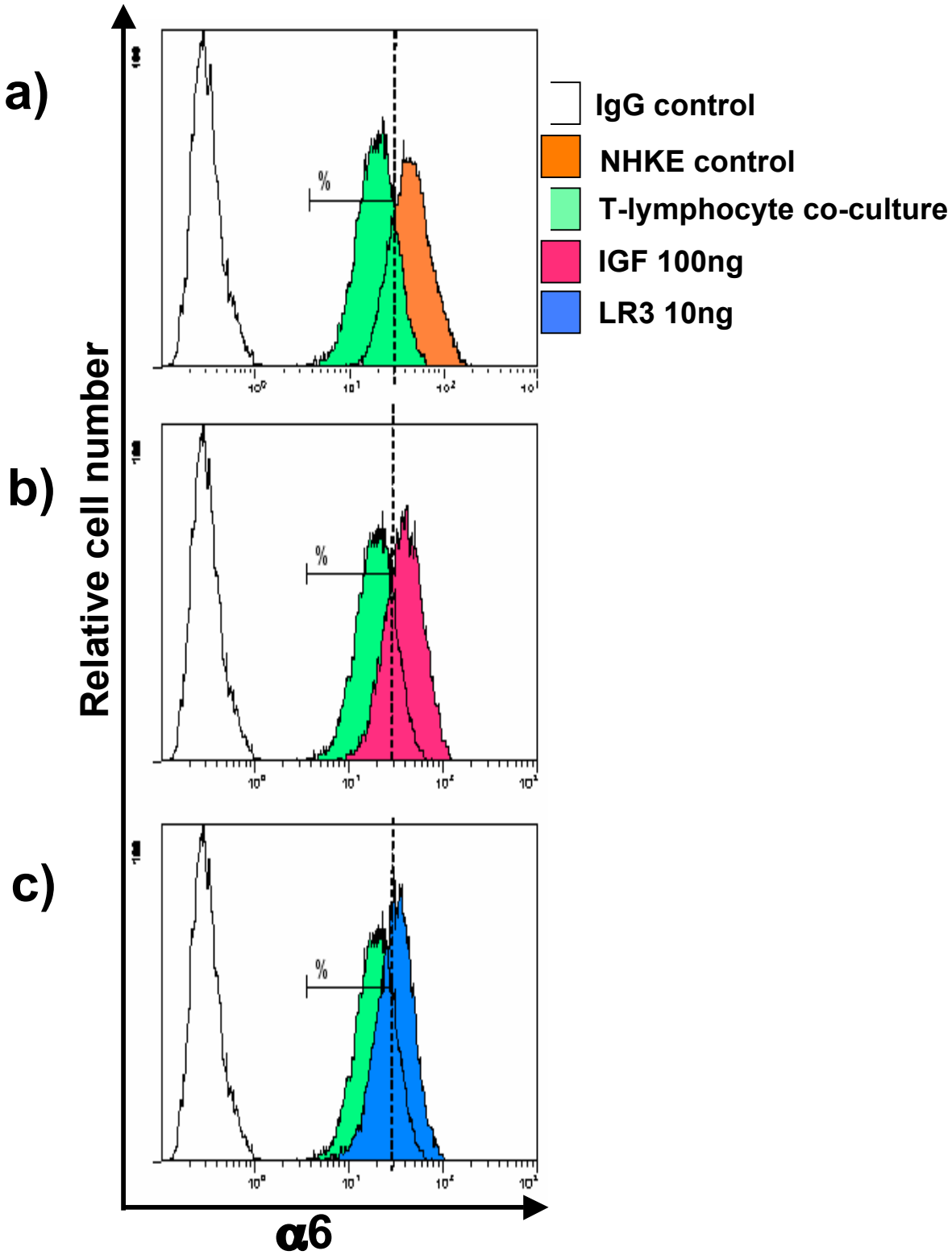

Figure 6.18. Effect of IGF-I and LR3-IGF on T-lymphocyte induced NHEK α6 expression. Expression of α6 integrin was assessed by flow cytometry on (a) NHEK controls (orange histogram) and T-lymphocyte co-cultures (green histogram). (b) T-lymphocyte co-cultures treated with IGF-I (100ng/ml; pink histogram) and (c) T-lymphocyte co-cultures treated with LR3-IGF (10ng/ml; blue histogram). Unfilled histograms represent staining with an isotype-matched control Ab.

6.4 Summary

In this chapter it was shown that a combination of TGFβ_1 and IGF-I was able to protect HaCaTs from T-lymphocyte induced apoptosis. The effect of TGFβ_1 and IGF-I appeared to be independent of changes in Fas expression, even though these growth factors were able to prevent IFNγ induced Fas, and reduce IFNγ levels in co-culture conditioned media.

The results in Chapter 3 together with other reports, suggest that the initiation of apoptosis following death receptor activation is due to initiator caspases such as caspase 8 becoming activated and inducing morphological changes of the cell (Hirata *et al.* 1998, Zhang *et al.* 1999). The changes in cell morphology and ultimately apoptosis can be reversed by inhibiting the activation of these initiator caspases (Griffith *et al.* 2000, Maianski *et al.* 2003). The studies in this Chapter demonstrate that T-lymphocyte induced HaCaT apoptosis was only be prevented when cells were incubated with TGFβ_1 and IGF-I at the time of initiation of the co-culture. Once apoptosis has been induced by the T-lymphocytes, HaCaTs could not be rescued by the subsequent addition of TGFβ_1 and IGF-I.

Basal keratinocytes are present in the proliferative layer of the skin and express high levels of anti-apoptotic survival factors such as Bcl-2 and Bcl-xl, which allows them to be resistant to apoptotic stimuli such as UV radiation (Taylor *et al.* 1999). It has been proposed also that basal keratinocytes may be protected from UV radiation-induced

apoptosis by integrin-mediated survival signals (Norris *et al.* 1997), however these mechanisms remain ill defined. As T-lymphocytes induced apoptosis of keratinocytes by inducing differentiation and consequently increasing their susceptibility to FasL mediated apoptosis via Fas (Chapter 5 Section 5.3.11), the capacity of TGFβ_1 and IGF-I to maintain the undifferentiated basal status of HaCaTs was examined.

T-lymphocyte induced differentiation of HaCaTs was prevented by combination of TGFβ_1 and IGF-I as α6 expression was not diminished in growth factor treated co-cultures. Unlike untreated co-cultures where there was a shift to a population of low expressing α6 keratinocytes (α6-dim) associated with apoptosis, HaCaTs retaining their high levels of α6 integrin did not become apoptotic. As such, this data indicates that the growth factors protected the keratinocytes from T-lymphocyte induced apoptosis by preventing the initiation of terminal differentiation. A different response was observed in primary cell co-cultures using NHEKs, where TGFβ alone or in combination with IGF-I did not affect T-lymphocyte induced apoptosis. IGF-I alone however prevented NHEK apoptosis and inhibited T-lymphocyte associated differentiation, consistent with the HaCaT results.

The ability of IGF-I to protect both HaCaTs and NHEKs was confirmed in studies using the IGF-I analogue; LR3-IGF. LR3-IGF demonstrated the same response as IGF-I but at a 10-fold lower concentration. This is consistent with the known ability of LR3 to activate IGF-IR more efficiently than IGF-I, due to it having less affinity for IGF-BP's, (Francis *et al.* 1992, Tomas *et al.* 1993). IGF-I mediated effects via IGF-IR in these

studies is supported by the finding that IGF-IR is highly expressed by HaCaT keratinocytes (Appendix 8).

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

- Combination of TGFβ_1 (10ng/ml) and IGF-I (100ng/ml) or IGF-I alone (100ng/ml), prevent T-lymphocyte induced keratinocyte apoptosis independently of any effects on Fas expression.
- The combination of TGFβ_1 and IGF-I or IGF-I alone, protect keratinocytes from T-lymphocyte induced apoptosis by preventing the induction of terminal differentiation.