

Insulin-like growth factor binding protein 3 (IGFBP-3) potentiates TRAIL-induced apoptosis of human colorectal carcinoma cells through inhibition of NF- κ B

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There is growing evidence that the insulin-like growth factor-binding protein 3 (IGFBP-3) can have IGF-independent effects on cell growth. However, despite the fact that IGFBP-3 has been reported to be both antiproliferative and proapoptotic, the molecular mechanisms underlying the action of IGFBP-3 have not been elucidated. We report that although addition of IGFBP-3 (either synthetic or secreted protein) had no effect on cell survival, IGFBP-3 (100 ng/ml) significantly enhanced TNF-related apoptosis-inducing ligand (TRAIL)-induced cell death in colonic carcinoma-derived cell lines (20–30% depending on cell line), whereas it had no effect on the survival of the TRAIL-resistant adenoma-derived cells. Both addition of IGFBP-3 protein to cell cultures or enforced expression of IGFBP-3 in the HT29 carcinoma cell line inhibited nuclear factor kappa B (NF- κ B) activation in response to the induction of apoptosis by TRAIL. We propose that IGFBP-3 is a non-toxic NF- κ B inhibitor, which could be used as an adjuvant in the treatment of colon cancer.

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Insulin-like growth factor binding protein-3 (IGFBP-3) is a member of a family of high-affinity binding proteins known to regulate the function of insulin-like growth factors (IGF-I and IGF-II) through modulating interactions with the signalling receptor IGF-I receptor (IGF-IR). However, there is growing evidence that IGFBP-3 can have direct IGF-independent effects on cell growth (reviewed in Baxter *et al.*¹). Supporting its role as a primary growth inhibitor, the anti-proliferative effects of retinoic acid, tumour necrosis factor α (TNF- α), wild-type p53, vitamin D, anti-estrogens, and transforming growth factor- β (TGF β) have all been shown to be mediated, in part, through IGFBP-3. Furthermore, there is now accumulating evidence to suggest that IGFBP-3 is involved in the induction of apoptosis (reviewed in Butt and Williams²). Interestingly, IGFBP-3 has been shown to increase ceramide-induced apoptosis in a breast cancer cell line,³ and to enhance both p53-dependent^{4,5} and p53-independent apoptosis.⁶ In these studies, addition of IGFBP-3 alone had no effect on cell growth or cell survival. These data suggest that rather than having a direct role in initiating apoptosis, IGFBP-3 may interact with mediators of apoptosis leading to enhanced levels of cell death.

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), is a member of the TNF family, capable of inducing apoptosis in a wide variety of cancer cells including colon,

breast, and lung.⁷ The importance of TRAIL as a potential therapeutic is demonstrated by the fact that it has been shown to selectively kill tumour cells, and not the majority of normal cells (reviewed in Ashkenazi⁸ and Leblanc and Ashkenazi⁹). TRAIL has been shown to reduce solid tumour growth and induce tumour regression in mouse xenografts.^{10–12} Unlike TNF- α and Fas ligand, TRAIL does not induce apoptosis in non-tumorigenic cells.^{13–15}

TRAIL can induce apoptosis by interaction with two of four membrane-bound receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5, KILLER) that contain a protein–protein interaction motif, termed the ‘death domain’ (reviewed in Ashkenazi and Dixit¹⁶). The other two receptors TRAIL-R3 and -R4, which either lack or contain an incomplete death domain, have been shown to attenuate TRAIL-induced apoptosis and are therefore often referred to as ‘decoy’ receptors.¹⁶ TRAIL signals death through formation of a death-inducing signal complex (DISC), leading to rapid activation of caspase 8. Caspase 8 mediates apoptosis either through direct activation of downstream effector caspases, or through cleavage of proapoptotic molecules such as the Bcl-2 homolog, Bid (reviewed in MacFarlane¹⁷).

TRAIL resistance was initially attributed to the presence of the decoy receptors, but more recently it has been suggested that resistance is regulated intracellularly. Several intra-

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Abbreviations: IGFBP-3, insulin-like growth factor-binding protein-3; NF- κ B, nuclear factor kappa B; I κ B, inhibitor of κ B; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF- α , tumour necrosis factor-alpha; TGF β , transforming growth factor-beta; c-FLIP, cellular Flice/caspase-8 inhibitory protein; c-IAPs, cellular inhibitors of apoptosis; TRAF, TNF receptor-associated factor; HEK293, human embryonic kidney cells; IGF-1R, insulin-like growth factor 1 receptor; XIAP, X chromosome-linked inhibitors of apoptosis; I κ B α -SR, HA-tagged mutant super repressor I κ B α gene; PARP, poly(ADP-ribose) polymerase; CPT11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin

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cellular proteins have been implicated including the (IAP) inactive caspase 8 homolog, c-FLIP, inhibitor of apoptosis proteins, and the Bcl-2 family members Bcl-2 and Bcl-X_L.¹⁷ A pro-survival signalling pathway implicated in resistance to members of the TNF family is activation of the transcription factor, nuclear factor kappa B (NF- κ B). In many cell types, TNF regulates its own cytotoxicity through upregulation of NF- κ B, leading to the transcriptional activation of anti-apoptotic genes such as XIAP, c-IAP1, and c-IAP2.^{18,19} TRAIL has also been shown to activate NF- κ B, mediated by the death receptors TRAIL-R1 and -R2.^{20–22} Furthermore, it has been reported that TRAIL sensitivity can be modulated by activation or inhibition of NF- κ B.²²

The term NF- κ B refers to a family of dimeric transcription factors binding a common DNA sequence motif known as the κ B site. In mammals, there are five NF- κ B family members, p50, p52, p65(RelA), RelB and c-Rel, which form various homo- and heterodimers. In most resting cell types NF- κ B is sequestered in the cytoplasm via binding to inhibitory proteins of the I κ B family. Upon exposure to stimuli such as TNF- α , I κ B proteins are phosphorylated and degraded allowing NF- κ B proteins to enter the nucleus and bind to κ B elements of target gene promoters. NF- κ B is considered to have a critical role in the regulation of apoptosis owing to its ability to activate the expression of many anti-apoptotic genes, for example, TRAF and IAP proteins, c-FLIP and Bcl-X_L (reviewed in Karlin *et al.*²³).

Previously, we have shown that IGFBP-3 is differentially expressed along the normal colonic crypt,⁴ and postulated that IGFBP-3 may be important in the regulation of colonic cell survival. However, despite the fact that IGFBP-3 has been reported to be both anti-proliferative and proapoptotic, the molecular mechanisms underlying the action of IGFBP-3 have not been elucidated. In the current report, we show that IGFBP-3 selectively enhances TRAIL-induced cytotoxicity in the sensitive colorectal carcinoma-derived cells, but has no effect on the survival of adenoma-derived cells that are resistant to TRAIL-induced cell death. Taken together with previous data,^{4,6} this finding led us to hypothesize that IGFBP-3 may be pro-apoptotic through regulation of survival pathways activated in response to the induction of apoptosis, potentially explaining why IGFBP-3 does not cause apoptosis when added directly to cell cultures. In this report, we show for the first time that IGFBP-3 can inhibit NF- κ B activation in response to the induction of apoptosis by TRAIL. Inhibition of NF- κ B activation has previously been reported to be important in increasing efficacy of both chemotherapy and radiotherapy.²⁴ Therefore, we propose that, through inhibition of the NF- κ B pro-survival pathway, IGFBP-3 may be a potent adjuvant to a number of cancer treatments.

Results

Tumorigenic colorectal cell lines are sensitive to TRAIL-induced apoptosis whereas adenoma-derived cell lines are not. A differential sensitivity to TRAIL between colorectal adenoma and carcinoma-derived cell lines has been

observed²⁵ where carcinoma-derived cell lines are sensitive to the induction of apoptosis, whereas the adenoma-derived cell lines are not. However, as experiments studying the effects of IGFBP-3 are carried out under serum-free conditions, we initially needed to verify that the differential sensitivity to TRAIL-induced apoptosis was retained under serum-free culture conditions. Therefore both adenoma and carcinoma-derived cells were treated with TRAIL (0.05–0.25 μ g/ml) for 24 h in serum-free medium (Figure 1A). In the three tumorigenic cell lines investigated, there was a significant decrease in attached cell yield and associated increase in floating cells (confirmed as apoptotic by nuclear morphology and poly(ADP-ribose) polymerase (PARP) cleavage, Figure 1B and C). In contrast, the three adenoma-derived cell lines remained relatively resistant to TRAIL with only an approximate 3% increase in TRAIL-induced apoptosis in cells treated with the highest dose of 0.25 μ g/ml TRAIL (Figure 1A). This finding was in accordance with the findings of our previous study.²⁵

IGFBP-3 potentiates TRAIL-induced apoptosis in carcinoma but not in adenoma-derived cells. As the pro-apoptotic IGFBP-3 protein has previously been shown to increase the sensitivity of colorectal cell lines to the induction of apoptosis,^{4,6} we investigated whether IGFBP-3 affected the response of the cells to receptor-mediated TRAIL-induced apoptosis. The levels of IGFBP-3 secreted by the cell lines was found to be variable (<1 ng/10⁶ cells for S/RG/C2 and HT29, 4–40 ng/10⁶ cells for PC/AA/C1, S/AN/C1 and SW620, and approximately 70 ng/10⁶ cells for the AA/C1/SB10 cell line,⁴ and unpublished data). Cells were treated with the physiologically relevant dose of 100 ng/ml IGFBP-3 (used in previous studies^{4,6}), which equates to an approximate dose of 50 ng/10⁶ cells. For the purpose of this study, we investigated two alternative sources of exogenous protein to control for the processing (glycosylation and proteolytic cleavage) of secreted protein *in vivo*. The first was a synthetic form of the protein (Gropep, AUS) added at a concentration of 100 ng/ml (effective reported dose⁴). The second was collected from the conditioned medium of human embryonic kidney cells (HEK293) cells, which were transiently transfected to express IGFBP-3 protein. Secreted protein was harvested from the culture medium (referred to as IGFBP-3 conditioned medium, BP3-CM). The resultant concentrated conditioned medium contained glycosylated protein at approximately 100 ng/ml. These experiments were controlled by treatment of parallel cultures with concentrated medium from mock-transfected HEK293 cells (referred to as conditioned medium, CM). Results are shown in Figure 2. Cancer-derived cells were treated with a dose of TRAIL previously shown to induce moderate apoptosis, the adenoma-derived cells were treated with the maximum dose of TRAIL used in these experiments. As previously reported, addition of either source of IGFBP-3 to the serum-free medium had no effect on the survival of either adenoma or carcinoma-derived cell lines (Figure 2).^{3,4} However, in the carcinoma-derived cell lines, increasing the amount of IGFBP-3 in the medium (both synthetic IGFBP-3 and IGFBP-3 from conditioned-medium) significantly increased the cellular sensitivity to TRAIL-induced apoptosis (shown in Figure 2A and B).

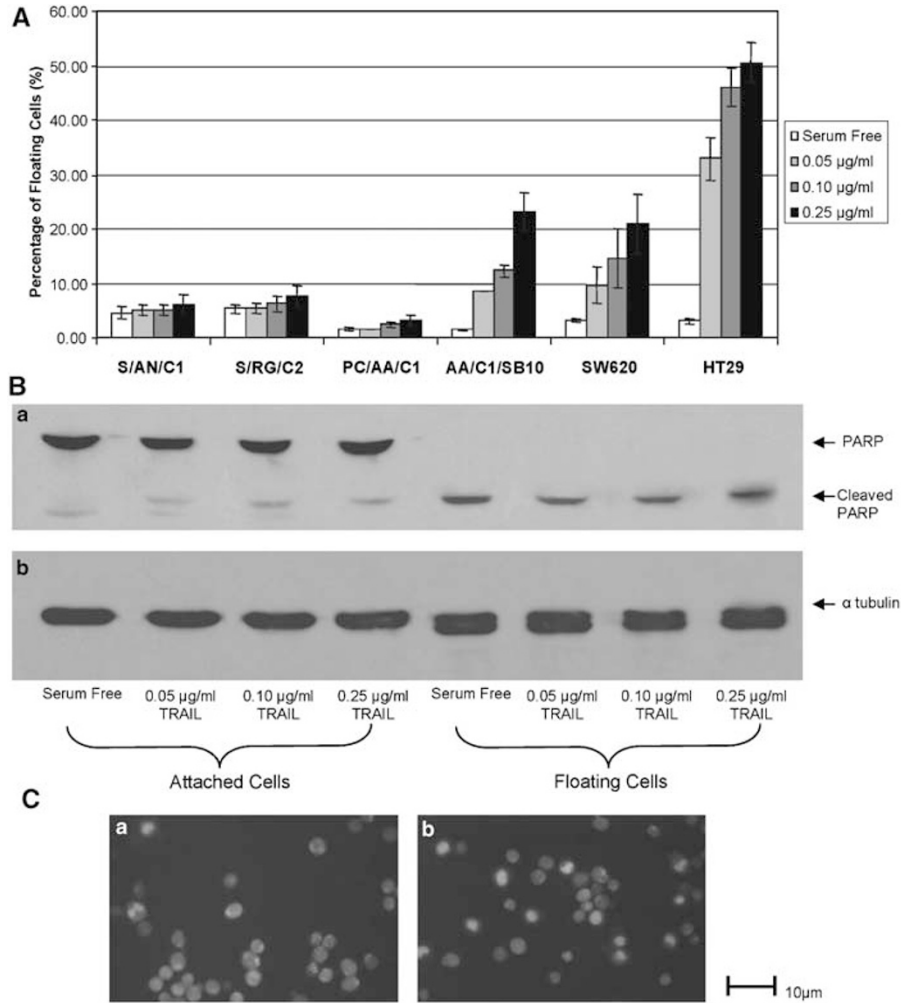


Figure 1 Tumorigenic colorectal cell lines are sensitive to TRAIL-induced apoptosis. (A) Apoptosis in adenoma and carcinoma-derived cell lines 24 h after treatment with different doses of TRAIL (0.05, 0.10 and 0.25 μ g/ml). The results shown are the mean of three separate experiments \pm S.E.M. S/AN/C1, S/RG/C2 and PC/AA/C1: adenoma-derived cell lines. AA/C1/SB10: tumorigenic cell line. SW620 and HT29: carcinoma-derived cell lines. (B) Western blot showing (a) PARP expression and (b) α -tubulin (loading control), in attached and floating HT29 cell populations 24 h after treatment with TRAIL (0.05, 0.10 and 0.25 μ g/ml). Cleavage of PARP indicates that the floating cell population is apoptotic. (C) DAPI staining of the combined attached and floating HT29 cells from (a) control untreated cultures and (b) cultures treated with 0.05 μ g/ml TRAIL for 24 h, showing an increase in the proportion of apoptotic cells in TRAIL-treated cells

(The higher level of apoptosis in the SW620 cell line when treated with BP3-CM as compared to synthetic IGFBP-3. (Figure 2B(b) as compared to Figure 2B(a)) is due to the sensitivity of the SW620 cells to the conditioned-medium.) Interestingly, the addition of IGFBP-3 to adenoma-derived cell lines had no effect on cell survival; the adenoma-derived cells remained relatively resistant to TRAIL-induced apoptosis, (Figure 2C; data not shown for two additional adenoma-derived cell lines, which were also resistant to TRAIL-induced apoptosis).

The IGF-1R blocking antibody IGF-IR α (5 μ g/ml, refer to Williams *et al.*⁴) failed to block IGFBP-3 potentiation of TRAIL-induced apoptosis, showing that the increase in cell death was not due to the ability of the IGFBP-3 protein to inhibit IGF-dependent signalling pathways (Figure 3). Furthermore, the potentiation by IGFBP-3 of TRAIL-induced apoptosis was completely blocked by the addition of neutralizing IGFBP-3

antibody to the medium confirming that IGFBP-3 increases TRAIL-induced apoptosis.

IGFBP-3 inhibits the activation of NF- κ B on induction of apoptosis by TRAIL. In this study, we have shown that IGFBP-3 potentiates TRAIL-induced apoptosis, but fails to increase the sensitivity of resistant adenoma-derived cells. Taken together with the previous findings that IGFBP-3 potentiates apoptosis induced by a number of different agents, this result suggests that rather than increasing TRAIL signalling, IGFBP-3 may act by suppressing survival signalling initiated in response to the induction of apoptosis. Death receptor mediated apoptotic signalling results in concurrent activation of the NF- κ B survival pathway.²² It is the balance between death and survival signalling that determines the resultant cellular response to death-inducing ligands. For example, inhibition of NF- κ B has been shown

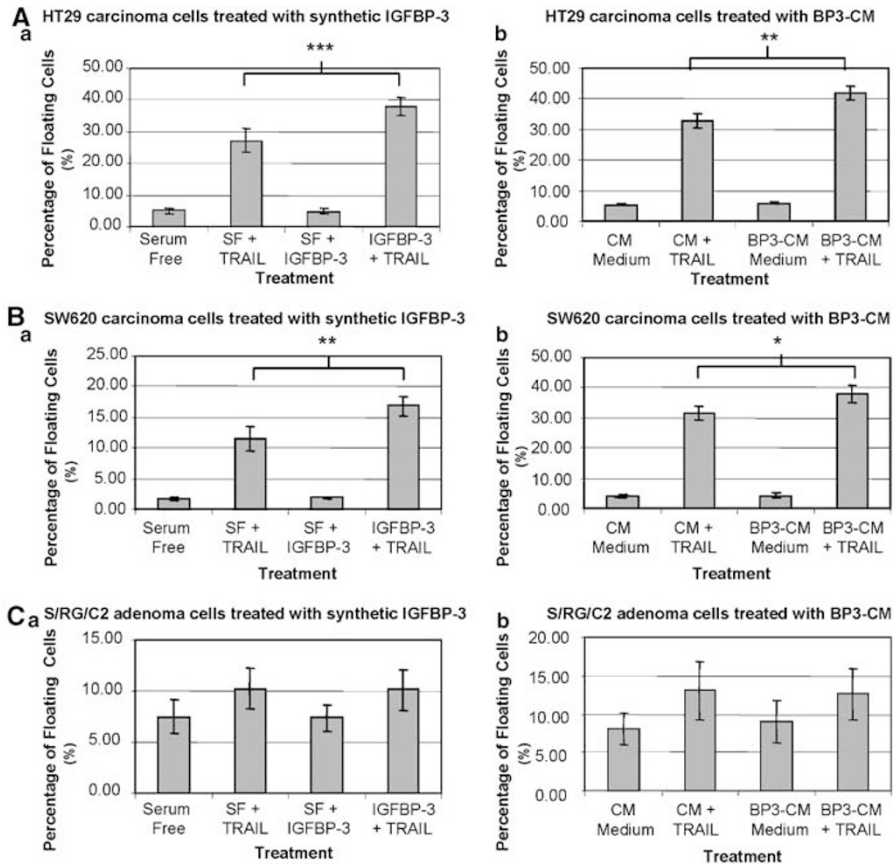


Figure 2 IGFBP-3 increases TRAIL-induced apoptosis in human carcinoma-derived cell lines. Apoptosis in carcinoma and adenoma-derived cell lines after 24 h treatment with TRAIL: (a) \pm 100 ng/ml synthetic IGFBP-3 protein (Gropep AUS), (b) \pm conditioned medium from mock transfected or IGFBP-3 protein expressing (approx. 100 ng/ml) HEK293 cell line. Cells were treated with different doses of TRAIL according to their sensitivity to TRAIL-induced apoptosis: (A) HT29 treated with 0.05 μ g/ml TRAIL, (B) SW620 treated with 0.10 μ g/ml TRAIL, (C) S/RG/C2 treated with 0.25 μ g/ml TRAIL. The results shown are the mean of three separate experiments \pm S.E.M. Statistical difference determined by two-way ANOVA with replication, * P < 0.05, ** P < 0.01, *** P < 0.001

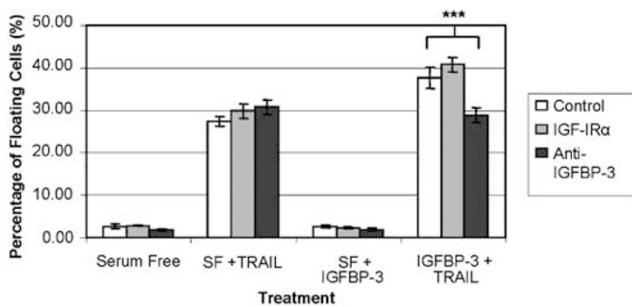


Figure 3 IGFBP-3 increases TRAIL-induced apoptosis in the presence of an IGF-1 receptor blocking antibody. Induction of apoptosis in HT29 cells treated with or without 0.05 μ g/ml TRAIL for 24 h in the presence of the anti-IGF-1 receptor antibody IGF-IR α (5 μ g/ml, Santa Cruz, USA) or the IGFBP-3 neutralising antibody (5 μ g/ml). Experiments were performed 3 times; results shown represent the mean \pm S.E.M. Statistical difference determined by two-way ANOVA with replication, * P < 0.05, ** P < 0.01, *** P < 0.001

to enhance TNF α -induced cytotoxicity in a number of different cell types including intestinal epithelial cells.^{26–28} Interestingly, IGFBP-3 has also been shown to enhance TNF α -induced cytotoxicity in breast cancer cells.²⁹ Therefore

to determine whether IGFBP-3 potentially enhances TRAIL-induced apoptosis through inhibiting the opposing actions of the NF- κ B pathway, we used a reporter assay to determine NF- κ B activation in the presence of the IGFBP-3 synthetic protein. Results are summarised in Figure 4. In the two carcinoma-derived cell lines HT29 and SW620, treatment with TRAIL resulted in increased NF- κ B activity. Furthermore, treatment with TRAIL in the presence of IGFBP-3 resulted in a significant decrease in NF- κ B activity when compared to TRAIL-treated cells in the absence of IGFBP-3 (detectable also at 6 h after treatment, data not shown). Again induction of NF- κ B and inhibition by IGFBP-3 correlated with the sensitivity of the two carcinoma-derived cell lines to TRAIL-induced cytotoxicity. In the adenoma-derived S/RG/C2, which is resistant to TRAIL-induced cell death, there was no activation of NF- κ B transcriptional activity, and no change in reporter activity in the presence or absence of IGFBP-3. Similar results were obtained for cells treated with BP3-CM (data not shown). Furthermore, when IGFBP-3 was stably expressed in the HT29 carcinoma-derived cell line (Figure 5A), the induction of NF- κ B activity by TRAIL was significantly inhibited (Figure 5B). In addition, IGFBP-3 expression in the HT29 cell line inhibited the induction of

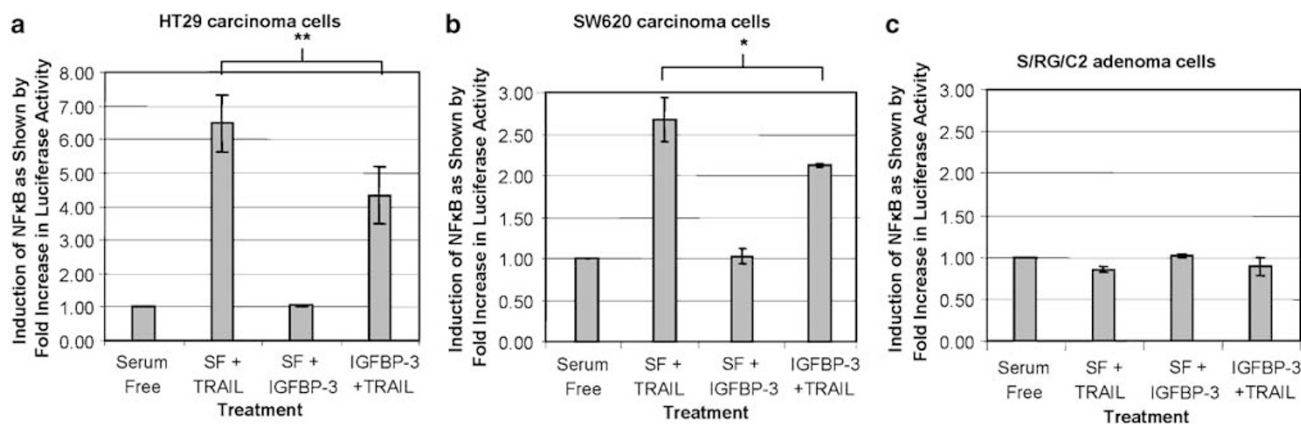


Figure 4 IGFBP-3 inhibits TRAIL-induced NF- κ B activation in the carcinoma-derived cells. The effect of 24 h treatment with TRAIL \pm 100 ng/ml IGFBP-3 protein (Gropep, AUS) on the luciferase reporter assay for NF- κ B activation. Results are expressed as pNF- κ B-TA-luc:firefly renilla ratios, expressed as a fold of control untreated cells (the range of readings for the NF- κ B-driven reporter in the control untreated cell lines was: HT29 19.0–40.0, SW620 116.0–145.0, RG/C2 4.0–19.0, the TA-luc (control) < 1 for all cell lines). Data represent the mean results from three independent experiments \pm S.E.M. Statistical difference was determined by two-way ANOVA with replication, * P < 0.05, ** P < 0.01, *** P < 0.001. (a) HT29 treated with 0.05 μ g/ml TRAIL, (b) SW620 treated with 0.10 μ g/ml TRAIL, (c) S/RG/C2 treated with 0.25 μ g/ml TRAIL

the NF- κ B target gene product X chromosome-linked inhibitor of apoptosis (XIAP) by TRAIL (Figure 5C), further supporting the role of IGFBP-3 as an inhibitor of NF- κ B activation.

Having shown that IGFBP-3 inhibits TRAIL-induced transcriptional activity using a reporter assay and target gene product, NF- κ B activity was also measured by using an enzyme-linked immunosorbent assay (ELISA)-based assay (Trans-Am NF- κ B p50 and p65 transcription factor assay kit). This assay detects the binding of p50 or the transcriptionally active p65 NF- κ B subunit (*RelA*) to a NF- κ B consensus sequence. Results show a reduction in p65 NF- κ B binding (as detected by the antibody) after treatment with TRAIL in the presence of IGFBP-3 (Figure 5D). These data further suggest that IGFBP-3 prevents TRAIL-induced NF- κ B transcriptional activity.

The increase in TRAIL-induced apoptosis through expression of the NF- κ B inhibitor I κ B α is equivalent to that caused by IGFBP-3. To determine whether inhibition of NF- κ B activity by a known inhibitor of its activity led to an equivalent increase in TRAIL-induced apoptosis as shown for addition of IGFBP-3, the HT29 carcinoma-derived cell line was transiently transfected with the NF- κ B super repressor (HA tagged mutant super repressor I κ B α -SR), Figure 6a). I κ B α -SR inhibited both basal and TRAIL-induced NF- κ B activity (Figure 6b). Interestingly, although expression of the repressor potentiated TRAIL-induced apoptosis, the level of apoptosis in the I κ B α -SR-expressing cells was equivalent to that in the cells treated with TRAIL in the presence of IGFBP-3 recombinant protein, (Figure 6c). Furthermore, there was no additive effect; treating with IGFBP-3 did not further increase the level of TRAIL-induced apoptosis in the I κ B α -SR expressing cells. These data show that the inhibition of NF- κ B potentiates TRAIL-induced apoptosis, and that the amount of cell death was equivalent to that after treatment with TRAIL in the presence of IGFBP-3. In addition, there was no additive effect when I κ B α -SR-expressing cells were treated with TRAIL in the presence of IGFBP-3,

further suggesting that IGFBP-3 potentiates TRAIL-induced apoptosis through inhibition of the NF- κ B-mediated survival pathway.

Discussion

IGFBP-3 is a pro-apoptotic protein, previously shown to potentiate apoptotic cell death in a number of cell systems. In the current report, we have used a panel of human colorectal non-tumorigenic adenoma-derived cells as well as carcinoma cell lines to show that IGFBP-3 increases TRAIL-induced apoptosis in colorectal carcinoma cell lines, while not affecting the response of the TRAIL insensitive adenoma-derived cells. This result is important as it suggests that IGFBP-3 may be used to increase TRAIL-induced apoptosis in cancer cells while having no effect on the survival of non-tumorigenic cells.

Apoptosis is induced by both extrinsic and intrinsic pathways initiated by the activation of death receptors and stress-inducing stimuli (reviewed in Adams³⁰ and Daniel and Korsmeyer³¹). Results from this study contribute to the emerging literature showing that IGFBP-3 can enhance both extrinsic and intrinsic apoptotic pathways (reviewed in Butt and Williams,²). Furthermore, IGFBP-3 has been shown to be pro-apoptotic in a range of different cell types (e.g. breast, oesophageal, colonic), although addition of IGFBP-3 directly to cells does not induce apoptosis.^{3,5,6} These observations led us to hypothesize that rather than being an integral part of cell death signalling, IGFBP-3 may modify the activity of pathways that modulate the cellular response to apoptosis. This would represent a more general mechanism by which IGFBP-3 is able to potentiate many different death-inducing pathways.

NF- κ B is widely considered to be a survival factor. NF- κ B anti-apoptotic activity has been reported to reduce cellular sensitivity not only to the TNF family,^{18–20} but also to other apoptosis-inducing agents such as ionizing radiation³² and therapeutically used drugs including CPT-11,³³ cisplatin, and etoposide (reviewed by Barkett and Gilmore³⁴). In the current report, we show that IGFBP-3 inhibits NF- κ B activation in

response to TRAIL-induced apoptosis, and that sensitivity to TRAIL-induced cell death is increased by suppression of the NF- κ B survival pathway. This is in concordance with previous reports where TRAIL sensitivity is modulated by inhibition of NF- κ B.²² Furthermore, the fact that IGFBP-3 suppresses the activation of the NF- κ B anti-apoptotic transcription factor would explain why IGFBP-3 is able to potentiate cell death induced by many different death-inducing agents.

However, it is of interest to note that IGFBP-3 has also been reported to potentiate sodium butyrate (NaBt)-induced

apoptosis,⁶ but that unlike TRAIL, NaBt is thought to induce apoptosis at least in part through inhibition of constitutive NF- κ B activity in cancer cells.^{35,36} Results presented here suggest that IGFBP-3 may potentiate NaBt-induced cell death through acting synergistically with NaBt, thereby increasing NF- κ B inhibition.

There is evidence that NF- κ B activity is deregulated in cancer, generally manifested as aberrant constitutive activity (reviewed in Rayet and Gelinás³⁷), and that NF- κ B inhibitors are currently undergoing clinical evaluation for use in treatment of cancer patients. Data from this study and others would suggest that addition of exogenous IGFBP-3 protein to colorectal carcinoma cells is unlikely to inhibit constitutive NF- κ B activity to a level that is sufficient to induce apoptosis, as addition of IGFBP-3 alone had no effect on cell survival.³⁻⁶ However, in combination with reagents whose cytotoxicity is diminished through co-activation of the NF- κ B survival pathway, addition of exogenous IGFBP-3 may be an effective way to increase the efficacy of such therapies.

The importance of TRAIL as a potential therapeutic agent is demonstrated by the fact that it has been shown to selectively kill tumour cells, and not the majority of normal cells (reviewed in Ashkenazi⁸). The data presented here show that, through inhibition of NF- κ B, IGFBP-3 can further increase TRAIL-induced apoptosis in carcinoma-derived cells while having no effect on the survival of non-tumorigenic cells. This suggests that in combination, IGFBP-3 may increase the therapeutic potential of TRAIL in the treatment of cancer. Interestingly, inhibition of NF- κ B has also been reported as a target for chemoprevention, where NF- κ B activation provides a critical survival function for pre-neoplastic lesions.²⁴ Furthermore, one mechanism of induced resistance in metastatic colon cancer to a number of chemotherapeutic reagents has been reported to be through activation of NF- κ B.²⁴ Hence, in summary, through inhibiting NF- κ B activation, IGFBP-3 increases the sensitivity of cancer cells to TRAIL-induced apoptosis and may also be important both in the prevention of colorectal cancers and as a potential adjuvant for a number of other therapeutic reagents currently used in the treatment of metastatic colon cancer.

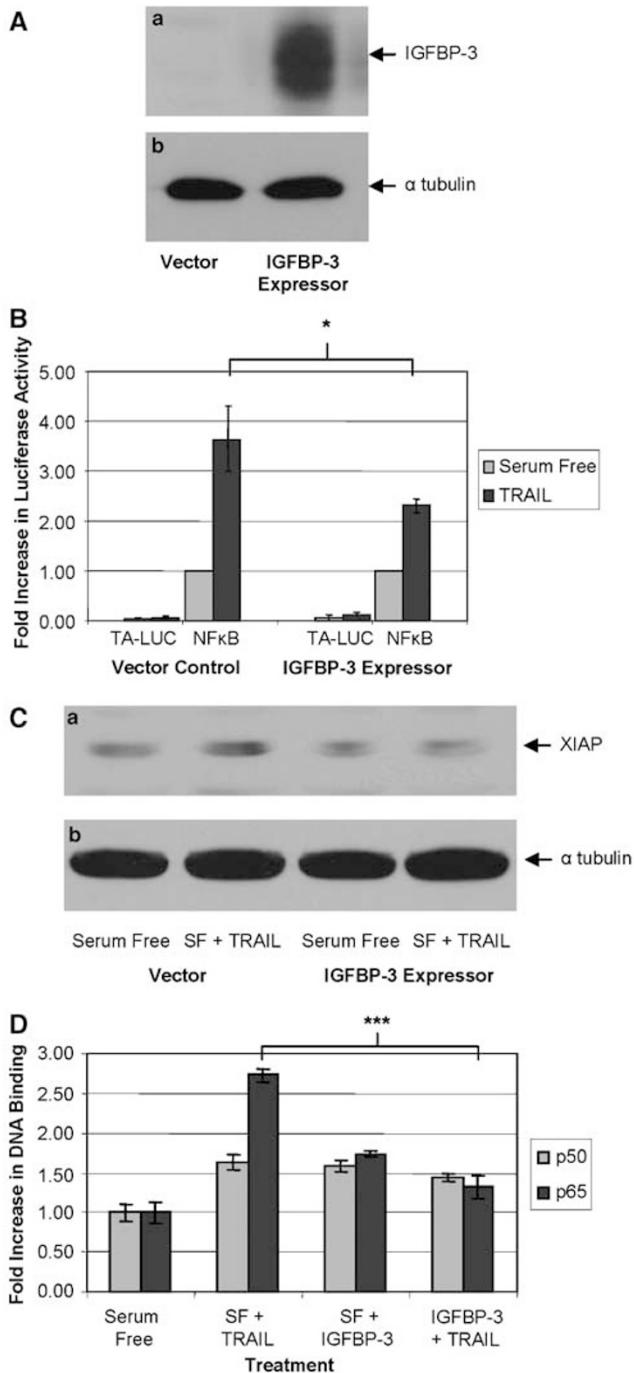


Figure 5 Expression of stable transfected IGFBP-3 in the HT29 carcinoma-derived cell line inhibits the induction of NF- κ B activity by TRAIL. **(A)** Western blot showing IGFBP-3 protein expression (a) from concentrated conditioned serum-free medium collected from 5×10^6 vector control or IGFBP-3 expressing HT29 cells. Western blot showing α -tubulin (b) used as a loading control for attached cell number from which conditioned medium was harvested. **(B)** The effect of 0.05 μ g/ml TRAIL on NF- κ B activation in HT29 cells in which IGFBP-3 has been stably expressed, pNF- κ B-TA-luc:firefly renilla ratios expressed as a fold of untreated vector only cells (the range of readings for the NF- κ B-driven reporter in the control untreated HT29 cells was 19.0–40.0, the TA-luc (control) < 1). Results represent the mean results from three independent experiments \pm S.E.M. Statistical difference determined by two-way ANOVA with replication, * P < 0.05, ** P < 0.01, *** P < 0.001. **(C)** Western blot showing the effect of 0.05 μ g/ml TRAIL on (a) XIAP expression and (b) α -tubulin (loading control), in HT29 cells transfected with: lanes 1 and 2, vector alone, and lanes 3 and 4 IGFBP-3. **(D)** DNA-binding activity of p65 NF- κ B protein associated with TRAIL-induced apoptosis is inhibited by IGFBP-3 in HT29 carcinoma-derived cells treated with 0.05 μ g/ml TRAIL \pm 100 ng/ml IGFBP-3 protein (Groppe, AUS) for 24 h. The experiment was performed in triplicate; results shown represent the mean \pm S.E.M. statistical difference determined by two-way ANOVA with replication, * P < 0.05, ** P < 0.01, *** P < 0.001

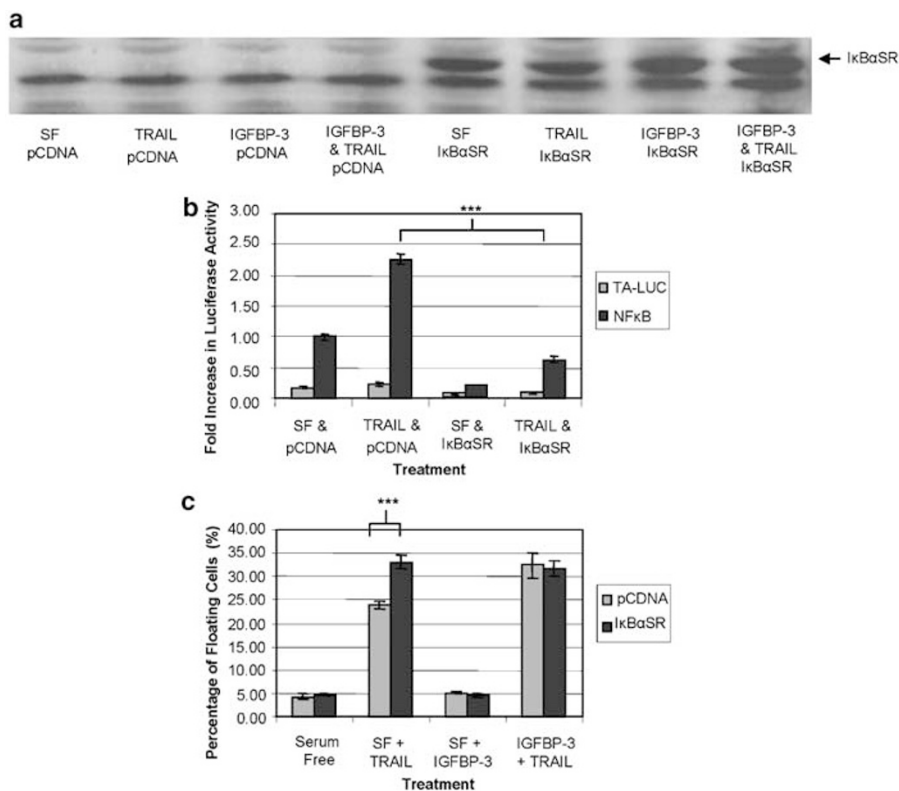


Figure 6 TRAIL-induced apoptosis in the presence of the NF- κ B repressor I κ B α is equivalent to apoptosis induced by TRAIL \pm 100 ng/ml IGFBP-3. **(a)** Western blot showing the presence of the NF- κ B super repressor HA-tagged plasmid (I κ B α) using the anti-HA polyclonal antibody (Clontech, BD Bioscience, Europe) in the HT29 cell line after 24 h treatment with 0.05 μ g/ml TRAIL \pm 100 ng/ml IGFBP-3 protein (Gropep, AUS). **(b)** Reporter assay to show TRAIL-induced NF- κ B activation is inhibited by I κ B α . The NF- κ B super repressor (I κ B α -SR) was transiently expressed in HT29 cells treated with 0.05 μ g/ml TRAIL, pNF- κ B-TA-luc:firefly renilla ratios expressed as a fold of untreated vector only cells representing the mean results from three separate flasks \pm S.E.M. Statistical difference was determined by two-way ANOVA with replication, * P < 0.05, ** P < 0.01, *** P < 0.001. **(c)** Induction of apoptosis in HT29 cell lines after 24 h treatment with 0.05 μ g/ml TRAIL in the presence or absence of the NF- κ B repressor (I κ B α) \pm 100 ng/ml IGFBP3 protein (Gropep, AUS). The results shown are the mean of three separate flasks \pm S.E.M. statistical difference determined by two-way ANOVA with replication, * P < 0.05, ** P < 0.01, *** P < 0.001

Materials and Methods

Cell lines and culture conditions. S/RG/C2 is a clonogenic, nontumorigenic human colonic adenoma-derived cell line hemizygous for the p53 gene, with the remaining allele having a 282 (Arg \rightarrow Trp) mutation. S/AN/C1 and PC/AA/C1 are clonogenic, nontumorigenic human adenoma-derived cell lines, which express wild-type p53. PC/AA/SB10 is a tumorigenic derivative of the PC/AA/C1 cell line shown to express increased levels of wild-type p53 protein as compared to the parental cell line (described in detail in Williams *et al.*³⁸). These cell lines were maintained on conditioned medium (refer to Williams *et al.*³⁸). The human colonic carcinoma-derived cell lines HT29 and SW620 (both mutant for p53) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (from Autogen Bioclear, UK) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine and 10% fetal bovine serum (referred to as 10% FBS DMEM medium). HT29 cells transfected with, and stably expressing the pcDNA3 expression vector containing IGFBP-3 (IGFBP-3 cDNA was a kind gift from Rob Baxter, Kolling Institute for Medical Research, Sydney, AUS) HT29/BP3, and a vector control, HT29/pcDNA3, were grown in 10% FBS DMEM supplemented with 200 μ g/ml G418 as previously described.³⁹

Treatment with TRAIL. Cells were seeded in triplicate flasks and grown under standard growth conditions until approximately 70% confluent. Preliminary investigations showed that all the cell lines studied were able to tolerate serum-free growth conditions (SFM) for up to 96 h (SFM = standard non-conditioned growth medium without addition of FBS). Cells were grown for 24 h in SFM to remove IGFBP-3 present in the serum and then grown for up to 24 h in SFM supplemented with or without TRAIL (0.05–0.25 μ g/ml), previously reported to induce apoptosis.²⁵

Assessment of apoptosis. We and others have previously shown that in routine culture of colorectal epithelial tumour cells, the majority of spontaneously occurring floating cells are apoptotic (approximately 90%), whereas the proportion of attached cells that are apoptotic is low (<3%). Treatment with agents such as TRAIL does not increase the proportion of attached cells with apoptotic morphology but does increase the proportion of floating cells, and this is due to induction of apoptosis.²⁵ Hence, in these cases, the level of apoptosis in cultured epithelial cells can be determined by measuring the proportion of the total cell population that has detached from the cell monolayer. In this study, apoptosis induced by TRAIL was determined essentially as previously described.²⁵ Briefly, after determining the proportion of cells that had detached from the cell monolayer and were floating in the medium, the attached and floating cell population was stained with 5 μ g/ml acridine orange and 1:10 000 2,6-diamidino-2-phenylindole (DAPI) (Sigma, UK) in PBS, and analysed by fluorescent microscopy for morphological features of apoptosis (most obviously the characteristically condensed chromatin). An example of DAPI staining is shown in Figure 1C. Biochemical confirmation of apoptosis was obtained by demonstration of PARP cleavage by Western blotting in the floating cell population (Figure 1B), PARP was detected by the anti-PARP monoclonal C-2-10 (Alexis Corporation, CHE), α -tubulin by an anti- α -tubulin monoclonal (Sigma, UK), using the ECL detection system (KPL, USA).

Treatment with TRAIL \pm IGFBP-3. Cells were seeded in duplicate flasks and grown under standard growth conditions until approximately 70% confluent, and then grown for 24 h in SFM to remove IGFBP-3 present in the serum. Cells were then grown for up to 24 h in SFM supplemented \pm TRAIL (0.05 μ g/ml HT29, 0.1 μ g/ml SW620 and 0.25 μ g/ml S/RG/C2) \pm IGFBP-3. Synthetic IGFBP-3 protein

was added at a concentration of 100 ng/ml (Gropep, AUS). Secreted protein was harvested from HEK293 cells transfected with IGFBP-3 in the pcDNA3 expression vector or mock transfected HEK293 cells. HEK293 cells had been transfected when 70% confluent using Lipofectamine 2000 (Invitrogen, UK). After 6 h, the medium was replaced by SFM which was harvested 48 h later. The IGFBP-3 concentration was adjusted to approximately 100 ng/ml (by Western analysis comparing to IGFBP-3 protein standards, Upstate Biotechnology, USA).

Treatment with TRAIL \pm IGF-IR α or anti-IGFBP-3 antibodies. Cells were incubated in SFM for 24 h, followed by SFM plus the anti IGF-IR antibody (Santa Cruz, USA) or the anti-IGFBP-3 antiserum (polyclonal from Diagnostic System Laboratory, USA) at concentrations of 5 μ g/ml for 24 h \pm 0.05 μ g/ml TRAIL in the presence or absence of IGFBP-3 (Gropep, AUS). The cell yield and floating cell population in the presence of either antibody were counted using a Neubauer counting chamber (VWR, UK).

NF- κ B reporter assays. Cells were transiently transfected with either the NF- κ B reporter plasmid pNF- κ B-TA-luc or with the control reporter plasmid pTA-luc (Clontech, BD, Europe). pNF- κ B-TA-luc contains four copies of a consensus NF- κ B binding sequence (GGGAATTTCC) in addition to a minimal promoter (P_{TA} , the TATA box from the herpes simplex virus thymidine kinase promoter) located upstream of the firefly luciferase (*luc*) gene. The consensus NF- κ B binding sequences are absent from the control vector pTA-luc. All transfections also included the renilla luciferase vector pRL-SV40 (Promega, UK) as an internal control for transfection efficiency.

For transient transfection, all cells were grown to 70% confluence in T12.5 flasks. Triplicate flasks were cotransfected with one of the two reporter constructs (pTA-luc or pNF- κ B-TA-luc) and with the renilla construct (pRL-SV40) in a ratio of 50:1. Each flask was incubated for 6 h with 2 μ g of plasmid DNA and 5 μ l of Lipofectamine 2000 (Invitrogen, UK) or Tfx-20 (Promega, UK) diluted in Opti-MEM serum-free medium according to the manufacturer's instructions. Following transfection, cells were allowed to recover overnight in SFM before experimental treatment as described above.

Luciferase reporter assay. Twenty four hours after treatment, cells were washed in phosphate buffered saline (PBS) and lysates prepared in 1 \times PLB (Promega, UK) according to the manufacturer's instructions. Reporter activity was measured using the Dual-Luciferase reporter assay system (Promega, UK) and a Jade Luminometer (Labtech, UK) set for a 10-s read. Sample readings were corrected for background autoluminescence using untransfected cells as a control. The NF- κ B activity in the untreated tumour cell lines was variable, ranging from an approximate 10–100-fold activation of the reporter plasmid depending on the cell line (refer to figure legends).

Stable transfection. HT29 cells were transfected with a 0.9-kilobase pair IGFBP-3 cDNA fragment in the expression vector pcDNA3. Cells were stably transfected using Lipofectamine 2000 (Invitrogen, UK) according to the manufacturer's protocol. After 48 h, the transfected cells were selected for using G418 (400 μ g/ml), cell lines grown up from single colonies and maintained in 10% FBS DMEM with 200 μ g/ml G418.

Assessment of IGFBP-3. Subconfluent monolayers were grown for 24 h in SFM. The cells were then grown for a further 24 h in SFM, with or without TRAIL, the media harvested, centrifuged to remove floating cells and stored at -70° C. Attached cell numbers were determined using a haemocytometer. Proteins from conditioned medium were concentrated using 'Microsep' 10 K centrifuge columns from Gelman Laboratory. The levels of IGFBP-3 were assessed by SDS-PAGE immunoblotting, the IGFBP-3 protein detected by anti-IGFBP-3 antiserum (polyclonal from Diagnostic System Laboratory, USA) using ECL detection system (KPL, USA).

NF- κ B ELISA. The DNA-binding activity of NF- κ B in cells quantified by ELISA by means of the Trans-Am NF- κ B p50 and p65 transcription factor assay kit (Active Motif, BEL), as described.⁴⁰ Briefly, cell extracts were prepared from treated cells and incubated in 96-well plates coated with immobilized oligonucleotide (5'-AGT TGAGGGGACTTTCCAGGC-3') containing a consensus (5'-GGGACTTCC-3') binding site for NF- κ B. NF- κ B binding to the target oligonucleotide was detected by incubation with primary antibodies specific for the activated form of either p50 or p65, visualized by anti-IgG horseradish peroxidase conjugate and developing

solution and quantified at 450 nm with a reference wavelength of 655 nm. Background binding, obtained by incubation with a 2-nucleotide mutant oligonucleotide (5'-AGTTGAGGCCACTTTCCAGGC-3'), was subtracted from the value obtained for binding to the consensus DNA sequence.

XIAP protein expression. The levels of XIAP were assessed by SDS-PAGE immunoblotting, with the XIAP protein detected by the hIPL/XIAP monoclonal antibody (BD Bioscience, Europe) using an ECL detection system (KPL, USA).

Enforced expression of the NF- κ B super repressor protein I κ B α . HT29 cells were transiently transfected with HA-tagged mutant super repressor I κ B α gene in the expression vector pcDNA3 (kind gift of Dr Reinhard Voll, Friedrich-Alexander University Erlangen-Nuremberg, Germany). Cells were transiently transfected using Tfx-20 (Promega, UK) according to the manufacturer's protocol. Expression was verified using anti-HA polyclonal antibody (Clontech, BD Bioscience, Europe).

Statistical analysis. The data represent the mean of three separate experiments. (Each experiment was carried out in duplicate or triplicate parallel flasks. The experiment was repeated three times, and results presented as the mean of the three separate experiments.) Statistical analysis was carried out using SPSS for Windows statistical software (release 10.0.5, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to determine differences among means. Pair-wise comparisons were made using Tukey's *post hoc* test for multiple comparisons.

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