Insulin-like growth factor binding protein-4 gene therapy increases apoptosis by altering Bcl-2 and Bax proteins and decreases angiogenesis in colorectal cancer

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Abstract. Insulin-like growth factors are known to inhibit apoptosis and promote tumour angiogenesis. Previously we have shown that insulin-like growth factor binding protein-4 (IGFBP-4) gene therapy increased apoptosis and decreased mitosis in colon cancer. In this experiment we used HT-29 colon cancer cells to induce subcutaneous cancers in nude mice and administered either the mammalian expression vector with IGFBP-4 insert or vector only around the tumour site. Three weeks after gene transfer, tumours were harvested and expressions of Bax, Bcl-2 and IGF-I receptor in tumours were determined by Western blotting and immunofluorescence. Micro-vessel counting was performed by immuno-staining with CD34 and von Willebrand antibodies. Results showed that tumours treated with IGFBP-4 gene had higher expression of Bax, lower expression of Bcl-2 and IGF-I receptor. Bcl-2 was localised to tumour cell cytoplasm while Bax was expressed both in the interstitial area and cytoplasm. IGFBP-4 treatment also decreased micro-vessel count in tumour tissues. Micro-vessels were mainly located in the periphery and interstitial area. This experiment shows that IGFBP-4 gene therapy increases tumour apoptosis via altering the expressions of Bcl-2 and Bax and decreasing the angiogenesis in colorectal cancer.

Introduction

It is well known that apoptosis and angiogenesis influence tumour growth. Insulin-like growth factors (IGFs) enhance tumour angiogenesis, by up-regulating VEGF (1), and are anti-apoptotic (2) in action. Insulin-like growth factor binding protein-4 (IGFBP-4) acts by binding to IGFs thereby reduces their local tissue bioavailability (3). Thus, IGFBP-4 can influence angiogenesis and apoptosis. Apoptosis is influenced by various intracellular proteins and enzymes. Two important proteins which influence apoptosis are B-cell CLL/lymphoma 2 (Bcl-2) which is an anti-apoptotic protein and Bcl-2-associated X protein (Bax) which promotes apoptosis. Bax exists in an inactive state in the cytoplasm of many cells. Secondary to various stimuli, Bax protein undergoes conformational changes and translocates to mitochondrial membranes where Bax releases cytochrome c (4). IGF-I not only down-regulates the Bax expression (5) but also prevents its translocation to mitochondria (6), inhibits activation of caspase-3 (7) and release of cytochrome 3 from the mitochondria (7). A fine balance between Bax and Bcl-2 proteins often determines the apoptotic process (8-10). Experiments show that an increase in the ratio of Bax/Bcl-2 proteins often proceeds apoptosis (11,12) and mutations of these genes are often associated with cancer (13-15). Insulin-like growth factors influence the levels of Bax and Bcl-2 (16) and thereby apoptosis (17). It has been shown that blockade of the IGF-I receptor with truncated mutated IGF-IR (18,19) inhibits angiogenesis in colon cancers.

Previously we have shown that IGFBP-4 gene transfers by local administration decreased mitosis and increased apoptosis of the tumour cells in HT-29 colon cancer. In this report we demonstrate how IGFBP-4 mediates apoptosis and its effect on angiogenesis in vivo.

Materials and methods

Plasmid. Mammalian expression vector pcDNA 3 (Promega, USA) containing myosin light chain enhancer and Cytomegalovirus (CMV) promoter was used in the experiment and IGFBP-4 cDNA was inserted between Kpn-1/EcoR1 sites. The plasmid was transformed using Easy vector kit (Promega) and purified with Endo Free Maxi kit (Qiagen, UK). Sequencing was verified (MWG, Germany) prior to the animal experiment.

Colon cancer cells. HT-29 colon cancer cells (European Cell Culture Society, UK) were grown under standard conditions using McCoy’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were serially
cultured and those with viability of >90% were used for animal experiments as determined by Trypan blue.

Animal model. The animal experiments were performed according to the Home Office (UK) guidelines. Four- to six-week-old male nu/nu MF-1 nude mice (n=13) were subcutaneously inoculated with 3x10^6 HT-29 cells in their flank and they were given free access to sterile food and water. The animals were maintained under standard condition. One week post inoculation 150 μg of either plasmid of vector alone or plasmid of vector with IGFBP-4 insert was administered locally around the tumour subcutaneously. The animals were observed for another 3 weeks and sacrificed by cervical dislocation. Tumour samples were collected for further examination.

Mechanism of apoptosis

Western immunoblot for Bcl-2 and Bax. The tumour tissues were crushed into powder in liquid nitrogen and the cells were lysed in reporter lysis-buffer (Promega). Total proteins in the solution were quantified by Modified Lowry protein assay method (Pierce Biotechnology, USA). Total protein (50 μg) (10 μl in volume) was mixed with an equal volume of Laemmi sample buffer (2X, Sigma, UK) concentration containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH ~6.8. The samples were then denatured by heating at 95˚C for 5 min. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins, which was then electro-blotted onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad Lab, USA). The membrane was blocked with 5% milk (Marvel semi-skimmed milk powder) and incubated with rabbit anti-mouse Bcl-2 antibody (Santa Cruz, USA) at 1/200 dilution or rabbit anti-mouse Bax polyclonal antibody (Santa Cruz, USA) at 1/200 dilution followed by incubation with goat anti-rabbit secondary antibody conjugate with horseradish peroxidase (Dako, UK) at 1/2000 dilution. The membrane was illuminated with Super Signal West Dura Extended Duration Substrate (Pierce, USA) and exposed to X-ray film for 10 sec (Fujifilm, Japan). The X-ray was scanned and the density of the protein bands were analysed with Bio-Rad densitometry software (Molecular Analyst, windows software for Bio-Rad's image analysis system version 1.5, USA).

Immuno-staining for Bcl-2 and Bax. Frozen sections (10 μm) were prepared from tumour tissues and fixed at 5% formalin at 4˚C for 5 min. The sections were washed 3 times with PBS and incubated with rabbit anti-Bax and Bcl-2 polyclonal antibody (Santa Cruz Biotechnology, USA, both at 1/200 dilution) overnight at 5˚C. Alexaflour red (Molecular Probes, USA, 1/200 dilution) or goat anti-rabbit FITC conjugate (Paris, France, 1/100 dilution) secondary antibodies were used for detecting the primary antibody. The immuno-staining of Bax and Bcl-2 were analysed under fluorescent microscope (Nikon, Japan).

Tumour angiogenesis. Micro-vessels in the tumour tissue were assessed by immunofluorescence using antibodies against von Willebrand factor and CD34.

Immuno-staining for CD34. Frozen tumour sections (10 μm) were fixed with acetone for 10 min at -20˚C, then air-dried for a few min at room temperature and rehydrated with PBS. After incubation with 0.03% H₂O₂ in methanol, the sections were washed with tap water and PBS 2 times each. Subsequently sections were blocked with 3% goat-serum for 30 min at room temperature. Excess serum was tapped off and rat anti-mouse CD34 polyclonal antibody (HyCult Biotechnology, UK) was applied with 1/50 dilution for 1 h at room temperature. The sections were briefly washed in PBS. The primary antibody was then detected by secondary goat anti-rabbit antibody (Alexafluor, Invitrogen, UK) with 1/200 dilution. Then the slides were washed with PBS for 2x5 min. The slides were mounted with AF-1 (Citiflour, UK) medium. Fluorescent microscope was used to assess the micro-vessels.

Immuno-staining for von Willebrand factor (vWF). The procedure for immuno-staining of von Willebrand factor was the same as described for CD34 except that primary rabbit anti-human polyclonal anti-vWF antibody (Dako Cytomation, UK, 1/200 dilution) and the secondary goat anti-rabbit antibody-FITC conjugate (Paris, France, 1/100 dilution) were used.

For comparison and counting of micro-vessel in the tumours, 5 different hotspots were identified in each tumour tissue section after staining with both CD34 and vWF (double staining) and vessels were counted under light microscopy at a magnification of x200. The average count was calculated as micro-vessel density for each tumour.

Western blotting for components of IGF-I system. The effect of the IGFBP-4 gene therapy on expressions of IGF-I and IGF-IR within tumours were assessed by Western blotting using rabbit anti-IGF-I polyclonal antibody (Santa Cruz, USA, 1/200 dilution) and rabbit anti-IGF-1 receptor polyclonal antibody (Santa Cruz, 1/200 dilution). The procedure and the secondary antibody for Western blotting was the same for Bax and Bcl-2.

Statistical methods. Results are expressed as mean ± SM. The unpaired student t-test (Prism 2003, USA) was used for statistical analysis and P<0.05 was considered as significant.

Results

Mechanism of apoptosis. Immunostaining showed that Bcl-2 was mainly localised to the cytoplasm of cancer cells while Bax was expressed in cytoplasm of the cancer cells as well as interstitial area of most tumours (Fig. 1). Higher immunofluorescence for Bax and lower immunofluorescence for Bcl-2 was observed in tumours treated with IGFBP-4 gene compared to control group tumours. Western blot densitometry (Fig. 2) showed that IGFBP-4 gene therapy significantly increased the expression of Bax (0.58±1.55, 5.18±0.37, BP-4 vs control; P<0.05) and decreased the expression of Bcl-2 (10.81±1.30, 12.27±2.91; BP-4 vs control; P=0.65) in tumour tissues.

Angiogenesis. Micro-vessels were identified as fluorescence staining of endothelium (Fig. 3) with anti-CD34 and vWF.
Figure 1. Immunostaining for Bax and Bcl-2 proteins from tumour of control and BP-4 groups (x200). The arrow indicates the protein. Increased Bax and decreased Bcl-2 protein expressions were seen in BP-4 group (A and D) when compared to control (B and C). Bax was expressed in both the cytoplasm and interstitial tissue of colon cancer while Bcl-2 was expressed mainly in the cytoplasm of cancer cells.

Figure 2. Western blot analysis for Bcl-2 (A) and Bax (C). B1-B6 represent the BP-4 group and M1-M6 represent the control group. Densitometry showed lower Bcl-2 expression (B) and significantly higher average Bax expression (D) by BP-4 group compared to control plasmid group (P<0.05).

Figure 3. Immunofluorescence for micro-vessels (x200). Both CD34 and von Willebrand factor staining showed less micro-vessels (arrow) in the BP-4 group (B and D) than in the control plasmid group (A and C).
antibodies. Double staining confirmed the presence of micro-
vessels when there was doubt. Histological examination
revealed that there was some gland formation and the micro-
vessels were mainly present between the glands in the
interstitium and periphery of the tumours. The average
number of micro-vessels per area (micro-vessel density) was
significantly higher in the control group than in the IGFBP-4
treated group (13.93±0.76 vs 9.10±1.27; control group vs
IGFBP-4 treated group; P<0.01) (Fig. 4).

IGF system. The IGF-IR levels were significantly increased
(5.08±0.21 vs 3.73±0.31; BP-4 vs control; P<0.01) in IGFBP-4
treated group (Fig. 5). IGF-I was not detectable in either group.

Discussion

This study presents in vivo evidence for the first time that
over-expression of IGFBP-4 increases apoptosis in colon
cancer by altering expression of both Bcl-2 and Bax proteins,
decrease angiogenesis and increase IGF-IR levels in tumour
tissues.

Apoptosis involves an autolytic cascade in the target cell
with DNA fragmentation preceding cell lysis. Mitochondria
play an important role in the regulation of cell death. Bcl-2
family anti-apoptotic proteins are located in the outer mito-
chondrial membrane and promote cell survival. Mitochondria
also have the ability to promote apoptosis through release
of cytochrome c (20), which in turn can activate caspase-9.
Caspases are cysteine proteases that exist within the cell as
inactive pro-forms or zymogens. These zymogens can be
cleaved to form active enzymes during apoptosis. Caspase-3
and caspase-6 are responsible for the cleavage of the key
 cellular proteins that leads to the typical morphological
changes observed in cells during apoptosis (21).

Previous in vitro experiments have shown that IGFBP-4
inhibits proliferating and growth promoting actions of IGFs in
both malignant and normal cells (22-25). It has been suggested
that IGFBP-4 may act by altering Bcl-2 and Bax levels (26,27). Results of both Western blotting and immunofluorescence of our experiment showed that there was a significant increase in Bax protein levels in IGFBP-4 treated tumours compared to the control group. Bcl-2 was decreased in IGFBP-4 treated tumours, although it was not statistically significant. Our hypothesis of the mechanism of IGFBP-4 affecting the apoptosis in tumour is summarized in Fig. 6. In theory IGFBP-4 has to compete with IGF-I receptor to bind free IGF-I. When over-expressed, IGFBP-4 binds IGF-I and reduces its free level which is available to bind to the IGF-I receptor. Therefore, IGFBP-4 influences the expression of Bax and Bcl-2 in tumour tissues. To compensate for the decrease in free IGF-I levels, the tumour has to produce either more IGF-I or IGF-1R by feedback mechanism. The fact that IGFBP-4 treatment increased the IGF-I receptor confirm our theory. In cancer cells Bax and Bcl-2 act on caspases which triggers apoptosis. Bax and Bcl-2 proteins show structural similarities with pore-forming proteins. It has been suggested that Bax can form a transmembrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and efflux of cytochrome c and the apoptosis inducing factor. It is thought that Bcl-2 can prevent this pore formation. Bcl-2 family proteins can also influence the levels of releasable Ca²⁺ in the endoplasmic reticulum, and can release Ca²⁺ to overload mitochondria and induce cell death (28). The hallmark of apoptosis is the cleavage of chromosomal DNA into nucleo-somal units which is mediated by an enzyme caspase activated DNase (29). It can be assumed that IGFBP-4 either acts by the local binding of IGF-I or an independent mechanism via its own receptors. Experiments have shown that IGF-I protect HT-29 cells from apoptosis (30) and the blockade of IGF-1R increases apoptosis in the same cells (19). This provides evidence for an IGF-dependent mediation of apoptosis of IGFBP-4. Thus, the alteration in Bax/Bcl-2 may be secondary to a decrease in free IGF-1.

Angiogenesis is essential for the viability and growth of solid tumours. Neovascularisation is mediated by angiogenic molecules released by tumour cells themselves and by accessory host cells such as macrophages, mast cells, and lymphocytes. Vascularity of the tumours is influenced by several factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and interleukin-8 (IL-8). New blood vessels will provide nutrients to proliferating cancer cells and favour tumour growth. The tumour releases angiogenic factors which diffuse into the surrounding tissue. Tumour angiogenesis is also associated with the production of highly permeable and poorly-formed vasculature. This is thought to be due to high expression of factors such as VEGF and angiopoietin-2.

The most widely used endothelial cell markers for studying angiogenesis are von Willebrand factor and CD34. The former is synthesized by endothelial cells and the latter is a transmembrane glycoprotein constitutively expressed on endothelial cells and haematopoietic stem cells. Results from our experiments showed that the micro-vessels were located mainly in the interstitium between cancer cells more towards the periphery of the tumour. It is in accordance with the fact that tumours receive their blood supply from the periphery.

IGFBP-4 treated tumours showed a significant reduction in the average numbers of micro-vessels per field. Previous studies have shown that IGF-I stimulates angiogenesis by inducing VEGF. In these experiments the decrease in angiogenesis may be due to a decrease in the local levels of IGF-I which in turn reduced VEGF levels. Thus, a decrease in angiogenesis may be contributing to the increase in cell death. The possibility exists that the increase in apoptosis induced by IGFBP-4 gene therapy may cause less IGF-I production by tumour cells as part of a negative feedback loop which then results in less micro-vessel formation.

In the IGFBP-4 treated group the tumour cells showed an increase in IGF-1R levels. The increase in IGF-IR may be due to another feedback mechanism whereby the receptor density is increased in order to decrease free IGF levels. The inability to detect IGF-I expression indicates in cancer cells that either these cells depend on external source of IGF-I or that by restricting this locally using BP-4 it is possible to kill these tumourigenic cells.

In conclusion our studies showed that IGFBP-4 causes apoptosis, which is mediated via altering expression of Bax/Bcl-2 in tumour tissues which in turn decreases tumour angiogenesis. The effect on IGF-IR may be due to a feedback mechanism to compensate for the decreased free IGFs. 

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References


