Insulin-like growth factor-binding protein-3 is partially responsible for high-serum-induced apoptosis in PC-3 prostate cancer cells

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Abstract

Cells are known to undergo apoptosis when cultured in high serum concentrations. However, the serum factors responsible for this induction of apoptosis have not been identified. The IGF-binding protein-3 (IGFBP-3), a negative growth regulator, is found at concentrations of 5 μg/ml in serum. We have recently demonstrated that IGFBP-3 induces apoptosis in PC-3 cells, a prostate cancer cell line, at a concentration of 500 ng/ml. In this communication, we demonstrate the role of IGFBP-3 as one of the apoptosis-inducing agents in high serum concentrations. Treatment of PC-3 cells with increasing concentrations (40% to 90%) of intact human serum (HS) resulted in a dose-dependent decrease in cell growth. Valinomycin, an ionophore, was used as a positive control to measure the induction of apoptosis by serum treatment in PC-3 cells. Treatment with 90% serum showed significant suppression of growth ($P<0{\cdot}001$) compared with the effect of 10% serum. Treatment with increasing concentrations of HS (40% to 90%) resulted in a dose-dependent increase in apoptosis. Treatment with 90% HS showed a 10-fold increase in apoptotic index compared with cells treated with 10% HS. Treatment of PC-3 cells with IGFs and IGFBP-3-depleted 90% human sera (depleted serum=DS) demonstrated significantly lower levels of apoptosis (50% reduction in the effect of 90% HS) suggesting a role of IGFBP-3 in inducing apoptosis in high serum concentration. Furthermore, treatment with DS supplemented with recombinant IGFBP-3 (500 ng/ml) brought the apoptotic index down close to the level of apoptosis induced by 90% intact serum treatment ($P<0{\cdot}001$). However, DS supplemented with physiological concentrations of IGFs (500 ng/ml) showed only partial recovery of cell survival demonstrated by 90% DS. This data indicates that IGFBP-3 is one of the factors in serum that is responsible for high-serum-induced apoptosis.

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Introduction

Programmed cell death (apoptosis) induced by a high serum concentration has previously been demonstrated *in vitro* in multiple cell lines (Kurita & Namiki 1994, Valentinis & Baserga 1996, Young *et al.* 1996). Expression of insulin-like growth factors (IGFs) and IGF-type 1 receptors (IGF-1R) have been shown to partially rescue cells from apoptosis induced by high serum concentrations (Valentinis & Baserga 1996). However, the actual molecule/s present in serum that induce apoptosis have not been defined.

IGF-binding protein-3 (IGFBP-3), a negative growth regulator, is found at a concentration of about 5 μg/ml in adult serum. We and others have previously demonstrated the effects of IGFBP-3 as a negative regulator of cell proliferation in prostatic and other tissues (Cohen *et al.* 1993*a*, 1994, Kaicer *et al.* 1993, Angelloz-Nicoud & Binoux 1995, De Mellow & Baxter 1998). Further, in support of its role as a negative regulator of cell growth and proliferation, IGFBP-3 gene expression has been shown to be induced by agents known to have growth inhibitory and apoptosis-inducing effects, such as transforming growth factor-β1 (TGF-β1) (Martin *et al.* 1992, Oh *et al.* 1995, Gucev *et al.* 1996), retinoic acid (Gucev *et al.* 1996), tumor necrosis factor-α (Yateman *et al.* 1993), and the tumor suppressor gene, p53 (Buckbinder *et al.* 1995). This negative growth regulation by IGFBP-3 has been proposed to involve a separate cellular signaling pathway from that of the IGF-mediated cell survival pathway (Oh *et al.* 1995, Valentinis *et al.* 1995). We recently demonstrated that the negative growth regulatory role of IGFBP-3 is due not only to its ability to regulate the availability of free IGFs and to induce growth arrest, but also to its ability to induce apoptosis (Rajah *et al.* 1997). Using an IGF-1R-negative (R(-)) mouse fibroblast cell line we further elucidated that this process involves an IGF-independent mechanism (Sell *et al.* 1993, Rajah *et al.* 1997). In this publication, we now demonstrate that IGFBP-3 is one of the factors that causes high-serum-induced apoptosis.

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Materials and Methods

Materials

Tissue culture supplies were purchased from Flow Laboratories (McLean, VA, USA), Corning (Corning, NY, USA), and Hyclone (Logan, UT, USA). Recombinant DNA-derived non-glycosylated (*E. coli*) human IGFBP-3 was the generous gift of A Sommer (Celtrix Inc., Santa Clara, CA, USA). The calcium ionophore (Cocktail A, Sigma #I-1772) was purchased from Sigma Chemicals (St Louis, MO, USA). Human sera were collected from blood obtained from volunteers of both sexes whose ages ranged from 25 to 40 years, and were pooled into three samples.

PC-3 cell cultures

The human PC-3 cells were purchased from ATCC (Rockville, MD, USA), and were initiated from a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian. The PC-3 cells were grown in 75 cm^2 flasks according to standard protocol (FK-12 supplemented with 10% human serum (HS) and 1% penicillin-streptomycin). For each experiment, cells were dissociated, pelleted, and resuspended in serum containing FK-12 media with antibiotics. Cells were inoculated at a density of 1 × 105 cells/cm^2 in 24-well or 6-well tissue culture dishes in a humidified atmosphere of 5% CO_2 at 37 °C and grown to confluence before treatment. After a quick wash with serum-free FK-12 media (SFM), the confluent cells were treated with various concentrations of serum, IGFBP-3, or other required agents. SFM with antibiotics was used as the control treatment.

Cell growth assays

For each experimental condition, the PC-3 cells were plated at 1 × 102 cells/cm^2 in 96-well plates. A non-radioactive CellTiter 96 assay was used to measure cell proliferation. Samples were treated in multiples of eight for each condition. This method measures the cellular conversion of the tetrazolium salt, MTS, into a formazan, which is measured at 490 nm directly in the plate (Promega, Madison, WI, USA). The absorbance reading is directly proportional to the number of viable cells/well. The resulting means and standard deviations were determined.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

In situ detection of apoptosis in cultured cells was performed with the use of direct immunoperoxidase detection of biotin-labeled genomic DNA in monolayer cells. In brief, following treatment with different conditions, the monolayer cultures were fixed in 3·7% paraformaldehyde solution for 10 min at room temperature followed by dehydration in 70% ethanol for 5 min at room temperature. Following this step, the endogenous peroxidase was quenched by treatment with 2% hydrogen peroxide in methanol for 5 min. The cells were incubated in the labeling mixture (Biotin dNTP mix, 50 × $MgCl_2$, TdT, and labeling buffer) for 60 min at 37 °C. The free 3′-OH DNA in the apoptotic cells were visualized using the streptavidin-horseradish peroxidase-diaminobenzidine (DAB) detection system. The apoptotic cells appeared as dark brown cells.

Apoptosis ELISA assay

Photometric ELISA or cell death detection ELISA (Boehringer-Mannheim, Indianapolis, IN, USA) was performed to quantitate the apoptotic index by detecting the histone-associated DNA fragments (mono- and oligonucleosomes) generated by the apoptotic cells. The assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones respectively for the specific determination of these nucleosomes in the cytoplasmic fraction of cell lysates. In brief, an equal number of cells were plated in 24-well culture plates (1 × 104/cm^2) in serum-supplemented FK-12 medium and grown to confluency for 72 h. The confluent cells were washed with PBS and treated with an ionophore, Valinomycin, as a positive control, various concentrations of IGFBP-3, serum, or other required agents for the designated time period. The cells were dissociated gently (PBS with 0·1 M EDTA) and pelleted together with the floating cells (mostly apoptotic cells) collected from the conditioned media. The cell pellets were used to prepare the cytosol fractions which contained the smaller fragments of DNA. Equal volumes of these cytosolic fractions were incubated in anti-histone antibody-coated wells (96-well plates) and the DNA fragments were allowed to bind to the anti-histone antibodies. The peroxidase-labeled mouse monoclonal DNA antibodies were used to localize and detect the bound fragmented DNA using photometric detection with 2,2′-azino-di-[3-ethylbenzathiazoline sulphonate] (ABTS) as the substrate. Samples were run in triplicate and each experiment was performed at least three times. The reaction products in each 96-well plate were read using a Bio-Rad microplate reader (Model 3550-UV; Bio-Rad, Melville, NY, USA). Average of the values with ± S.E.M. from double absorbance measurements of the samples were plotted.

Immunodepletion of IGFBP-3 from HS

The HS samples were depleted of IGFBP-3 by running the samples through an IGFBP-3 antibody column. The serum samples before and after passing through the

IGFBP-3 antibody column were quantitatively assessed using IGF-I and IGF-II, and IGFBP-3 IRMA (Diagnostic Systems, Webster, TX, USA). This procedure removed both IGFBP-3 and the IGFs bound to it.

Western immunoblots (WIB)

The WIB analysis was performed as previously described (Rajah *et al.* 1996). HS and IGFBP-3-depleted HS were used as samples. Samples of 100 µl (equivalent to 2 µl serum) were electrophoresed through 12·5% non-reducing SDS-PAGE overnight at constant voltage, electroblotted onto nitrocellulose, blocked with 5% non-fat dry milk in Tris–buffered saline, probed with specific affinity-purified IGFBP-3 antibodies, and detected using a peroxidase-linked enhanced chemiluminesence detection system (Pierce, Rockford, IL, USA).

Densitometric and statistical analysis

Densitometric measurement of immunoblots was performed using a Bio-Rad GS-670 imaging densitometer (Bio-Rad). Protein levels were estimated by comparing the optical density of each specific protein band from control (intact serum) conditions to that of the IGFBP-3 immunodepleted conditions. All experiments were repeated at least three times. When applicable, means ± S.E.M. are shown. Student's *t*-tests were used for statistical analyses.

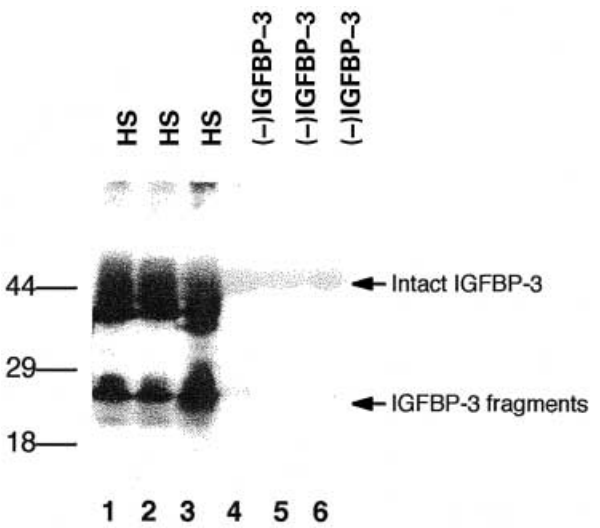

Figure 1 Depletion of IGFBP-3 from serum. Serum samples were passed through the IGF affinity column to remove IGFBP-3. The serum samples before and after passing through the IGFBP-3 antibody column were quantitatively assessed using IGF-I and IGF-II, and IGFBP-3 IRMA. IGFBP-3, IGF-I and IGF-II were significantly reduced after passing through the affinity column ($P<0·0001$). Samples from intact HS (HS) and IGFBP-3-depleted human sera ((−)IGFBP-3) were separated on 12·5% SDS-PAGE overnight at constant voltage, electroblotted onto nitrocellulose, blocked with 5% non-fat dry milk in Tris–buffered saline, probed with affinity purified IGFBP-3 specific antibodies, and detected using a peroxidase-linked enhanced chemiluminescence detection system.

Results

Confirmation of IGFBP-3 depletion from intact serum

IGFBP-3 depletion from serum was confirmed by two methods. The serum samples before and after passing through the IGFBP-3 antibody column were quantitatively assessed using IGF-I and IGF-II, and IGFBP-3 IRMA. The successful depletion of IGFBP-3 was demonstrated by IGFBP-3 IRMA which demonstrated less than 25 ng/ml IGFBP-3 in the depleted serum (DS) samples. IGF-I levels were 200 ng/ml in intact serum and <10 ng/ml in depleted sera. IGF-II levels were 500 ng/ml in intact sera and <10 ng/ml in depleted sera. Depletion of IGFBP-3 and IGFs from HS was also confirmed by western immunoblotting for IGFBP-3 using intact HS and the IGFBP-3-depleted HS. Complete depletion of IGFBP-3 from these serum samples is shown in Fig. 1. Although this method showed very slight staining for intact IGFBP-3 in DS samples (lanes 4–6), no labeling was detected from similar samples using western ligand blotting (data not shown).

The in situ *detection of apoptotic cells*

The apoptotic cells were detected *in situ* in monolayer cell cultures of PC-3 cells treated with SFM and increasing concentrations of intact HS using TUNEL. The dramatic difference between 10% HS and 90% HS is shown in Fig. 2. The DNA fragments bound to the peroxidase-DAB reaction product in apoptotic cells were visualized *in situ* as dark brown cells. Cells in 10% HS displayed no detectable apoptotic cells (Fig. 2A) while cells treated with 90% HS treatment revealed numerous apoptotic cells (Fig. 2B).

Dose–response effect of serum concentration on cell growth and apoptosis

Quantitative analyses with the 96-well cell growth assay demonstrated the ideal concentration of serum for cell survival to be 10 to 20 percent (Fig. 3*a*). Increased concentrations of intact serum resulted in significant growth suppression at 60% and 90% ($P<0·0001$). The quantitative analysis with photometric ELISA (Fig. 3*b*) revealed a basal level of apoptosis in serum-free conditions. This basal level of apoptotic index was suppressed at 10% HS concentration. Further increase in HS concentration (from 30% to 90%) increased the apoptotic index in a dose-dependent manner with a significant

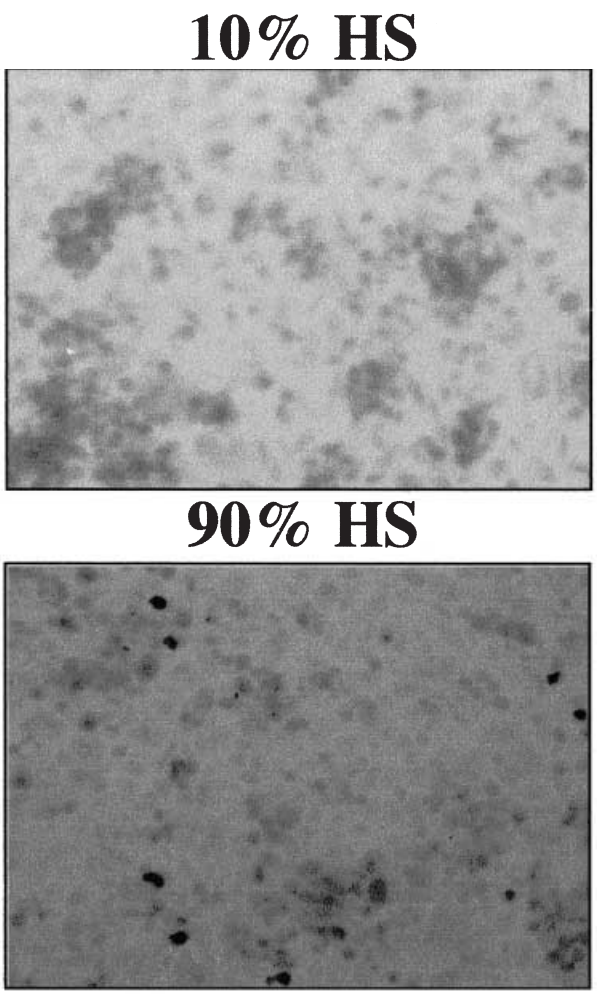

Figure 2 Detection of serum-induced apoptosis in PC-3 cells: TUNEL of cells grown in 10% and 90% HS. The cytoplasmic DNA fragments were detected *in situ* in the 10% and 90% serum-treated PC-3 cells after incubation with biotinylated dNTP mix and TdT. The free 3′-OH DNA fragments were visualized using a streptavidin-horseradish peroxidase detection system with the positive cells appearing black.

($P<0{\cdot}01$–$P<0{\cdot}0001$) increase in the number of apoptotic cells with increasing serum treatment (ANOVA).

Comparative analysis of IGFBP-3 depletion and supplementation on cell growth and apoptosis

Figure 4 shows that the toxic concentration of serum starts with 40% HS at which the growth suppression (data not shown in this graph) as well as apoptosis induction is significantly higher compared with 10% HS. In addition even at 60% HS concentration, depletion of IGFBP-3 removed more than 50% of the apoptosis-inducing effect of intact serum and supplementation of physiological concentrations (500 ng/ml) of IGFBP-3 brought back more than half of the effect of IGFBP-3 depletion. Replenishing physiological levels of IGF-I and IGF-II did not completely suppress the apoptosis induced by DS.

IGFBP-3 induces apoptosis

Using the quantitative ELISA method we demonstrated that cells treated with IGFBP-3 (500 ng/ml) show a significant ($P<0{\cdot}001$) increase in the level of apoptotic index relative to the serum-free condition (Fig. 5), which is similar to that of the effect of the ionophore, Valinomycin, a positive control. Higher concentrations of IGFBP-3 (5 μg/ml) showed a further increase in the apoptotic index. This degree of apoptosis was similar to that seen in cells treated with 90% HS.

Apoptosis induction by IGFBP-3 in high serum concentrations

The quantitative ELISA was used to demonstrate the effect of DS in inducing apoptosis in PC-3 cells (Fig. 6). While treatment with 90% intact HS demonstrated a degree of apoptosis similar to the Ca^{++} ionophore treatment ($P<0{\cdot}001$), DS showed a 33% lower degree of apoptosis ($P<0{\cdot}01$ relative to 90% HS). However, the repletion of IGFBP-3 (500–5000 ng/ml) without IGFs to the DS brought back the apoptotic index to the level seen with treatment with 90% HS ($P<0{\cdot}01$ relative to DS treatment). Suppression of apoptosis by exogenous IGF-I and IGF-II (each 500 ng/ml) added to IGFBP-3-depleted human sera (90%) was not statistically significant.

Discussion

Five to thirty percent serum concentrations are commonly added to chemically defined medium for the purpose of optimizing the growth of cells *in vitro*. When serum is highly concentrated, however, cell death is induced. This cell death-inducing activity of serum is seen regardless of the source of the sera in terms of species or age (Kurita & Namiki 1994). The nature of the apoptosis-inducing activity of serum has not been clearly defined but has previously been shown to be partially prevented by thiols and IGFs. We now demonstrate IGFBP-3 to be involved in this phenomenon.

IGFs have been shown to protect cells from undergoing apoptosis through an IGF-1R-mediated cell survival pathway (Resnicoff *et al.* 1995, Sell *et al.* 1995, Stewart & Rotwein 1996, Werner *et al.* 1996). Both the effect of a decrease in the number of IGF-1Rs causing apoptosis and an over-expression of IGF-1R protecting cells from apoptosis have been demonstrated *in vivo* (Resnicoff *et al.* 1995). The role of IGFs and the IGF-1R as autocrine survival factors (Stewart & Rotwein 1996) and as protective agents that prevent apoptosis induced by other agents, such as etoposide, has also been shown (Sell *et al.* 1995).

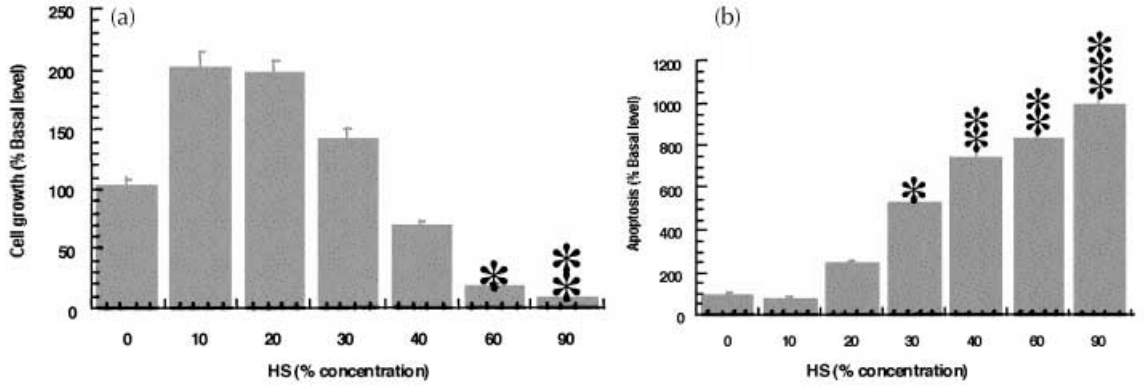

Figure 3 Quantitative analysis of cell growth and apoptosis. (a) Quantitative analysis and dose-dependent response of serum on cell growth. PC-3 cells were plated at 1×10^2 cells/cm^2 in 96-well plates. A non-radioactive CellTiter 96 assay was used to measure cell proliferation. Samples were treated in multiples of eight for each condition. The absorbance reading (490 nm) is directly proportional to the number of viable cells/well. The resulting means and standard deviations were determined and presented as a percentage of the serum-free (basal) level. Cell growth suppression by 90% serum treatment was significant relative to serum-free conditions. (b) Quantitative analysis and dose-dependent response of serum-induced apoptosis in PC-3 cells. Photometric ELISA was used to determine the change in apoptotic index in cells treated with increasing concentrations of serum. The fractions of small DNA fragments extracted from the samples were used for this experiment. The histones of the DNA fragments bound to anti-histone antibodies on the 96 wells were detected using a peroxidase-labeled mouse monoclonal DNA antibody and photometric reaction. The amount of fragmented DNA present in each sample was estimated and the results were plotted as a bar graph. Each value represents the mean ± S.E.M. of triplicate experiments. Results are presented as a percentage of the serum-free (basal) level. ANOVA analysis demonstrated significant dose dependence (*P<0·01, **P<0·001, ***P<0·0001).

Mutant versions of p53 protein, commonly associated with malignant states, have been shown to de-repress the IGF-I-R promoter, with ensuing mitogenic activation by locally produced or circulating IGFs (Werner *et al.* 1996). In addition, the IGF-1R has been shown to partially protect cells from apoptosis induced by high serum concentration (Valentinis & Baserga 1996). All the above mentioned studies indicate the important role of IGFs and the IGF-1R in preventing cells from undergoing apoptosis through a cell survival pathway.

We previously demonstrated a pathway for induction of apoptosis by IGFBP-3 independent of IGF and IGF receptor effects (Rajah *et al.* 1997). In this study, we have shown a similar effect of IGFBP-3 on cells *in vitro* when presented in high concentrations of serum. IGFBP-3 in serum is mostly found bound to acid labile subunit (ALS). This raises questions such as whether bound IGFBP-3 is capable of inducing apoptosis and whether free IGFBP-3 is presented to cells by some means when provided in high concentrations. Data from this study allows us to presume both possibilities i.e. that IGFBP-3 bound to ALS is capable of inducing apoptosis and that IGFBP-3 may be found in free form under these conditions in culture and therefore is capable of inducing apoptosis *in vitro*. This presumption is made possible by the observation that addition of free IGFBP-3 is capable of replenishing the levels of apoptosis that was suppressed by the removal of IGFBP-3 together with IGFs from the serum.

The availability of free IGFBP-3 in the serum relies on phenomena such as its affinity to IGF, its putative

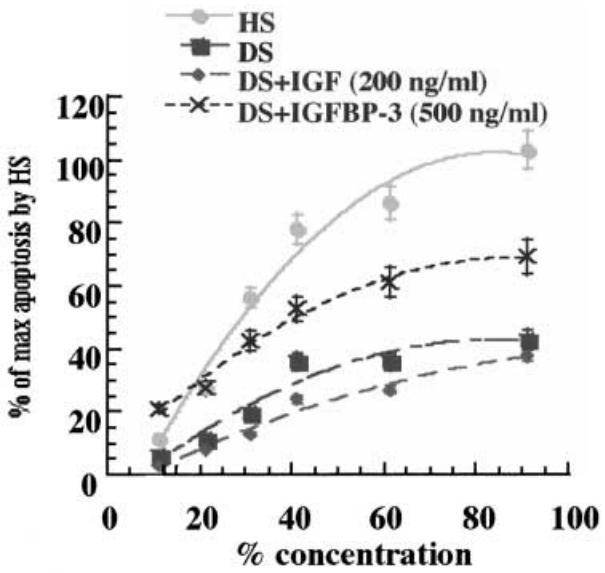

Figure 4 Analysis of serum IGFBP-3 depletion and supplementation on apoptosis. Cell growth and apoptosis were measured as detailed in Materials and Methods. Intact serum induces apoptosis in a dose-dependent manner. Depletion of IGFBP-3 from serum in increasing concentrations surpassed the apoptosis induced by intact serum of that concentration. Supplementation of IGFBP-3 alone brought the apoptosis level back close to that of serum-induced apoptosis, but not completely. Supplementation with IGFs (IGF-I and -II 500 ng/ml each) did not completely suppress apoptosis induced by DS. Each value represents the mean ± S.D. of triplicate experiments.

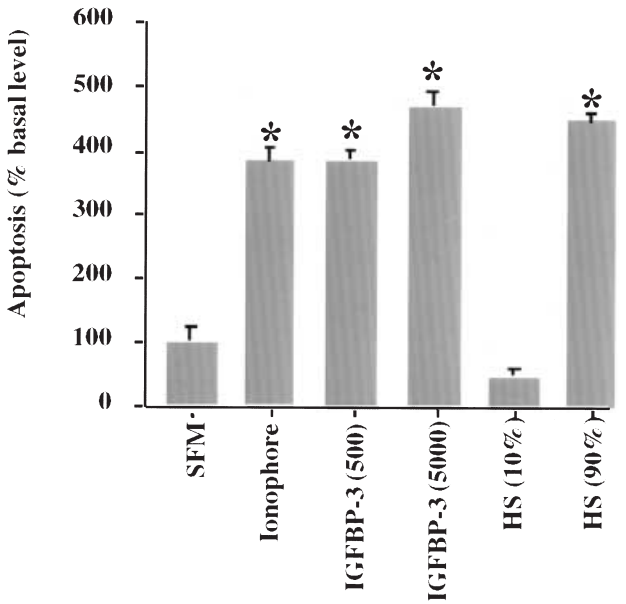

Figure 5 Potency of serum-induced apoptosis. Photometric ELISA was used to determine the change in apoptotic index of cells treated with SFM, the positive control (Valinomycin, an ionophore), IGFBP-3 (500–5 μg/ml), and 10% and 90% HS. Each value represents the mean ± S.E.M. of triplicate experiments. Results are presented as a percentage of the basal level (SFM=basal level). **P*<0·001 relative to 10% serum-treated condition.

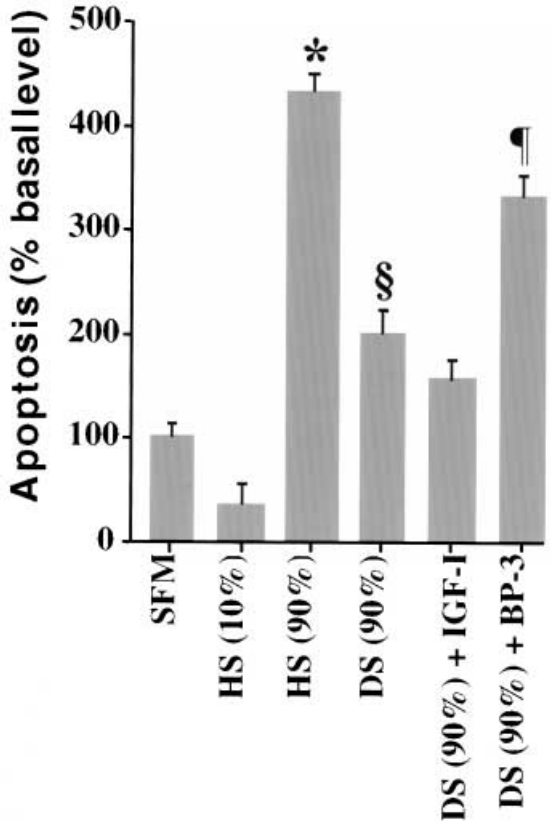

Figure 6 Effects of IGFBP-3 depletion on serum-induced apoptosis. Photometric ELISA was used to determine the change in apoptotic index of cells treated with SFM, 10% and 90% HS, 90% DS and 90% DS replenished with either IGFs (IGF-I or -II 200 ng/ml) or recombinant IGFBP-3 (500 ng/ml) (DS+BP-3). Each value represents the mean ± S.E.M. of triplicate experiments. Results are presented as the percentage of the basal level (SFM=basal level). **P*<0·001 compared with 10% HS-treated condition; §*P*<0·001 compared with intact 90% HS-treated condition; ¶*P*<0·001 compared with DS.

receptors and other association proteins. An IGF-independent action of IGFBP-3 has been demonstrated in cellular systems. We and others have previously demonstrated the role of IGFBP-3 as a growth inhibitory protein in various cell types (Goldstein *et al.* 1991, Cohen *et al.* 1993*a*,*b*, 1994, Kaicer *et al.* 1993, Moerman *et al.* 1993, Velez-Yanguas *et al.* 1996, De Mellow & Baxter 1998). Initially, IGFBP-3 was thought to inhibit growth by binding to IGFs and sequestering them from their receptors. Later, it was suggested that the cell growth inhibitory effect of IGFBP-3 was also IGF independent and involved cell growth arrest (Moerman *et al.* 1993, Velez-Yanguas *et al.* 1996). IGFBP-3 expression is induced by p53 activation and expression of IGFBP-3 has been shown to be essential for the ability of p53 to induce apoptosis (Buckbinder *et al.* 1995). We recently demonstrated an apoptosis-inducing role for IGFBP-3 in a p53-independent pathway, and also demonstrated that this mechanism was IGF/IGF-1R independent and mediated the effect of TGFβ on cell death. Observations from the study presented here further extend the possible role of IGFBP-3 as an apoptosis-inducing protein *in vivo*.

In serum, IGFBP-3 is found mostly bound to ALS and to IGFs as a 150 kDa ternary complex (Baxter 1994). This large complex does not cross the capillary barrier and is found only in the intravascular space. The existence of IGFBP-3 in serum as a non-diffusable complex may represent a means of protection to prevent this apoptosis-inducing agent from reaching cells in high concentrations. The effects of serum on the induction of apoptosis in transplanted tissues directly exposed to serum have recently been proposed (Kurita & Namiki 1994). Certain cells, particularly endothelial cells and circulating white blood cells are presumably protected from serum-induced apoptosis. Other cell types may be physiologically programmed to die in the presence of serum as a mechanism of limiting metastatic seeding of tumor cells through the circulation. The reduced levels of IGFBP-3 in the sera of aging individuals may contribute to the increased incidence of tumors in the elderly.

Observations from this study clearly demonstrate that IGFBP-3 is one of the factors that induces apoptosis in high serum concentrations. IGFs also appear to modulate

serum-induced apoptosis. However, even after the removal of IGFBP-3 from serum, it appears that there may be additional factors found in serum that may also play a role in mediating serum-induced apoptosis. One such factor could be tumor necrosis factor-α which has been shown to induce apoptosis in numerous models (Vanhaesebroeck *et al.* 1993, Flugy *et al.* 1995, Halicka *et al.* 1995). Other agents, yet to be identified, may also play a role.

From these observations it is evident that the ratio of free IGFs and IGFBP-3 regulate cell growth not only by balancing the rate of cell proliferation and cell growth arrest, but also by regulating the rate at which the cells might be induced to undergo apoptosis. In summary, IGFBP-3 in serum is capable of inducing apoptosis at high concentrations. The effects of serum on apoptosis, however, involve additional factors including IGFs, which may operate in concert with IGFBP-3 to regulate cell survival in the circulation.

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