EXPERIMENTAL STUDY

Effects of IGF-I and -II, IGF binding protein-3 (IGFBP-3), and transforming growth factor- β (TGF- β) on growth and apoptosis of human osteosarcoma Saos-2/B-10 cells: lack of IGF-independent IGFBP-3 effects

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Abstract

Objective: Insulin-like growth factor binding protein-3 (IGFBP-3) inhibits cell growth. Previous reports have suggested the existence of plasma membrane IGFBP-3 receptors that could mediate direct, IGF-independent effects. Thus far, however, the only well-defined putative IGFBP-3 receptor is the type V transforming growth factor- β (TGF- β) receptor, a membrane glycoprotein that mediates TGF- β -induced growth inhibition in selected cells. The aim of the study was to test whether IGFBP-3 and TGF- β exert short-term effects in an osteosarcoma cell line that produces no IGF but contains type 1 IGF receptors.

Design: DNA synthesis and apoptosis in Saos-2/B-10 cells were measured in response to IGF-I, IGF-II, IGFBP-3 and TGF- β_2 , and to type 1 IGF receptor ligands with poor affinity for IGFBP-3 ([QAYL]-IGF-I and insulin).

Results: IGF-I and IGF-II stimulated thymidine incorporation into DNA and suppressed apoptosis in a dose-dependent manner with maximal effects at 1 and 3 nM respectively. TGF- β_2 slightly increased thymidine incorporation into DNA but had no effect on apoptosis. IGFBP-3 had no effect by itself. Whereas it blocked the above effects of 1 nmol/l IGF-I, it did not block those of 1 nmol/l [QAYL]-IGF-I or 100 nmol/l insulin.

Conclusions: IGFBP-3 does not affect DNA synthesis or apoptosis in an IGF-independent manner in IGF-responsive osteosarcoma cells. It therefore appears to act essentially by sequestration of IGF.

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Introduction

Increased levels of insulin-like growth factor (IGF-I) have been associated with breast, prostate and colon cancer growth. IGF-I stimulates growth of a variety of human malignant cells in tissue culture, including breast cancer, prostate carcinoma, and osteosarcoma cells. It is a mitogen for several osteosarcoma cell lines, such as MG-63 (1, 2), Saos-2 (3–5) and OHS-4 (6), and inhibits apoptosis (4). Type 1 IGF (and insulin) receptors function as tyrosine kinases and signal through these receptors to trigger mitogenic and anti-apoptotic pathways. Treatment of Saos-2 cells with α IR-3, a blocking monoclonal antibody against the type 1 IGF receptor, or with an antisense oligonucleotide to the type 1 IGF receptor decreased [3 H]thymidine incorporation and cell proliferation, suggesting that signaling via this receptor was necessary for growth and survival of these cells *in vitro* (7). IGF binding protein-3 (IGFBP-3), the main GH-dependent IGFBP in adult human serum, blocks IGF-stimulated thymidine

incorporation into human fibroblasts (8) and Saos-2 cells (3, 5) in a dose-dependent manner. It induces apoptosis in MCF7 breast cancer cells when tested in the presence of 0.5% FCS over a period of 72 h (9). Recent studies have indicated that IGFBP-3 may directly inhibit DNA synthesis and promote apoptosis in Hs578T cells, a human breast cancer cell line (10, 11) and in PC-3 human prostate carcinoma cells (12). IGFBP-3 has, therefore, been suggested to bind to specific IGFBP-3 receptors (13). However, these receptors have not yet been cloned, their signaling pathway has remained elusive, and short-term biological effects have not been clearly documented. In Hs578T breast cancer cells, IGFBP-3 binding was found to correlate with its ability to inhibit growth, and IGF-I (which, by itself, did not stimulate DNA synthesis in this particular cell line) decreased cell surface binding of IGFBP-3 and prevented IGFBP-3-induced growth inhibition (10). In PC-3 prostate carcinoma cells, IGFBP-3-induced apoptosis was reversed by IGF-I but not by an IGF analog that does not bind IGFBP-3, again suggesting that

growth inhibition and apoptosis were caused by IGFBP-3 and not by IGF-I–IGFBP-3 complexes. Moreover, IGFBP-3 has been reported to mediate effects of TGF- β on apoptosis in this cell line (12). IGFBP-3 has also been shown to predispose Hs578T breast cancer cells to ceramide-induced apoptosis in an IGF-independent manner (11, 14). Transfection of the human IGFBP-3 gene into murine fibroblasts inhibited cellular proliferation (15), and inhibited growth of fibroblast cell lines derived from IGF-I receptor-knockout embryonic mice, animals which are not viable after birth (16). The latter cells exhibit low growth rates, and their apoptosis is enhanced when rhIGFBP-3 is added to the culture medium (12). Most studies used long-term incubations (24 h and more) to show potentially IGF-I-independent effects of (intact) IGFBP-3, and/or they used genetically manipulated cells, such as IGF-I-unresponsive, type 1 IGF receptor-deficient cells, i.e. cells that exhibit rather unusual features. In human cancer, however, absence of p53 may be much more common than absence of type 1 IGF receptors. Most of these experimental systems are also complicated by the fact that IGFs and IGFBPs may be locally produced and processed. Therefore, we looked at whether IGFBP-3 and TGF- β affect growth and apoptosis in short-term experiments independently of type 1 IGF (or insulin) receptor signaling in an IGF-responsive cell line that produces no detectable amounts of IGF-I and IGF-II.

Materials and methods

Cells and peptides

Saos-2/B-10 cells (17) were kindly provided by Drs S and GA Rodan (Merck, Sharp & Dohme, Westpoint, PA, USA). Recombinant human (rh) IGF-I and IGF-II were from the former Ciba-Geigy, Basel, Switzerland. [Gln³, Ala⁴, Tyr¹⁵, Leu¹⁶]-IGF-I ([QAYL]-IGF-I) was a generous gift from Dr M Cascieri (Merck, Sharp & Dohme, Rahway, NJ, USA). Insulin was from Novo Nordisk, Gentofte, Denmark. Glycosylated rhIGFBP-3 expressed in Chinese hamster ovary cells (IGFBP-3^{CHO}), non-glycosylated rhIGFBP-3 expressed in *Escherichia coli* (IGFBP-3^{E. coli}) and rabbit antihuman IGFBP-3 antiserum were kindly provided by Dr A Sommer, Celtrix, Santa Clara, CA, USA. rh transforming growth factor (TGF- β_2) was from R&D Systems, Abingdon, UK. Fatty acid-free bovine serum albumin (BSA) was from Sigma (St Louis, MO, USA).

Cell culture

Cells were passaged in Falcon tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin (50 μ g/ml), glutamine (2 mmol/l) and 10% FCS at 37 °C in an atmosphere of 5% CO₂ in air. Cell cultures between passages 10 and 40 were used. Cells grown to confluence were detached

from the dishes with 0.25% trypsin and replated either in Falcon six multiwell tissue culture plates (9.6 cm² surface area) at a density of 2×10^5 cells per well or in 24 multiwell (2 cm² surface area) dishes at a density of 4×10^4 cells per well and grown for 3 days in 5% FCS-containing medium, rinsed with serum-free medium, and incubated for 4 or 18 h in serum-free Ham's F12 medium containing 2 mmol/l glutamine, 50 μ g/ml gentamicin and 1 g/l BSA.

Proliferation assay

Thymidine incorporation into DNA After 3 days of growth, the confluent cultures were washed in serum-free Ham's F12 medium and incubated in 1 ml serum-free F12 medium supplemented with 2 mmol/l glutamine, 50 μ g/ml gentamicin, 1 g/l BSA and test agents for 18 h, pulsed with methyl-[³H]thymidine (Amersham, 85 Ci/mmol; 1 μ Ci per dish) and further incubated for 3 h at 37 °C. They were then rinsed with ice-cold PBS, and DNA was precipitated with 10% trichloroacetic acid. Incorporated radioactivity was measured in a liquid scintillation counter.

DNA fragmentation assay

Photometric enzyme-immunoassay for determination of cytoplasmic histone-associated DNA fragments An ELISA kit (Cell Death Detection ELISA^{PLUS} from Boehringer Mannheim) was used to measure cytosolic oligonucleosome-bound DNA. The test allows quantitative determination of histone-associated DNA fragments (mono- and oligonucleosomes) and is based on a sandwich-enzyme immunoassay which detects enrichment of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by monoclonal antibodies directed against DNA and histones. A total of 4×10^4 cells were seeded in 24 multiwell (2 cm² surface area) dishes and grown for 3 days in FCS-containing medium, rinsed with serum-free medium and incubated for 4 h in 200 μ l of serum-free Ham's F12 medium containing 1 g/l BSA. Media were removed and the cell layers were washed twice with ice-cold PBS, then directly lysed into 400 μ l of kit lysis buffer and centrifuged for 10 min at 200 *g* at room temperature. A 20 μ l volume was used for analysis following the manufacturer's instructions. Results are expressed as the difference in absorbance at 405 and 490 nm ($A_{405\text{ nm}} - A_{490\text{ nm}}$). High values reflect high amounts of DNA fragments.

¹²⁵I-IGF-I binding studies

A total of 4×10^4 cells were grown in multiwell plates (2 cm² surface area) as described above, kept in serum-free medium for 24 h, rinsed with saline, and incubated over night at 4 °C in 200 μ l Dulbecco's buffer containing 1 g/l BSA and ~50 000 c.p.m. of monoiodinated

(3-[¹²⁵I]iodotyrosyl) IGF-I [~ 2000 Ci/mmol, from Amersham], and unlabeled IGF-I as indicated. Cells were rinsed, lysed, and bound radioactivity was counted in a gamma counter.

Processing and analysis of cell culture media

Ligand and immunoblot analysis of IGFBPs Conditioned media were collected after 4 and 18 h, centrifuged at 250 *g* for 10 min, and the supernatants were stored at -20°C . ¹²⁵I-IGF-II ligand blot and IGFBP-3 immunoblot analysis were performed to screen for the production of IGFBPs and to check for the recovery of added rhIGFBP-3. Unprocessed media aliquots were used to check for the recovery of rhIGFBP-3, whereas media were concentrated prior to analysis for detection of secreted IGFBPs. To this end, 10^6 cells were plated in 55 cm² dishes in serum-containing medium as described above, rinsed with serum-free medium and kept for 18 h in 10 ml serum-free Ham's F12 medium containing 1 g/l BSA. Conditioned media were collected, dialyzed against 0.1 mol/l ammonium bicarbonate, lyophilized and dissolved in 200 μl of distilled water. Aliquots (15 μl) were electrophoresed on 15% SDS polyacrylamide gels under non-reducing conditions (except ¹⁴C-labeled molecular weight markers; Rainbow marker, Amersham, UK) and transferred to nitrocellulose membranes. The air-dried filters were washed in Tris-buffered saline (TBS; 0.15 mol/l NaCl and 0.01 mol/l Tris, pH 4), containing 0.1% Tween 20 and then incubated with ¹²⁵I-labeled IGF-II (4×10^6 c.p.m. per plastic bag) for 6 h at room temperature. After washing with TBS containing 0.1% Tween 20, the filters were exposed for 1 day at -80°C to an X-ray film (Kodak X-OMAT AR) in cassettes equipped with intensifying screens as described (18). For immunoblot analysis, 30 μl media aliquots were loaded per lane and electrophoresed under reducing conditions. The air-dried filters were washed in TBS, pH 7.6, containing 0.1% Tween 20. The first antibody (anti-IGFBP-3) was used at a dilution of 1:1000, the second (anti-rabbit, horseradish peroxidase-conjugated, from Anawa, Wangen, Switzerland) at a dilution of 1:500. Bands were detected by enhanced chemiluminescence with RPN 2106 following the prescription of the supplier (Amersham, UK) as described (4), and their relative intensities were evaluated by densitometry (GS-700, BioRad, Glattbrugg, Switzerland).

Statistical analysis

The results are presented as means $\pm$ S.E.M.S. The difference between means was evaluated by the Wilcoxon rank order test. A probability level of a random difference of $P < 0.05$ is considered statistically significant.

Results

Effects of IGF-I and IGF-II

DNA synthesis IGF-I and IGF-II stimulate [³H]thymidine incorporation into DNA of Saos-2/B-10 cells (Fig. 1A). Both peptides are effective at low concentrations. At 0.3 nmol/l, IGF-I was more effective than IGF-II ($P < 0.01$), and maximal stimulation was observed at 1 nmol/l IGF-I or at 3 nmol/l IGF-II. IGF-I was two to three times more potent than IGF-II and equally potent as [QAYL]-IGF-I (not shown). To achieve maximal stimulation of [³H]thymidine incorporation into DNA of Saos-2/B-10 cells by insulin, 100 nmol/l was required (not shown).

Apoptosis Microscopic examination of the cell cultures showed that a large number of cells detached from the dishes after serum withdrawal, i.e. during the test period. Less cells detached when they were kept in medium supplemented with IGF-I or IGF-II. Therefore, we analyzed DNA extracted from detached cells for fragmentation. As assessed by gel electrophoreses after 4 and 18 h, DNA was fragmented to a larger extent in control cells, whereas IGF-I and IGF-II prevented DNA fragmentation (not shown).

Since analysis of extracted DNA by gel electrophoresis does not allow us to reliably distinguish apoptosis from necrosis, enrichment of oligonucleosomes in the detached and adherent cells was monitored by using monoclonal antibodies directed against DNA and histones (4). The cell layer lysates were analyzed after 4 h of exposure of the cells to test medium. The data, expressed as absorbance ($A_{405\text{nm}} - A_{490\text{nm}}$), show that IGF-I and IGF-II are potent inhibitors of apoptosis (Fig. 1B). As described for thymidine incorporation into DNA, there is a clear dose-response relationship; IGF-II was less potent than IGF-I by a factor of about 5.

¹²⁵I-IGF-I binding ¹²⁵I-IGF-I binding studies showed that $\sim 20\%$ of the added counts was bound. Over 97% of the bound ¹²⁵I-IGF-I could be displaced by 1 $\mu\text{mol/l}$ cold IGF-I, i.e. non-specific binding was less than 3% of the total binding. Half-maximal displacement was observed at 0.4 nmol/l IGF-I (Fig. 2) (or with threefold higher concentrations of IGF-II [not shown]).

Effects of IGFBP-3 and TGF- β_2

DNA synthesis IGFBP-3 (both rhIGFBP-3^{E. coli} and rhIGFBP-3^{CHO}) by itself had no significant effect on [³H]thymidine incorporation into DNA of Saos-2/B-10 cells at 1, 3 (not shown) and 10 nmol/l (Table 1), in line with previous reports (3, 4). IGFBP-3 (10 nmol/l) prevented stimulation by 1 nmol/l IGF-I and IGF-II but not stimulation by 100 nmol/l insulin or 1 nmol/l [QAYL]-IGF-I (Table 1a). [QAYL]-IGF-I is an IGF analog

Figure 1 (A) Effects of rhIGF-I and rhIGF-II on [³H]thymidine incorporation into DNA of Saos-2/B-10 cells. Cells were grown for 3 days in 5% FCS, then exposed to serum-free test medium containing IGF-I or IGF-II for 18 h at 37 °C as indicated, and pulsed with [³H]thymidine for additional 3 h as described in Materials and Methods. Data are means $\pm$ S.E.M.s from six different experiments, $n = 19$. (B) Inhibition of apoptosis by IGF-I and IGF-II: detection of nucleosomes (arbitrary absorbance values) in cytoplasmic fractions of Saos-2/B-10 cells after 4 h of exposure to serum-free test medium containing IGF-I and IGF-II. Cells were grown for 3 days in 5% FCS and exposed to serum-free test medium containing IGF-I and IGF-II for 4 h at 37 °C as described in Materials and Methods. The medium was removed, the cells were rinsed, lysis buffer was added directly to the cell layer, and aliquots of the lysate were analyzed (see Materials and Methods). Data show the means $\pm$ S.E.M.s of five different experiments in triplicate (assayed in duplicate), $n = 15$, for absorbance ($A_{405\text{ nm}} - A_{490\text{ nm}}$).

that binds normally to type 1 IGF receptors but has a 600-fold reduced affinity for IGFBP-3 (19).

TGF- β_2 was tested under the same conditions, at concentrations up to 0.2 (Table 1b) and 1 nmol/l (not

Figure 2 Competitive inhibition of the binding of ¹²⁵I-IGF-I by unlabeled IGF-I in Saos-2/B-10 cells. Cells were grown in multiwell plates (2 cm² surface area) and exposed to serum-free medium for 24 h before test incubations overnight at 4 °C in buffer containing ¹²⁵I-IGF-I and unlabeled IGF-I as described in Materials and Methods. Total binding of ¹²⁵I-IGF-I is shown as a function of added cold IGF-I and expressed in percent (100%=tracer binding by cells in the absence of cold IGF-I). Means $\pm$ S.E.M.s from three experiments carried out in triplicate are shown.

shown), both alone and in combination with 1 nmol/l IGF-I. A slight stimulatory effect on [³H]thymidine incorporation into DNA was observed at 0.01, 0.1 and 0.2 nmol/l; however, TGF- β_2 did not modify the stimulatory effect of IGF-I (Table 1b).

Apoptosis IGFBP-3^{E. coli} at a concentration of 10 nmol/l had no effect on DNA degradation as assessed by DNA gel electrophoresis after 4 or 18 h in serum-free test medium (not shown) and no significant effect on apoptosis unless 1 nmol/l IGF-I or 5% FCS were present (Table 2). The data summarized in Table 2 also show that IGFBP-3 had no significant effects by itself (in the absence IGF-I) and no effect when cells were kept in the presence of 100 nmol/l insulin or 1 nmol/l [QAYL]-IGF-I. IGFBP-3^{E. coli} at a concentration of 10 nmol/l completely blocked the effects of 1 nmol/l IGF-I, but only partially those of 5% FCS (Table 2a). Identical results were obtained when 10 nmol/l (glycosylated) IGFBP-3^{CHO} (not shown) were used instead of (non-glycosylated) IGFBP-3^{E. coli}. TGF- β_2 at a concentration of 0.1 nmol/l had no effect on DNA degradation after 18 h of incubation (not shown) and it had no effect on apoptosis after 4 h of incubation (not shown).

Processing, production and recovery of endogenous and added IGFBP-3 Added rhIGFBP-3 can be recovered from the medium after 18 h of incubation,

Table 1 Effects of IGFBP-3 and TGF- β_2 on basal and IGF-I-stimulated thymidine incorporation into DNA of Saos-2/B-10 cells. Cells were handled as described in legend to Fig. 1A. (a) Effects of 10 nmol/l IGFBP-3^{E. coli}, 1 nmol/l IGF-I, 1 nmol/l [QAYL]-IGF-I and 100 nmol/l insulin. Data are means $\pm$ S.E.M.s from three experiments in triplicate. (b) Effects of TGF- β_2 and 1 nmol/l IGF-I. Data are means $\pm$ S.E.M.s from four experiments in triplicate.

(a) Effects of rhIGFBP-3^{E. coli}, IGF-I, [QAYL]-IGF-I and insulin				
	Control	1 nmol/l IGF-I	1 nmol/l [QAYL]-IGF-I	100 nmol/l insulin
Control	3595 $\pm$ 541	16 421 $\pm$ 3239*	14 839 $\pm$ 2317*	15 682 $\pm$ 2756*
IGFBP-3, 10 nmol/l	3549 $\pm$ 508	3530 $\pm$ 555	15 101 $\pm$ 2546*	15 509 $\pm$ 2659*

* $P < 0.05$ treated versus control.

(b) Effects of TGF-β_2 and IGF-I					
	Control	TGF- β 0.001 nmol/l	TGF- β 0.01 nmol/l	TGF- β 0.1 nmol/l	TGF- β 0.2 nmol/l
Control	2469 $\pm$ 168	2709 $\pm$ 246	2944 $\pm$ 228*	2896 $\pm$ 222*	2953 $\pm$ 186*
IGF-I, 1 nmol/l	9008 $\pm$ 1016*	10 164 $\pm$ 1135*	9619 $\pm$ 1203*	9070 $\pm$ 1012*	9484 $\pm$ 971*

* $P < 0.05$ treated versus control.

as shown by ¹²⁵I-IGF-II ligand blot of unprocessed conditioned medium. A stronger 33 kDa band (reflecting IGFBP-3^{E. coli}) is found in medium from cells treated with IGF-I than in medium from control-treated cells (Fig. 3A). IGFBPs produced by the cells were only detectable when the media were concentrated (Fig. 3B). The ligand blot then showed several IGFBPs apart from the exogenous rhIGFBP-3, including the 45/42 kDa doublet (which is also increased in response to IGF-I as compared with the control)

characteristic for IGFBP-3, as also observed in adult human serum (Fig. 3A and B).

By Western blot analysis using specific polyclonal IGFBP-3 antibodies and chemiluminescence, as little as ~ 1 ng of IGFBP-3 could be detected. Added rhIGFBP-3^{CHO} could be almost completely recovered after 18 h of incubation, indicating a long half-life. By contrast, IGFBP-3^{E. coli} was less well recovered (Fig. 4), probably due to partial losses by rapid adherence to plastic and/or absorption or (slow) processing by cells, as assessed

Table 2 Effects of rhIGFBP-3^{E. coli} on apoptosis of Saos-2/B-10 cells in control-, 1 nmol/l IGF-I- 5% FCS-, 100 nmol/l insulin- and 1 nmol/l [QAYL]-IGF-I-supplemented medium. Cells were handled as described in legend to Fig. 1B and analyzed for enrichment of oligonucleosomes 4 h after exposure to test media containing rhIGFBP-3^{E. coli} as indicated. Data (expressed in $A_{405\text{ nm}} - A_{490\text{ nm}}$, means $\pm$ S.E.M.s) are shown from five experiments (carried out in triplicate) for comparison of rhIGF-I with 5% FCS (a), from three experiments (carried out in triplicate) for comparison of rhIGF-I with insulin (b) and from three experiments (carried out in triplicate) for comparison of 1 nmol/l IGF-I and 1 nmol/l [QAYL]-IGF-I (c).

(a) Effects of rhIGFBP-3, IGF-I and FCS			
	Control	1 nmol/l IGF-I	5% FCS
Control	0.557 $\pm$ 0.032	0.074 $\pm$ 0.008*	0.147 $\pm$ 0.015*
IGFBP-3, 1 nmol/l	0.685 $\pm$ 0.039	0.179 $\pm$ 0.055*	0.201 $\pm$ 0.010*
IGFBP-3, 3 nmol/l	0.620 $\pm$ 0.030	0.500 $\pm$ 0.034	0.296 $\pm$ 0.026*
IGFBP-3, 10 nmol/l	0.502 $\pm$ 0.029	0.445 $\pm$ 0.038	0.329 $\pm$ 0.038*

* $P < 0.05$ treated versus control.

(b) Effects of rhIGFBP-3, IGF-I and insulin			
	Control	1 nmol/l IGF-I	100 nmol/l insulin
Control	0.518 $\pm$ 0.047	0.048 $\pm$ 0.002*	0.058 $\pm$ 0.007*
IGFBP-3, 10 nmol/l	0.418 $\pm$ 0.032	0.398 $\pm$ 0.023	0.037 $\pm$ 0.002*

* $P < 0.05$ treated versus control.

(c) Effects of rhIGFBP-3, IGF-I and [QAYL]-IGF-I			
	Control	1 nmol/l IGF-I	1 nmol/l [QAYL]-IGF-I
Control	0.648 $\pm$ 0.038	0.066 $\pm$ 0.008*	0.063 $\pm$ 0.007*
IGFBP-3, 10 nmol/l	0.734 $\pm$ 0.063	0.599 $\pm$ 0.038	0.054 $\pm$ 0.004*

* $P < 0.05$ treated versus control.

Figure 3 ^{125}I -IGF-II ligand blot analysis of IGFBPs in culture media of human osteoblastic osteosarcoma (Saos-2/B-10) cells 18 h after exposure to 10 nmol/l rhIGFBP-3^{E. coli} in the absence and presence of 1 nmol/l rhIGF-I. Comparison with normal human serum (2 µl; lane S). Cells were grown on 10 cm diameter dishes as described in Materials and methods. In (A), 20 µl (i.e. 1/500 of the material) aliquots were analyzed directly (without further processing), whereas in (B), media were dialyzed, lyophilized, reconstituted in 200 µl water and 10 µl (i.e. 1/20 of the material) were loaded per lane. Exposure time for (A) was 3 days and for (B) 1 day. Numbers indicate molecular masses of the markers.

by time course experiments (not shown). The decrease of intact rhIGFBP-3^{E. coli} over time is significantly attenuated by the addition of 1 nmol/l IGF-I but not by the addition of 1 nmol/l [QAYL]-IGF-I (Fig. 4) or of 100 nmol/l insulin (not shown). There is no significant loss of rhIGFBP-3^{CHO} within 18 h of incubation, whereas the fraction of rhIGFBP-3 remaining in the medium after 4 and 18 h as measured by densitometry was significantly below the initial concentration set 1.0 at time zero: 0.36 ± 0.04 , 0.57 ± 0.02 , 0.28 ± 0.02 and 0.32 ± 0.03 after 18 h of incubation in the absence of hormone (control) or in the presence of 1 nmol/l IGF-I, 1 nmol/l [QAYL]-IGF-I or 100 nmol/l insulin respectively (four experiments). The net amount

of IGFBP-3 secreted into the medium by the cells was by far exceeded by the amounts of added rhIGFBP-3 (Figs 3 and 4).

Discussion

Cell characteristics

The primary goal of the present study was to characterize medium- and short-term effects of IGFBP-3 in human Saos-2 cells on DNA synthesis and apoptosis, and to look for potential actions of TGF- β in this cell line. Saos-2 cells, a human osteogenic osteosarcoma cell line, express high alkaline phosphatase activity in a constitutive manner and produce low amounts of collagenous matrix (20, 21). As compared with non-transformed osteoblasts, both p53 alleles are deleted in Saos-2 cells (5). The p53 tumor-suppressor gene is the most commonly mutated (or deleted) gene in human cancer. p53 transfected into Saos-2 cells decreases type 1 IGF receptor expression by repressing the IGF receptor promoter activity (22, 23). p53 overexpression was also found to induce IGFBP-3 in colon carcinoma cells (5). Both findings are consistent with the observation that Saos-2/B-10 cells express high type 1 IGF receptor levels and only small amounts of IGFBP-3 (4). Since wild-type, hyperphosphorylated p53 functions as a brake, the IGF system in p53-deficient cells participates in enhancing progression through the cell cycle. Moreover, p53-deficient cells display a poor cell cycle control and poor cell cycle arrest upon serum withdrawal, which may predispose them to undergo apoptosis (see below).

The high number of IGF-I receptors ($\sim 100\,000/\text{cell}$) in Saos-2/B-10 cells and the fact that these cells do not express IGF-I and IGF-II (4, 24) may well explain why they are exquisitely sensitive to IGF-I (K_d for IGF-I binding and action ~ 0.3 nM; Figs 1 and 2), why they

Figure 4 IGFBP-3 immunoblot analysis of rhIGFBP-3^{CHO}- and rhIGFBP-3^{E. coli}-containing culture media before and after 18 h of exposure to Saos-2/B-10 cells: effect of 1 nmol/l IGF-I and of 1 nmol/l [QAYL]-IGF-I on the recovery of 10 nmol/l rhIGFBP-3^{E. coli} and rhIGFBP-3^{CHO}. Cells were grown as described in Materials and methods and exposed to serum-free test medium containing rhIGFBP-3 as indicated. Aliquots (30 µl) were analyzed directly (without further processing), i.e. the lanes loaded with 30 µl of 1 nmol/l of IGFBP-3 contain ~ 1 ng IGFBP-3. Numbers on the left side indicate molecular masses of the markers. On top, the letter 'b' designates before (time point: 0 h) and the letter 'a' after (time point: 18 h) incubation with cells. The letter 'C' denotes control (no IGF-I), 'I' denotes IGF-I, and 'Q' denotes [QAYL]-IGF-I.

critically depend on the addition of IGF-I or serum for survival and why the addition of IGFBP-3 in the presence of IGF-I potentially abolishes the effects of the latter, most likely by IGF sequestration (4). As shown by the two dose-response curves in Fig. 1A and B, Saos-2 cells were even approximately threefold more sensitive to IGF-I in terms of inhibition of apoptosis than with regard to stimulation of DNA synthesis. The potencies measured during short-time experiments, e.g. stimulation of phosphate and glucose uptake and glycogen synthesis (3) and inhibition of apoptosis (as shown in this study), may more closely reflect signaling through the type 1 IGF receptor than the potencies measured during longer-term incubations, e.g. in thymidine incorporation or cell replication studies. In the latter, IGF-II and even insulin and its analogs become relatively more potent as compared with IGF-I, and the dose-response curves are broader and no longer strictly parallel to the IGF-I dose-response curves obtained for short-term effects (3, 25 and C Schmid, unpublished observations).

In contrast to many reports using other cell types, neither toxic agents nor prolonged incubation times are required for Saos-2 cells to demonstrate an anti-apoptotic effect of IGF-I. IGF-I at a concentration of 1 nmol/l is at least as effective as 5% FCS in blocking apoptosis within 4 h (Table 2a). These findings as well as those presented in Tables 1 and 2 are in line with the observation that signaling through the type 1 IGF receptor is critical for both proliferation and survival of Saos-2 cells (7).

Effects of IGFBP-3

IGFBP-3 has been reported to exert short-term inhibitory and longer-term potentiating effects on IGF-I-stimulated DNA synthesis in human (8) and on aminoisobutyric acid uptake in bovine fibroblasts (26). We had previously found that intact but not truncated IGFBP-3 blocked IGF-I-stimulated DNA synthesis in Saos-2/B-10 and rat osteoblastic cells (3). We now investigated the effects of IGFBP-3 on apoptosis in addition to those on DNA synthesis. Although we found specific ^{125}I -IGFBP-3^{CHO} binding to Saos-2 cell layers (not shown), neither DNA synthesis nor apoptosis were affected by IGFBP-3 in the absence of IGF-I or in the presence of [QAYL]-IGF-I which barely binds IGFBP-3. Thus, IGFBP-3 *per se* has no effect in short-term (less than 24 h) studies with Saos-2/B-10 cells (Tables 1 and 2). These results are in agreement with the observation of Nickerson *et al.* (9) that IGFBP-3, even over longer term (3 days), overcomes the anti-apoptotic effect of IGF-I in MCF-7 breast cancer cells, but not that of long R³ IGF-I, which has a low affinity for IGFBP-3. These latter as well as our findings are at variance with the report that IGFBP-3 interacts with type 1 IGF receptors in Chinese hamster lung fibroblasts (CCL39) and thereby inhibits cell growth

(27). The identification of IGFBP-3 as a p53-inducible gene (5) and the finding that IGFBP-3 can increase apoptosis have led to the proposal that IGFBP-3 may serve a protective role against cancer cell growth (28), and that part of the IGFBP-3 effects are IGF-independent and mediated by specific cell surface IGFBP-3 receptors (10–16, 28). So far, however, the latter have not been chemically identified or cloned. Our data provide evidence at least against direct short-term effects of exogenous IGFBP-3 and thus against specific IGFBP-3 cell surface receptors in osteosarcoma cells. They are rather compatible with the well-established notion that IGFBP-3 acts by sequestering IGFs.

In order to test whether the lack of IGFBP-3 effects in the absence of IGF-I was due to IGFBP-3 degradation we checked whether this was the case. However, we found little degradation of IGFBP-3^{E. coli} (fragments were detectable by immunoblot only after prolonged exposure, not shown), and degradation of IGFBP-3^{CHO} was not detectable. It has been reported that IGF-I regulates IGFBP-3 in fibroblasts by a non-receptor-mediated mechanism (29, 30), a finding also reported for human breast cancer cells (31). Indeed, IGF-I stimulated IGFBP-3 production also in Saos-2/B-10 cells. But again, our observation that IGF-I-stimulated IGFBP-3 production is suppressed by rhIGFBP-3^{E. coli} (Fig. 3B) may well be explained by sequestration of the added IGF-I. This is also in line with the data presented in Tables 1 and 2, which show that IGFBP-3 acts in Saos-2 cells not by itself, but by sequestering IGF. IGFBP-3^{E. coli} and IGFBP-3^{CHO} have equal IGF binding properties and, therefore, act identically on IGF-I sequestration despite marked differences in half-life and adsorption characteristics. IGFBP-3^{E. coli} is more rapidly adsorbed to plastic surfaces than IGFBP-3^{CHO}, as also observed by Conover (32).

Effects of TGF- β

Leal *et al.* recently reported that among several IGFBP-3 binding sites found on extracellular matrix and cell surface structures, the type V TGF- β receptor was the putative IGFBP-3 receptor (33). We, therefore, examined whether TGF- β_2 affected DNA synthesis in Saos-2 cells. Previous studies had indicated that TGF- β_1 and TGF- β_2 inhibit growth with similar potencies and that the type V TGF- β receptor is specific for TGF- β_1 and TGF- β_2 binding (34). This 400 kDa membrane glycoprotein mediates TGF- β -induced growth inhibition in mink lung epithelial cells. ^{125}I -IGFBP-3^{E. coli} binds to the type V TGF- β receptor with an apparent K_d of 6 nmol/l (33). The same authors also found that several epithelial tumor cell lines, such as human breast cancer MCF-7, HepG2 hepatoma and colorectal carcinoma RII37 and HCT116 cells, lack the type V TGF- β receptor (33, 34), and that growth of these cells is not decreased by TGF- β . It was, therefore, suggested that lack of the type V TGF- β receptor contributes to an

escape from growth inhibition. In Saos-2/B-10 cells, TGF- β_2 does not inhibit DNA synthesis (Table 1b).

On the other hand, IGFBP-3 has been implicated as a mediator of the actions of TGF- β and p53 (5, 35), particularly because antisense deoxynucleotide to IGFBP-3 decreased growth inhibition induced by TGF- β_2 in human breast cancer cells (35) and because TGF- β stimulated the expression of IGFBP-3 in fibroblasts (36) and in breast and prostate cancer cells (12, 35). We have also found that 0.1 nmol/l TGF- β_2 is a potent stimulator of IGFBP-3 expression (as assessed by IGFBP-3 mRNA expression and accumulation of intact immunoreactive IGFBP-3 in the medium) in normal human fibroblasts under conditions where it did not affect IGFBP-3 production in Saos-2/B-10 cells (C Schmid, unpublished observations). TGF- β_2 strongly induces IGFBP-3 production and inhibits proliferation of normal prostatic stromal cells, but much less in cells from benign prostatic hyperplasia (37). IGFBP-3 does not *per se* inhibit growth of rat osteoblasts (38) and of Saos-2 cells (this study) but does so only by sequestering locally released or externally provided IGF. Our data are, therefore, fully compatible with the speculation put forward by Leal *et al.* (33) that locally released IGFBP-3 may cause growth inhibition by sequestering IGFs from binding to type 1 IGF receptors in IGF-responsive cells, although they do not exclude the possibility that IGFBP-3 elicits a growth-inhibitory response mediated by the type V TGF- β receptor.

We conclude that both IGFBP-3 and TGF- β do not exert growth-inhibitory effects in Saos-2/B-10 cells, whereas type 1 IGF (and possibly insulin) receptors and their ligands (IGFs, insulin and their analogs) are critical for both growth and survival. There is no increased apoptosis and growth inhibition in response to IGFBP-3, strongly arguing against the existence of specific IGFBP-3 cell surface receptors. In the presence of IGF, sequestration of the latter appears to be the major mechanism of IGFBP-3 action on apoptosis and cell growth.

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