IGF-independent effects of IGFBP-2 on the human breast cancer cell line Hs578T

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Abstract

There is evidence that insulin-like growth factor-binding protein (IGFBP-2), a modulator of the actions of IGFs, also has IGF-independent effects in human tumor cell lines. These involve specific binding of IGFBP-2 to $\alpha5\beta1$-integrin, followed by alterations in the phosphorylation status of downstream signaling molecules. Previously, IGFBP-2 has also been shown to be associated with cell proliferation, adhesion and migration. Here, we investigated direct effects of IGFBP-2 on apoptosis and alterations in the expression of related proteins. The breast cancer cell line Hs578T, which shows no IGFBP-2 production of its own and is independent of the IGF-I receptor, was treated with human recombinant IGFBP-2 in order to study the changes in gene expression induced by IGFBP-2. The methods employed for this purpose were oligonucleotide microarrays, real-time RT-PCR, western blotting, and immunoassays. Out of the 440 genes covered by the Oligo GEArray Human Cancer Microarray OHS-802, the expression of 77 genes was directly influenced by IGFBP-2. By the use of real-time quantitative RT-PCR, the gene expression of Nuclear Factor (NF)κB, p53, transforming growth factor β (TGF β-1), LAMB1 (Laminin, Beta 1), Bcl-2, and IIp45 was found to be significantly upregulated (by 1·2- to 3·05-fold; all $P<0·001$). Accordingly, NFκB, p53, and TGF β-1 proteins, as measured by Western blotting and immunoassay, were upregulated $>1·5$-fold. By using an ELISA-based and a flow cytometry-based apoptosis assay, IGFBP-2 was found to have a pro-apoptotic effect on Hs578T cells. Our results suggest that IGFBP-2-induced gene expressions are of functional significance for proliferation, cell adhesion, cell migration and apoptosis, and showed that IGFBP-2 can promote apoptosis in tumor cells independent of IGF.

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Introduction

As part of the insulin-like growth factor (IGF) system, the IGF-binding proteins (IGFBPs) play an important role in the regulation of a multitude of cellular processes (Clemmons 1997, Firth & Baxter 2002). IGFBP-2, the second most frequent IGFBP in the human circulation, is involved in many physiological and pathological conditions and processes: IGFBP-2 is highly expressed during the perinatal period (Blum *et al.* 1993, Lindenbergh-Kortleve *et al.* 1997), while in adults the highest concentrations of IGFBP-2 are found in cerebrospinal fluid (Binoux *et al.* 1991, Arnold *et al.* 1999), mother's milk (Elmlinger *et al.* 1999) and seminal plasma (Rosenfeld *et al.* 1990). IGFBP-2 is also often highly expressed in malignant tumor cells but decreases upon remission (Muller *et al.* 1994, Elmlinger *et al.* 1996, Hoeflich *et al.* 2000, Elmlinger *et al.* 2001, Moore *et al.* 2003, Ranke *et al.* 2003). The elevated expression is reflected by elevated serum concentrations of IGFBP-2 in tumor patients, which is often correlated with tumor malignancy (Boulle *et al.* 2001, Elmlinger *et al.* 2001, Baron-Hay *et al.* 2004). This makes IGFBP-2 a potential progression-dependent tumor biomarker and suggests a functional role for IGFBP-2 in tumor progression.

IGFBP-2 possesses multiple biological functions beyond the modulation of IGF activity through sequestration of IGFs (Hoeflich *et al.* 2001*a*). These, for example, include the regulation of GH-stimulated growth (Hoeflich *et al.* 2001*b*, Eckstein *et al.* 2002), of proliferation and both cell adhesion and migration (Hoeflich *et al.* 1998, Schutt *et al.* 2004). This has been demonstrated by studies on human cell culture models

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as well as animals. IGFBP-2 actions themselves have been shown to be modulated by limited proteolysis of IGFBP-2 resulting in fragments with specific activities and/or affinities. In particular, limited proteolysis of IGFBP-2 could be induced by plasminogen (Menouny *et al.* 1997), basic fibroblast growth factor (Russo *et al.* 1999), and retinoic acid (Bernardini *et al.* 1994, Chambery *et al.* 1998).

As pointed out above, IGFBP-2 has a multitude of effects, which can be mediated either by its IGF-dependent or by its IGF-independent actions. IGF-dependent actions are considered to be due to any type of modulation of IGF activity, whereas IGF-independent actions are those mediated by other mechanisms in which IGFs are not involved. The identification of possible pathways through which IGFBP-2 can act IGF-independently has shed new light on the cellular functioning of this protein. To date, there is evidence that IGFBP-2 can initiate cellular signaling through specific binding of its RGD-motif to integrin receptors, e.g. α5β1 (Schutt *et al.* 2004), and modulate IGF and epidermal growth factor signaling through bimolecular interactions with αvβ3 integrins (Pereira *et al.* 2004). As a second pathway, IGFBP-2 has also been shown to bind to glycosaminoglycans, heparin, and proteoglycans (Arai *et al.* 1996, Russo *et al.* 1997, Conover & Khosla 2003), amongst others, by means of its heparin-binding domain (Russo *et al.* 2005). In addition, IGFBP-2 was found to be localized on the cell surface (Pereira *et al.* 2004, Schutt *et al.* 2004, Russo *et al.* 2005), in the cytoplasm, on the nuclear surface (Hoeflich *et al.* 2004) and even within the nucleus (Terrien *et al.* 2005).

While the regulation of proliferation, cell migration and adhesion could at least in part be ascribed to IGF-independent signaling through integrin receptors (Schutt *et al.* 2004), IGF-independent effects of IGF BP-2 on apoptosis have not been studied in detail so far.

One possible approach towards the identification of IGF-I-independent pathways of IGFBP-2 actions involves the human breast cancer cell line Hs578T, which possesses no functional IGF-I receptor, thereby excluding IGF-I-dependent effects (De Leon *et al.* 1992, Gill *et al.* 1997). By adding human recombinant IGF BP-2 to this cell line, which produces no IGFBP-2 on its own, we studied the effects of IGFBP-2 on gene expression with special interest in apoptosis-related genes and proteins.

Expression studies were performed on mRNA and protein level using oligonucleotide microarray analysis, real-time RT-PCR, Western immunoblotting, and an immunoassay. To study the effect of IGFBP-2 on apoptosis, the apoptotic status of the Hs578T cell cultures was determined with two different cell death-detection assays.

Materials and methods

Cell culture

The breast cancer cell line Hs578T was purchased from the European Collection of Cell Cultures (No. 86082104 ECACC, Porton Down, Salisbury, UK) and cultured in DMEM:F12 (1:1) + GlutaMAX I supplemented with HEPES (12·5 mM) and 10% fetal bovine serum in a humidified incubator at 37 °C and 5% CO_2. Unless indicated otherwise, all reagents and media used for cell culture were obtained from GIBCO Invitrogen, Karlsruhe, Germany. Hs578T cells were grown up to ~70% confluency, the standard medium was replaced by serum-free medium 24 hours before harvesting, and equal numbers of cell cultures were left either untreated or were treated with 2000 ng/ml IGFBP-2, overnight. Cells ($0{\cdot}5\times10^6$) were seeded into T25 polystyrene culture flasks (BD Biosciences Discovery Labware, Heidelberg, Germany) for mRNA isolation and 100×20 mm polystyrene culture dishes (BD Biosciences Discovery Labware) for producing protein lysates. Cells were detached using Trypsin-EDTA (1X) and counted with the Advia 120 cell counter (Bayer, Leverkusen, Germany).

Microarray expression analysis

mRNA from Hs578T cells treated with 2000 ng/ml IGFBP-2 and untreated Hs578T cells were isolated with the ArrayGrade mRNA purification kit (Superarray, Frederick, USA). Using the TrueLabeling-AMP Linear RNA amplification kit (Superarray), the mRNA was reversely transcribed to obtain cDNA and converted into biotin-labeled cRNA using biotin-16-UTP (Roche, Mannheim, Germany) by *in vitro* transcription. Prior to hybridization, the cRNA probes were purified with the ArrayGrade cRNA cleanup kit (Superarray). The purified cRNA probes were then hybridized to the pretreated Oligo GEArray Human Cancer Microarrays OHS-802 (Superarray), which cover 440 cancer-related genes. Following several washing steps, array spots binding cRNA were detected using alkaline phosphatase-conjugated streptavidin and CDP-Star as chemiluminescent substrate. Chemiluminescence was detected with the Raytest CCD camera system DIANA II (Raytest, Straubenhardt, Germany). The image data were transformed into numerical data using a software called ScanAlyze v2·50 (http://rana.lbl.gov/EisenSoftware.htm). The numerical data were then further evaluated with Microsoft Excel 97. Data evaluation included background correction (subtraction of minimum value) and median normalization. Data filtering criteria were as follows: at least one of the spot intensities to be compared had to be more than twice the background intensity, and the spot intensity ratios had to be higher

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than 1.5 (for upregulation) or lower than −1·5 (for downregulation).

Real-time RT-PCR

Total RNA was isolated from Hs578T cells with the RNeasy mini kit (Qiagen, Hilden, Germany) and was used for real-time quantitative RT-PCR. In order to compensate for biological variations, RNA samples from six cultures grown in parallel were pooled for IGFBP-2 treated and untreated Hs578T cells, respectively. The pooled RNA samples were reversely transcribed using the Omniscript kit (Qiagen) according to the manufacturer's instructions. An oligo-dT primer was used for priming the reverse transcription. β_2-Microglobulin (B2M) was chosen as the reference gene for normalization of the results. Technical variations were compensated by running 12 parallel reactions for each cDNA sample pool. The real-time PCR was performed in a Bio-Rad iCycler using SYBR Green I as the detection system (iQ SYBR Green Supermix; Bio-Rad, Munich, Germany). The results were analyzed with the Bio-Rad iCycler Software 3·0 and Microsoft Excel 97. PCR primers were designed using Primer3 at the Whitehead Institute for Biomedical Research (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) under consideration of the special design criteria for real-time PCR primers. The PCR products were analyzed by melt curve analysis and agarose gel electrophoresis to determine product size and to confirm that no by-products were formed.

Preparation of cell lysates and determination of protein concentration

Cell lysates were prepared by directly adding 500 µl lysis buffer (10 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl, 30 mM $Na_4P_2O_7 \cdot 10\,H_2O$, 50 mM NaF, 1% (w/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, complete mini EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany), pH 7·6) onto the cell cultures previously washed with PBS. After 15 min incubation on ice, the lysed cells were scraped off, triturated, and transferred to 1·5 ml polypropylene tubes. Insoluble cell debris was removed by centrifugation (20 000 *g*, 20 min, 4 °C). Protein concentrations were determined photometrically using the commercial BCA-200 Protein assay kit (Pierce, Rockford, IL, USA).

Western immunoblot analysis

Soluble fractions of the cell lysates were separated on a 6% stacking/12% separating SDS-polyacrylamide gel for approximately 2 h at 200 V. The separated proteins were then blotted onto a PVDF membrane (0·45 µm; Millipore, Schwalbach, Germany) at 1 mA/cm^2 by a semi-dry blotting procedure (Trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad) using CAPS (3-(cyclohexylamino)-1-propane sulfonic acid) buffer (10 mM CAPS, 0·005% SDS, 10% methanol, pH 11·0). After blocking for 1 h in TBS-T (0·01 M Tris, 0·15 M NaCl, 0·1% Tween-20, pH 7·6) containing 10% Rotiblock (Roth, Karlsruhe, Germany), the blots were incubated with either p53 or nuclear factor (NF)κB p105/p50 primary antibody (all 1:4 000 in TBS-T, 10% (v/v) Rotiblock) overnight at 4 °C (both antibodies were from Cell Signaling Technology, Beverly, MA, USA). After washing the membranes three times for 15 min with TBS-T, the blots were incubated for 2 h in secondary antibody conjugated to horseradish peroxidase (anti-rabbit IgG, HRP-linked antibody; Cell Signaling Technology) diluted 1:5000 in TBS-T with 10% (v/v) Rotiblock. This allowed detection of secondary antibody using the ECL Plus chemiluminescence detection kit (Amersham Pharmacia, Freiburg, Germany). The blots were then exposed to Kodak Biomax film for 5 to 10 min at room temperature. The bands were analyzed densitometrically using the Aida 2·1 software (Raytest). The results are based on 12–16 technical replicates (i.e. individual band densities).

TGF β-1 immunoassay

The level of secreted transforming growth factor β-1 (TGF β-1) in cell culture supernatants was measured using a commercial human TGF β-1 ELISA kit from Anogen (Ontario, Canada). The immunoassay was performed using biological triplicates for samples (Hs578T cell cultures treated with IGFBP-2) and controls (Hs578T cells left untreated). In addition, each biological sample was measured in duplicate. In order to compensate for differences in cell number affecting the amount of secreted TGF β-1, the cells were counted and the final results are given as mean amount of TGF β-1 produced per 10^3 cells.

Cell death detection assays

Both the Cell Death Detection ELISA^{PLUS} and the Annexin-V-FLUOS staining kit (both from Roche) were used to quantitate apoptosis for IGFBP-2-treated and untreated Hs578T cells respectively. The Cell Death Detection ELISA^{PLUS} is a photometric enzyme immunoassay for the quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death. In the case of the Cell Death Detection ELISA^{PLUS}, each stimulation and control group consisted of three cell cultures grown in parallel. The resulting samples were then measured in triplicate, so that the overall number of

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replicate measurements for one condition was nine. The relative apoptotic values were corrected for cell numbers. Flow cytometric detection of Annexin-V-labeling of phosphatidylserine was performed on a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) using the software CellQuest 3·3 (BD Biosciences). The FACS data were then evaluated with the software WinMDI 2·8 (http://facs.scripps.edu/software.html). The experiment was performed three times under identical conditions.

In both assays, cell cultures treated with camptothecin served as positive controls.

Statistical analysis

Replicate measurements were used to calculate arithmetic means and standard errors of the mean (S.E.M). Data are presented as means ± S.E.M. In order to assess the significance of differences, a two-tailed Student's *t*-test was performed. Differences were considered to be significant for $P<0{\cdot}05$ (*), $P<0{\cdot}01$ (**), and $P<0{\cdot}001$ (***) with increasing degrees of significance. The software used for statistical analysis was Microsoft Excel 97.

Results

IGFBP-2 regulates the expression of multiple genes at the mRNA level

In order to test our hypothesis that exposure to IGFBP-2 alone can induce changes in gene expression in the absence of IGF-I, we performed microarray experiments with the IGF-insensitive breast cancer cell line Hs578T. Out of the 440 genes covered by the Oligo GEArray Human Cancer Microarray OHS-802, 77 genes were found to be regulated by IGFBP-2 according to the chosen filter criteria. These genes comprised a variety of ontological groups, most of which could be ascribed to cellular functions such as cell growth or maintenance and cell death, suggesting a functional role of IGFBP-2 in these processes (Table 1).

Genes with relevance for the role of IGFBP-2 in proliferation, cell adhesion and apoptosis were further analyzed by real-time quantitative RT-PCR. NFκB and LAMB1 (laminin, beta 1) were selected from the aforementioned Oligo GEArray Human Cancer Microarray results, while p53 and TGF β-1 were selected from an Affymetrix microarray analysis (K W Frommer, M W Elmlinger, unpublished observations) performed earlier in our laboratory. Apart from LAMB1, which is involved in cell adhesion, all of these genes are related to apoptosis. Bcl-2 was chosen due to its up-regulation through α5β1 integrin (Zhang *et al.* 1995), which is a receptor for IGFBP-2. In addition, IIp45 was more closely investigated because it was recently shown to antagonize IGFBP-2 stimulation of glioma cell invasion by binding IGFBP-2 (Song *et al.* 2003). To a different extent, all the genes examined showed a highly significant increase in expression ($n=12$; $P<0{\cdot}001$) (Fig. 1). However, the size of the change in IIp45 expression was so small that its biological significance is rather questionable.

IGFBP-2 regulates NFκB, p53 and TGF β-1 protein levels

To determine whether IGFBP-2 addition to Hs578T cells also has a regulatory effect on the protein levels of NFκB, p53 and TGF β-1, we performed quantitative Western immunoblot analysis for NFκB and p53 as well as an immunoassay for TGF β-1. In response to IGFBP-2, NFκB and p53 protein levels were found to be upregulated by a factor of $1{\cdot}43\pm0{\cdot}11$ ($n=12$; $P<0{\cdot}001$) and $1{\cdot}50\pm0{\cdot}17$ ($n=16$; $P<0{\cdot}001$) respectively (Fig. 2). The amount of TGF β-1 secreted by the Hs578T cells, as normalized by the cell number was increased by a factor of $1{\cdot}52\pm0{\cdot}18$ ($n=9$; $P<0{\cdot}05$) in response to IGFBP-2 (Fig. 3).

IGFBP-2 has a pro-apoptotic effect on Hs578T breast cancer cells

Based on the observation that, following exposure to IGFBP-2, the expression of several apoptosis-related genes was significantly altered, we further investigated the immediate effects of IGFBP-2 on apoptosis by two different approaches. The Cell Death Detection ELISA^{PLUS} and the Annexin-V flow cytometry-based apoptosis assay were employed to determine the occurrence of apoptotic DNA degradation prior to plasma membrane breakdown and the appearance of phosphatidylserine on the outer surface of the plasma membrane. In the presence of IGFBP-2, both assays detected an increase in apoptosis. The Cell Death Detection ELISA^{PLUS} indicated a 1·82-fold increase ($P<0{\cdot}001$) of the relative apoptotic values of IGFBP-2 treated versus untreated Hs578T cells (Fig. 4A). Using the Annexin-V apoptosis assay, we measured a higher percentage of apoptotic cells for Hs578T cells treated with IGFBP-2 as compared with non-treated Hs578T cells. According to this assay, the percentage of apoptotic cells increased on average from 6·2% to 15·8%, which corresponds to a ratio of 2·6 ($P<0{\cdot}05$) (Fig. 4B).

Discussion

IGFBP-2 has been shown to be involved in a variety of key biological functions such as cell proliferation, migration and adhesion. However, the effects of IGFBP-2 observed *in vitro* vary depending on the cell lines observed

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Table 1 Selection of functionally classified genes whose expression was induced by IGFBP-2

Gene name	Gene symbol	GenBank accession number	Fold change
Cell proliferation/cell growth			
Transforming growth factor, alpha	TGFA	NM_003236.1	1·81
Non-metastatic cells 1, protein expressed in	NME1	NM_000269.2	1·65
stratifin (14-3-3-Sigma)	SFN	NM_006142.2	1·90
Tumor protein p73	TP73	NM_005427.1	2·52
v-yes-1 Yamaguchi Sarcoma viral oncogene homolog 1	YES1	NM_005433.2	2·02
v-myc myelocytomatosis viral oncogene homolog (Avian)	MYC	NM_002467.2	2·25
v-raf-1 murine leukemia viral oncogene homolog 1	RAF1	NM_002880·1	1·74
DNA-damage-inducible transcript 3	DDIT3	NM_004083.3	2·49
Polo-like kinase 2 (Drosophila)	PLK2	NM_006622.1	2·32
Retinoblastoma 1 (including osteosarcoma)	RB	NM_000321.1	2·06
Tumor susceptibility gene 101	TSG101	NM_006292.2	1·72
Insulin-like growth factor-binding protein 3	IGFBP-3	NM_000598.2	1·58
Suppressor of cytokine signaling 1	SOCS1	NM_003745.1	2·15
Integrin-linked kinase-2	ILK	NM_004517.1	2·03
Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	CDKN2B	NM_004936.2	1·97
Proliferating cell nuclear antigen	PCNA	NM_002592.2	1·72
Notch homolog 2 (Drosophila)	NOTCH2	NM_024408.2	2·73
Neurofibromin 2 (bilateral acoustic neuroma)	NF2	NM_000268.2	1·90
Tumor necrosis factor (ligand) superfamily, member 7	TNFSF7	NM_001252.2	1·69
Apoptosis/necrosis (cell death)			
CD27-binding (siva) protein	SIVA	NM_006427.2	1·80
Tumor protein p73	TP73	NM_005427.1	2·52
Nuclear factor of κ light polypep gene enhancer in B-Cells 1	NFKB1	NM_003998.2	2·23
Tumor necrosis factor (TNF superfamily, member 2)	TNF	NM_000594.2	2·34
Tumor necrosis factor receptor superfamily, member 6	TNFRSF6	NM_000043.3	1·65
Tumor necrosis factor (ligand) superfamily, member 7	TNFSF7	NM_001252.2	1·69
Tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A	NM_003844.2	1·71
v-raf-1 murine leukemia viral oncogene homolog 1	RAF1	NM_002880·1	1·74
Notch homolog 2 (Drosophila)	NOTCH2	NM_024408.2	2·73
v-myc myelocytomatosis viral oncogene homolog (Avian)	MYC	NM_002467.2	2·25
Non-metastatic cells 3, protein expressed in	NME3	NM_002513.2	1·67
Cell adhesion			
Laminin, beta 1	LAMB1	NM_002291.1	2·21
Tissue inhibitor of metalloproteinase 1	TIMP1	NM_003254.1	2·92
Integrin-linked kinase-2	ILK	NM_004517.1	2·03
Integrin, alpha 3 (antigen CD49C)	ITGA3	NM_002204.1	1·84
Ras-related C3 botulinum toxin substrate 1	RAC1	NM_006908.3	1·90

This table shows selected results from the oligonucleotide microarray analysis of 440 genes in Hs578T cells treated with 2000 ng/ml IGFBP-2 relative to untreated controls. It contains the IGFBP-2-induced genes belonging to the ontological groups of proliferation, cell death, and cell adhesion, which were of most interest here because the effects of IGFBP-2 on these cellular processes have previously (Schutt *et al.* 2004) and are now been studied in our laboratory. From the ontological groups of most interest, genes were selected for which most useful information was available and/or which are more commonly known so that the reader can more easily understand the association of genes with a certain ontological group.

and whether IGFs are involved or not. There have been several theories put forward to explain the positive and negative effects of IGFBP-2. As a carrier of IGFs, IGFBP-2 may increase IGF activity by protecting this growth factor from proteolysis and/or transporting it to the IGF-I receptor (Ranke & Elmlinger 1997). On the other hand, IGFBP-2 in both soluble and membrane-associated form has been shown to compete with IGFs for their receptor (Reeve *et al.* 1993), which may reduce IGF effects. The IGF-independent effects are thought to be due mainly to interactions of IGFBP-2 with the extracellular matrix (ECM). Therefore, ECM-connected signal transduction induced by IGFBP-2 interaction with the ECM is very likely (Schutt *et al.* 2004, Russo *et al.* 2005), but also combined signals from the ECM and the IGF-I receptor have to be taken into account.

In order to investigate how IGFBP-2 affects gene expression *in vitro* in an IGF-independent manner, we treated the IGF-insensitive cell line Hs578T with 2000 ng/ml human recombinant IGFBP-2. This concentration may appear to be very high compared with normal physiological concentrations. However, serum levels of IGFBP-2 can definitely reach those dimensions. For example, serum IGFBP-2 levels of 1240 ± 270 ng/ml were measured in patients with Type 1 diabetes (Frystyk *et al.* 1999). Ovarian cancer patients

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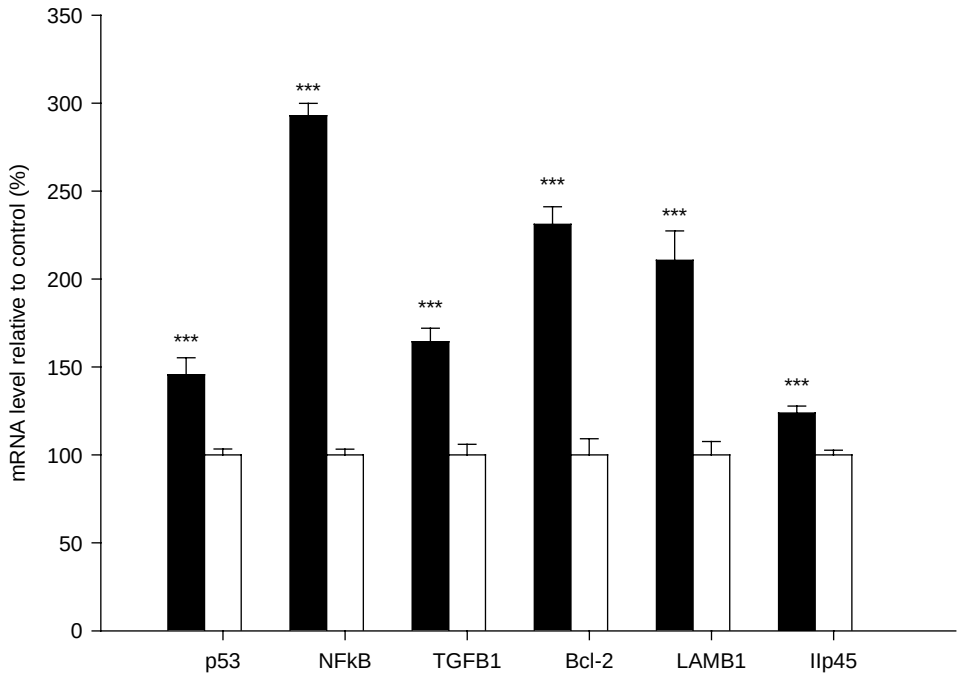

Figure 1 Total RNA was isolated from Hs578T cells treated with IGFBP-2 and from untreated controls. The mRNA was selectively reverse transcribed into cDNA using an oligo-dT primer and was quantitated by real-time PCR in a Bio-Rad iCycler with SYBR Green I as fluorescent dye. The determination of mRNA expression was performed as relative quantification using β2-microglobulin as the reference gene for normalization. The mRNA levels are given in percent relative to the control, with the error bars indicating the standard error. Black bars represent mRNA levels in IGFBP-2 treated Hs578T cells, whereas white bars represent mRNA levels in untreated controls. The number of measured replicates for each group and gene was 12. The differences were all statistically significant ($n=12$; $^{***}P<0{\cdot}001$).

showed very high serum IGFBP-2 levels of 1773±168 ng/ml with a range of 841 to 3493 ng/ml (Flyvbjerg *et al.* 1997) as measured by RIA. Patients with metastatic adrenocortical tumors had serum IGFBP-2 levels of 1314±554 ng/ml (Boulle *et al.* 2001). There are several other studies which also showed that IGFBP-2 is significantly increased in tumors (Cohen *et al.* 1993, Kanety *et al.* 1993, Zumkeller *et al.* 1993, Muller *et al.* 1994, Baron-Hay *et al.* 2004). In addition, with such high serum levels, local concentrations within and around tumors might even be higher. Therefore, concentrations of 2000 ng/ml IGFBP-2 can actually represent the conditions found with tumors *in vivo*.

In this study, we were able to identify three sets of genes related to proliferation, migration/adhesion, and apoptosis, which are regulated by IGFBP-2 in our IGF-insensitive breast cancer cell model Hs578T.

IGFBP-2 induction of genes related to proliferation

The most extensively studied biological end point after IGFBP-2 stimulation is cell proliferation, which nicely exemplifies the opposing effects that IGFBP-2 can have on different cell types. In IGFBP-2 overexpressing Y-1 adrenocortical tumor cells (Hoeflich *et al.* 2000), A673 Ewing sarcoma cells (Schutt *et al.* 2004) and SKN-SHEP neuroblastoma cells (Russo *et al.* 2005), as well as IGF BP-2 treated LAPC-4 prostate cancer cells (Moore *et al.* 2003), IGFBP-2 is clearly a pro-proliferative factor. In contrast, inhibition of proliferation occurred in IGFBP-2-treated fetal podocytes (Bridgewater & Matsell 2003) and Hs578T breast cancer cells (Schutt *et al.* 2004), as well as IGFBP-2 overexpressing HEK-293 embryonic kidney cells (Hoeflich *et al.* 1998). In relation to its proliferative effect, IGFBP-2 has also been found to correlate positively with tumor malignancy, which has been shown *in vitro* for the previously mentioned Y-1 adrenocortical tumor cells (Hoeflich *et al.* 2000) and *in vivo* for human gliomas (Elmlinger *et al.* 2001). In the present study, we investigated the molecular basis of the previously shown anti-proliferative effects of IGFBP-2 on Hs578T cells (Schutt *et al.* 2004) by way of identifying changes in the expression of genes known to be involved in proliferation. Since the Hs578T cells under study are IGF-insensitive, the effects of IGFBP-2 are very likely to represent

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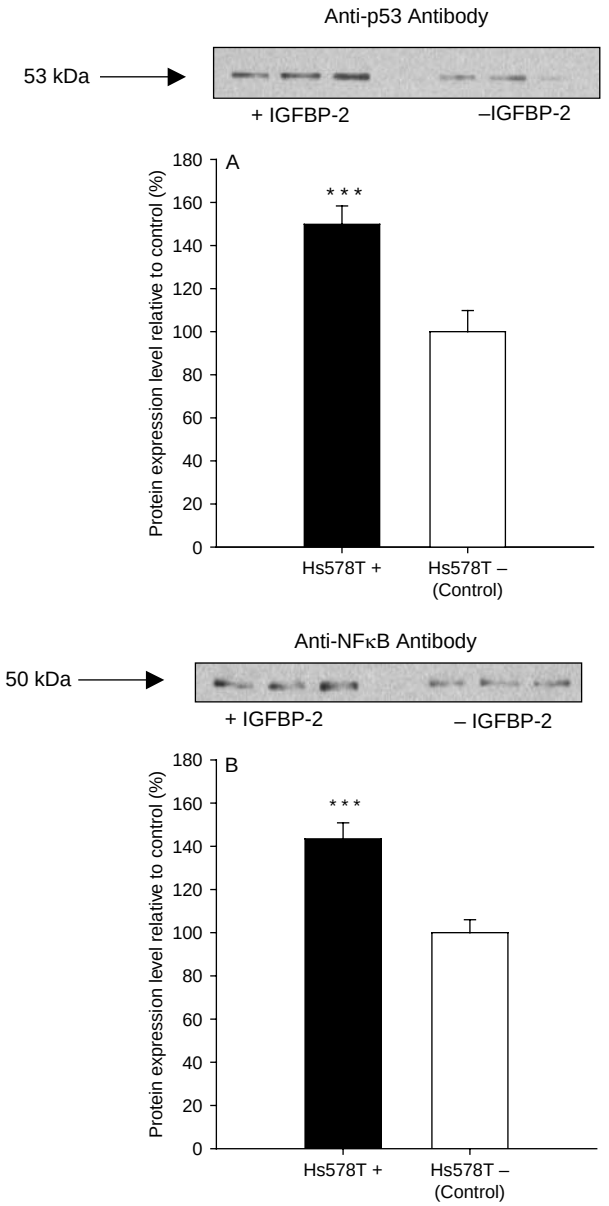

Figure 2 The soluble fractions of Hs578T cell lysates were separated by means of SDS-PAGE. Using a semi-dry blotting procedure, the separated proteins were transferred onto a PVDF membrane. The blots were incubated with either p53 or NF-κB p105/p50 as primary antibody and anti-rabbit IgG conjugated to horseradish peroxidase as secondary antibody. Detection of the secondary antibody was performed with the ECL Plus chemiluminescence detection kit. Finally, the bands were analyzed densitometrically. An exemplary blot is shown for both p53 (A) and NFκB (B). The protein levels are given in percent relative to the control, with the error bars indicating the standard error. Black bars represent protein levels in IGFBP-2-treated Hs578T cells, whereas white bars represent protein levels in untreated controls. The number of replicate bands that were analyzed was 16 for p53 and 12 for NFκB. The differences between treated samples and controls were statistically significant (***$P<0{\cdot}001$) for both proteins.

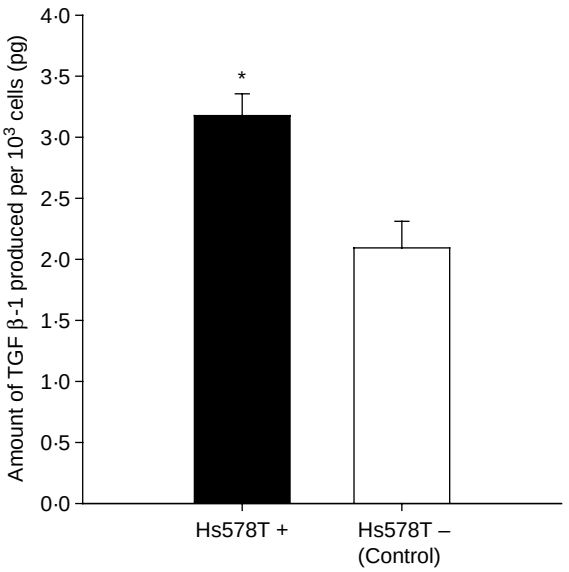

Figure 3 Secreted TGF β-1 levels in cell culture supernatants were quantified using the commercial human TGF β-1 ELISA kit from Anogen. The results are given as mean amount of TGF β-1 produced per 10^3 cells. The error bars indicate the standard error. The amount of TGF β-1 secreted by the Hs578T cells was increased by a factor of $1{\cdot}52 \pm 0{\cdot}18$ ($n=9$; *$P<0{\cdot}05$) in response to IGFBP-2.

IGF-independent effects of IGFBP-2. In agreement with the above-mentioned anti-proliferative effect of IGFBP-2 on Hs578T cells, we found that IGFBP-2 upregulates the expression levels of TGF β-1, NME1, SFN (14-3-3-sigma), TP73, RB, TSG101, CDKN2B, NF2, and TNFSF7, all of which represent proteins known to be capable of inhibiting cell proliferation.

IGFBP-2 induction of genes related to migration/adhesion

Another biological end point that IGFBP-2 is involved in is cell migration and adhesion. It has been shown that cell adhesion was increased in A673 cells and decreased in Hs578T cells (Schutt *et al.* 2004). Enhanced invasiveness was observed with SKN-SHEP cells (Russo *et al.* 2005) and SNB19 glioblastoma cells (Wang *et al.* 2003). In all studied cell models except for Hs578T cells, IGF-dependent effects as explained above cannot be excluded, but interaction of IGFBP-2 with the ECM has been proven in these cases. In this study, different genes like ILK (integrin-linked kinase-2), RAC1 (Ras-related C3 botulinum toxin substrate 1), ITGA3 (integrin, alpha 3), TIMP1 (tissue inhibitor of metalloproteinase 1), and LAMB1 were directly or indirectly upregulated by IGFBP-2. These genes are all related to cell adhesion and/or migration, but the interpretation of their regulation is not always straightforward, since the upregulation of some of these genes is not able to

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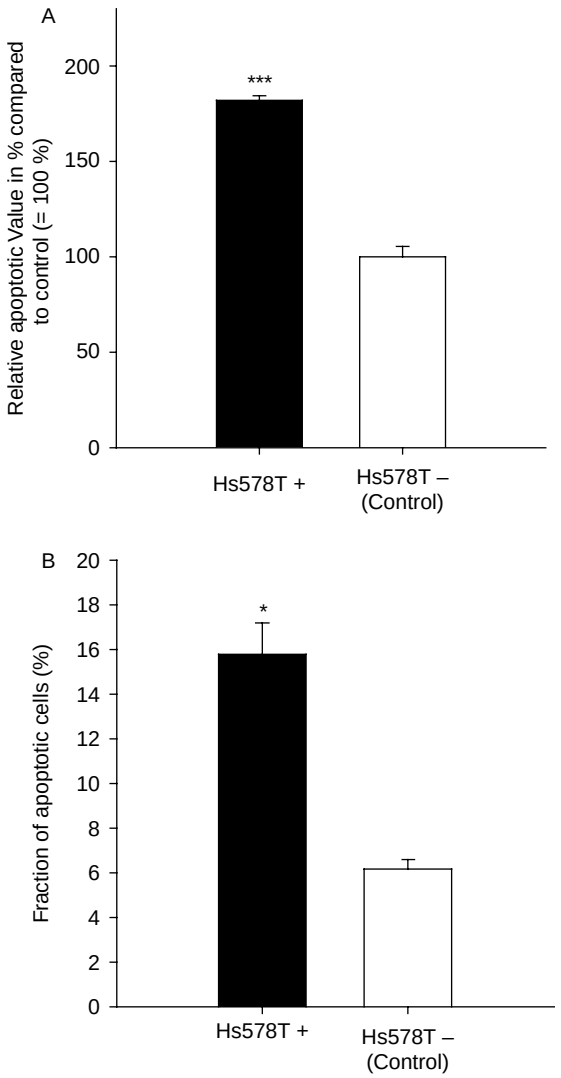

Figure 4 Apoptosis in IGFBP-2-treated (black bars) and untreated (white bars) Hs578T cells was quantitated using the commercial Cell Death Detection ELISA^{PLUS} (A) and the Annexin-V-FLUOS staining kit (B). Using the Cell Death Detection ELISA^{PLUS}, the relative apoptotic values of IGFBP-2-treated versus untreated Hs578T cells showed a ratio of 1·82 ($n=9$; $^{***}P<0·001$) (A). Measurements with the Annexin-V-FLUOS staining kit were performed three times under identical conditions. On average, by adding IGFBP-2, the percentage of apoptotic cells increased from 6·2% to 15·8% (B), which corresponds to a ratio of 2·6 ($n=9$; $^{*}P<0·05$).

directly explain the decrease in adhesion observed previously in the Hs578T cell line (Schutt *et al.* 2004). In agreement with the observed effects, an upregulation of ILK and RAC1 was found after IGFBP-2 stimulation. ILK is a regulator of integrin-mediated signal transduction and a component of focal adhesions, which are formed upon ligand binding to integrins (Sakai *et al.* 2003). In the context of adhesion, Hannigan *et al.* (1996) showed that overexpression of ILK indeed inhibited adhesion to integrin substrates in their epithelial cell model, which, in turn, might explain the decreased adhesion seen after IGFBP-2 treatment of Hs578T cells. RAC1, a GTPase belonging to the RAS family of small GTP-binding proteins, plays a role in the regulation of actin polymerization and in cooperation with CDC42 is able to increase cell motility and invasiveness through an integrin-mediated mechanism (Nobes & Hall 1995, Keely *et al.* 1997, Berrier *et al.* 2002). This corresponds with the enhanced invasiveness observed with SKN-SHEP cells (Russo *et al.* 2005) and SNB19 glioblastoma cells (Wang *et al.* 2003). The IGFBP-2 upregulated gene ITGA3 encodes the α3 subunit of certain integrin receptors, such as the integrin heterodimer α3β1, which is a receptor for fibronectin, laminin, collagen, epiligrin, thrombospondin, and CSPG4. Although most integrins are generally associated with an increase in adhesion, it has been found that, in some ITGA3 transfectants, the expression of this integrin α3 subunit resulted in a decrease in cell–cell adhesion (Weitzman *et al.* 1995). Another observation supporting the anti-adhesive effect of the integrin α3 subunit is that α3β1 integrin is required for the disruption of intercellular adhesion within epithelial cells (Kawano *et al.* 2001). Deadhesion of cells is certainly not promoted by TIMP, which acts as an inhibitor of specific metalloproteinases. Nonetheless, it appears to be upregulated by IGFBP-2. So its upregulation must be due to a different mechanism. It may be a regulatory cellular response antagonizing the loss of cell adhesion. LAMB1 is a subunit of the heterotrimeric laminin glycoproteins, which are part of the ECM and are able to bind to integrins (Belkin & Stepp 2000). Amongst others, the laminins play a role in cell adhesion, but the upregulation of LAMB1 does not directly explain the decrease in cell adhesion observed for Hs578T cells. This suggests that the upregulation of LAMB1 may also be a cellular counter response against excessive loss of cell adhesion.

IGFBP-2 induction of genes related to apoptosis

Finally, our study revealed that IGFBP-2 is directly involved in apoptosis, which – to our knowledge – has not been shown before. However, IGFBP-2 has been shown to positively correlate with another conceptually related process that limits a cell's lifetime: senescence. Several *in vitro* studies showed a marked upregulation of IGFBP-2 in correlation with replicative senescence using quantitative Northern and microarray analyses (Hjelmeland *et al.* 1999, Matsunaga *et al.* 1999, Shelton *et al.* 1999). The genes SIVA, TP73, TNF, TNFRSF6, TNFSF7, TNFRSF10A, NOTCH2, MYC, NME3, 14-3-3-sigma, NFκB, TGF β-1, and p53 found to be upregulated after stimulation of the Hs578T cells with IGFBP-2 are all known to have pro-apoptotic effects. In agreement with these gene-array data, we were able to confirm

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a pro-apoptotic impact of IGFBP-2 on the Hs578T cells by applying two different apoptosis assays. Some of the regulated genes appear to be part of a regulatory network. 14-3-3-sigma, for example, has a positive feedback effect on p53 activity (Yang *et al.* 2003). MYC affects the outcome of a p53 response in favor of apoptosis (Seoane *et al.* 2002). ILK (Li *et al.* 2003) as well as CDKN2B (p15INK4B) (Hannon & Beach 1994) have been shown to be a part of the pro-apoptotic response of cells exerted by TGF-β, which itself is upregulated by IGFBP-2 stimulation. SIVA, a pro-apoptotic protein, binds to TNFSF7 (CD27), through which it is believed to exert its effect on apoptosis (Prasad *et al.* 1997). The upregulation of the transcription factor NFκB in response to IGFBP-2 stimulation can also be related to the pro-apoptotic response seen in our cell model, since it has been observed in different studies that NFκB is not only involved in anti-apoptotic effects or in the counterbalancing of pro-apoptotic stimuli (Van Antwerp *et al.* 1996, Mayo *et al.* 1997) but that NFκB activity is also required for the development of pro-apoptotic activity of p53 (Ryan *et al.* 2000). Interestingly, the NFκB upregulation can result in an autocrine loop, since the promoter region of IGFBP-2 possesses four distinct positions that can putatively bind NFκB, which would suggest IGFBP-2 promoter activation (Cazals *et al.* 1999).

Although the IGF insensitivity of the Hs578T cell model studied here excludes IGF-dependent effects, the exact mechanism by which IGFBP-2 induces the genes involved in proliferation, migration/adhesion and apoptosis found in the present study has not been determined yet. According to the data available in the literature, various interactions of IGFBP-2 with the ECM of the target cell might account for the effects seen after stimulation with IGFBP-2. So far, a direct binding to integrin receptors (Pereira *et al.* 2004, Schutt *et al.* 2004) and resulting cell signaling via the RGD motif of IGFBP-2 (Schutt *et al.* 2004) as well as binding to glycosaminoglycans, heparin, and proteoglycans (Arai *et al.* 1996, Russo *et al.* 1997, Conover & Khosla 2003) have been observed. Not only proliferation or migration but also apoptosis is known to be induced by integrin signaling and, like IGFBP-1 (Perks *et al.* 1999), IGFBP-2 might induce apoptosis through dephosphorylation of focal adhesion kinase and the downstream MAPK pathway (Schutt *et al.* 2004). By using different IGFBP-2 mutants, a direct role of the heparin-binding domain of IGFBP-2 on proliferation and migration has been demonstrated in a recent study (Russo *et al.* 2005). Therefore, different signaling events mediated by the α5β1 integrin binding site (i.e. RGD motif), the heparin-binding domain and possibly other yet unknown ECM binding sites of IGFBP-2 might be responsible for the IGF-independent gene induction seen in this study. In addition, IGFBP-2 is able to bind different components of the ECM such as vitronectin, laminin, collagens, and fibronectin (Russo *et al.* 2005). So, it is reasonable to assume that there other alternative mechanisms of IGFBP-2 action, like modulation of the dynamics of the reservoir of ECM components and ECM associated proteins; even IGFBP-2 as an integrin ligand competitor acting through binding of other integrin ligands is conceivable (Russo *et al.* 2005). Besides the explanations for direct IGFBP-2-induced cell responses, further possible mechanisms must be taken into account for IGF-responsive cells. Here, ECM-associated IGFBP-2 can act as a competitor for the IGF-I receptor (Pereira *et al.* 2004), or the association of IGFBP-2 with the ECM itself may function as a cell targeting mechanism for IGFs. However, the findings that IGFBP-2 was also localized in the cytoplasm and on the nuclear surface (Hoeflich *et al.* 2004) and interacts with the intracellular cell cycle inhibitor p21CIP1/WAF1 (Terrien *et al.* 2005) show that IGFBP-2 exerts some of its actions directly within the cell. IGFBP-2 appears to have varying effects on different cell lines. Possible explanations are certainly based on the fact that every cell type has its own specifically composed ECM and expression profile. The gene expression patterns can also considerably differ depending on whether cells are malignant or non-malignant. Differences in gene expression can, amongst others, involve other IGFBPs or IGFBP proteases, which then affects limited IGFBP proteolysis. With different cellular components, there can be different interactions of IGBFP-2 with other molecules and different signaling pathways that can be induced. Last but not least, the type of administration, i.e. whether IGFBP-2 is overexpressed intracellularly or added externally, may also influence the effect that IGFBP-2 exerts on a certain cell type.

Our results suggest that IGFBP-2-induced gene expressions are of functional significance for proliferation, cell adhesion, cell migration and apoptosis. It might be concluded from our data regarding apoptosis that blockade of IGFBP-2 as a strategy to block tumor growth as suggested previously might – at least in certain cell types – represent a fatal error. The data presented here shed a new light on the biological consequences of locally produced IGFBP-2 as an IGF-independent modulator of proliferation, migration/adhesion and apoptosis. Certainly, however, further studies aimed at the dissection of the different IGFBP-2-mediated effects by its interaction with or modulation of the ECM via its integrin-binding domain, its heparin-binding domain, or even by its ability to bind and possibly target IGFs to the ECM are necessary to fully understand the mode of action of IGFBP-2.

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