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Alteration of the Insulin-Like Growth Factor Axis During In Vitro Differentiation of the Human Osteosarcoma Cell Line HOS 58

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Abstract The insulin-like growth factors I and II (IGF-I, IGF-II), their receptors, and high affinity binding proteins (IGFBPs) represent a family of cellular modulators that play essential roles in the development and differentiation of cells and tissues including the skeleton. Recently, the human osteosarcoma cell line HOS 58 cells were used as an in vitro model of osteoblast differentiation characterized by (i) a rapid proliferation rate in low-density cells that decreased continuously with time of culture and (ii) an increasing secretion of matrix proteins during their in vitro differentiation. In the present paper, HOS 58 cells with low cell density at early time points of the in vitro differentiation (i) displayed a low expression of IGF-I and -II; (ii) synthesized low levels of IGFBP-2, -3, -4, and -5, but (iii) showed high expression levels of both the type I and II IGF receptors. During the in vitro differentiation of HOS 58 cells, IGF-I and -II expressions increased continuously in parallel with an upregulation of IGFBP-2, -3, -4, and -5 whereas the IGF-I receptor and IGF-II/M6P receptor mRNA were downregulated. In conclusion, the high proliferative activity in low cell density HOS 58 cells was associated with high mRNA levels of the IGF-IR, but low concentrations of IGFBP-2. The rate of proliferation of HOS 58 cells continuously decreased during cultivation in parallel with a decline in IGF-IR expression, but increase of mitoinhibitory IGFBP-2. These data are indicative for a role of the IGF axis during the in vitro differentiation of HOS 58 cells. J. Cell. Biochem. 102: 28–40, 2007.

Key words: osteoblast-like cells; differentiation; IGF; IGF binding proteins; IGF-receptor

The insulin-like growth factors I and II (IGF-I and -II), their receptors, and binding proteins (IGFBPs) represent a family of different components that play essential roles in the regulation of growth and development of various organs including the skeleton. The insulin-like and cell-proliferative effects of the IGFs are mediated by the IGF-I and insulin receptor [LeRoith et al., 1995]. The IGF-II/mannose-6-phosphate (M6P) receptor is involved in M6P-dependent transport of lysosomal enzymes as well as in binding and internalization of IGF-II [Braulke, 1999]. The amount of locally available IGFs is modulated by the presence of six high affinity IGF binding proteins (IGFBPs) [Jones and Clemmons, 1995; Clemmons, 1997; Firth and Baxter, 2002; Mohan and Baylink, 2002] that differ in molecular mass, binding properties for IGFs and post-translational modifications such as phosphorylation and glycosylation [Shimasaki and Ling, 1991]. IGFBPs not only regulate the bioavailability of IGFs, but also inhibit or enhance their action on target tissues [Clemmons, 1997]. The extracellular concentrations of IGFBPs can be regulated at the level of gene expression as well as post-transcriptionally by limited proteolysis. Thus, several studies have reported on the role of IGFBP proteases in the regulation of IGF-dependent physiological

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and pathophysiological processes [Maile and Holly, 1999].

IGF-I and IGF-II are among the most abundant growth factors in the skeleton and are synthesized by osteoblasts in vitro [Conover, 2000; Mohan and Baylink, 2002; Govoni et al., 2005]. Both IGF-I and IGF-II stimulate osteoblast proliferation and differentiation in vitro [Conover, 2000]. The process of new bone formation includes recruitment, proliferation, differentiation of cells, and their maturation to specialized matrix-producing osteoblasts. There is substantial evidence from both in vitro and in vivo studies for the involvement of the components of the IGF axis, but it remains an important goal to define the precise role of the IGFs in regulating the steps of this process. Recently, the in vitro differentiation of the human osteosarcoma cell line HOS 58 has been reported [Siggelkow et al., 1998; Siggelkow et al., 2002]. This cell line expresses a differentiated phenotype and undergoes phenotypic maturation for at least 14 days of culture. The proliferation rate, secretion of matrix proteins, and the sequence of maturation observed in HOS 58 cells is comparable with that described for primary rat osteoblasts [Owen et al., 1990]. However, HOS 58 cells are lacking a proper mineralization of the extracellular matrix [Owen et al., 1990; Siggelkow et al., 1998; Siggelkow et al., 2002]. In the present study, the potential role of IGFs, the IGF receptors, IGFBPs as well as of IGFBP proteases during the in vitro differentiation of the human osteosarcoma cell line HOS 58 was investigated.

MATERIALS AND METHODS

Materials

Sodium [^{125}I] iodide (carrier-free, specific activity 16.85 mCi/μg), [^{32}P]-labeled deoxycytidine-triphosphate (specific activity 700 Ci/mmol/L), [^{3}H]-thymidine (specific activity, 94 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Braunschweig, Germany). Recombinant human IGF-I was from GroPep Ltd (North Adelaide, Australia). IGF-I was iodinated by the chloramine-T method to a specific activity of about 60–80 μCi/μg. Recombinant human non-glycosylated IGFBP-3 (rhIGFBP-3) was a gift of Drs. A. Sommer and C. Maack (Celtrix, Santa Clara, CA), rhIGFBP-4 and -6 produced in yeast [Kiefer et al., 1992] were kindly provided by Dr. J. Zapf (Zurich, Switzerland). Recombinant IGFBP-1, -2, and -5 were purchased from UBI (Lake Placid, NY) and from GroPep, respectively. rhIGFBPs were labeled with Na[^{125}I] using IODO-GEN (Pierce Chemical Co, Rockford, IL) to specific activities of 30-60 μCi/μg. Materials and kits were obtained from commercial sources as indicated: ECL detection kit and Hybond membranes (Amersham Pharmacia Biotech); nitrocellulose membranes (Sartorius, Göttingen, Germany); random priming kit (Roche, Mannheim, Germany); nick translation kit (Life Technology, Eggenstein, Germany); hybridization solution QuickHyb (Stratagene, Heidelberg, Germany); X-ray films X-Omat AR (Kodak, Rochester, NY). Chemicals were reagent grade and obtained from commercial sources as indicated: Dulbecco's modified Eagle medium, trypsin, bovine serum albumin, and L-glutamine (PAA, Linz, Austria); DNase I (Roche, Mannheim, Germany); bovine serum albumin (BSA) and antibiotics (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany).

Antibodies

Polyclonal rabbit antibodies against human IGFBP-1, -2, -3, -4, and -5 were purchased from GroPep. Biotinylated mouse monoclonal antibodies against rabbit IgG were supplied by Dianova (Hamburg, Germany) and horseradish peroxidase-conjugated streptavidin by Zymed Laboratories (San Francisco, CA).

cDNA Probes

The following complementary DNA (cDNA) probes were used for Northern blot analysis: a 1.5 kb fragment of human IGFBP-1 cDNA, a 1.4 kb fragment of human IGFBP-2 cDNA, a 2.3 kb fragment of human IGFBP-3 cDNA, a 2 kb fragment of human IGFBP-4 cDNA [Kiefer et al., 1991], and a 1.6 kb fragment of IGFBP-5 cDNA were kindly provided by Dr. J. Zapf (Zürich, Switzerland). A 2.8 kb fragment of human IGF-IR cDNA clone p igfr 85 [Ullrich et al., 1986] was obtained by Dr. A. Gray (South San Francisco, CA). cDNAs coding for human IGF-I and for human IGF-II [Bell et al., 1985] were donated by Dr. G. Bell (Chicago, IL), and a cDNA specific for human IGF-II/M6PR by Dr. W. Sly (St. Louis, MO). The oligonucleotides for IGF-I used for RT-PCR analysis (sense: 5′-GCTGGTGGATGCTCTTCA-3′; antisense: 5′-CTGACTTGGCAGGCTTGA-3′) and for IGF-II

(sense: 5′-AGTCGATGCTGGTGCTTCTCA-3′; antisense: 5′-GTGGGCGGGGTCT TGGGTG-GGTAG-3′) were synthesized by MWG (Ebersberg, Germany).

Cell Culture

The human osteosarcoma cell line HOS 58 derived from an osteosarcoma of the leg of a 21-year-old man represents an osteoblastic phenotype with the capability to further differentiate in culture [Siggelkow et al., 1998; Siggelkow et al., 2002]. These HOS cells have been shown to represent the phenotype of the mature osteoblasts. The cells (plating density: 12,500 cells/cm^2) were grown at 37°C and maintained in phenol red-free ISCOVE's medium supplemented with 10% charcoal-stripped fetal calf serum (cs-FCS) from Allgaeu BioTech Service (Goerisried, Germany), 1% glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. HOS 58 cells from passage number 11 were cultured in serum-free MEM supplemented with 0.125% (w/v) bovine serum albumin (BSA) for 2 days prior to RNA isolation and protein analysis. Cultures were analyzed for up to 25 days after reaching confluence by Day 7. Medium was changed three times weekly. Gene expression was investigated at Days 2, 4, 8, 12, 18, and 25.

Detection of IGFBPs by Western Ligand and Immunoblotting

Western ligand and immunoblotting were essentially performed as described recently [Hossenlopp et al., 1986; Scharf et al., 1996].

RNA Isolation and RT-PCR Analysis

Total cellular RNA was isolated using the RNeasy total RNA extraction kit from Qiagen (Hilden, Germany). Reverse transcription was performed with 1 μg of total RNA as previously described [Siggelkow et al., 1999; Viereck et al., 2003]. Each cDNA sample was run in triplicate for each PCR reaction. Competitive RT-PCR was performed using exogenous DNA competitors ("mimics") as internal control [Viereck et al., 2003] that were synthesized with the PCR mimic construction kit from Clontech (Palo Alto). PCR reactions were carried out in 15 μl volumes using primer sequences as previously described [Rickard et al., 1996; Viereck et al., 2003] and cycle numbers ensuring a linear amplification profile. The ribosomal housekeeping gene L7 mRNA was analyzed as reported elsewhere [Siggelkow et al., 2004]. IGF-I was analyzed using a protocol of 75 s at 94°C, 27 cycles (of 45 s at 94°C, 45 s at 60°C, 2 min at 72°C), 10 min at 72°C. For detection of IGF-II mRNA, a protocol of 2 min at 94°C, 20 cycles (of 60 s at 94°C, 60 s at 54°C, 2 min at 72°C), 10 min at 72°C and for type I collagen (COL-1) mRNA a protocol of 2 min at 94°C, 23 cycles (of 30 s at 94°C, 2 min at 55°C, 2 min at 72°C), 10 min at 72°C were used. For analysis of alkaline phosphatase (AP) and osteocalcin (OCN) mRNA, a protocol of 2 min at 94°C, 26 and 30 cycles (of 30 s at 94°C, 45 s at 55°C, 90 s at 72°C), respectively, and 10 min at 72°C was performed. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. The expression of each gene was quantified as target to mimic ratio and normalized to the L7 mRNA level. The specificity of the PCR products was examined by sequencing of the amplification product using the Abi Prism system from Perkin Elmer (Weiterstadt, Germany).

Northern Blot Analysis

Northern blot analyses were essentially performed as described before [Scharf et al., 1996; Scharf et al., 1998a].

Cell-Free IGFBP Protease Assay

IGFBP protease assays were performed as described recently [Kubler et al., 1998; Shalamanova et al., 2001]. Briefly, [^{125}I]-rhIGFBP (approximately 30,000 cpm) were added to 50 μl of conditioned media, adjusted to the indicated pH and incubated at 37°C for 20 h. Samples were solubilized and subjected to SDS–PAGE (12.5% (w/v) polyacrylamide) under non-reducing conditions. Radiolabeled intact rhIGFBPs and proteolytic fragments were detected by autoradiography.

Cell Proliferation

DNA synthesis was assessed by incorporation of [^{3}H]-thymidine as described previously [Scharf et al., 1998b]. In brief, HOS 58 cells kept under serum-free conditions for 1 h were incubated with 1 μCi [^{3}H]-thymidine per dish for 24 h in the presence or the absence of the indicated growth factor. Experiments were performed in triplicate, and means ± standard error of the means (SEM) of three independent experiments were expressed as a percentage of control.

Statistical Analysis

Unless otherwise stated, all experiments were reproduced at least three times. Autoradiographs of Western ligand and Northern blots were scanned using a laser densitometer (Epson GT-8000, Biometra, Göttingen, Germany). The relative densities of bands were expressed as a percentage of control. Values are expressed as the mean ± SEM of triplicate measurements and data obtained from representative experiments are shown. Student's paired *t*-test was used to evaluate differences between the sample of interest and its respective control. For analysis of time courses, multiple measurement ANOVA followed by Newman-Keuls post-test analysis was performed. A P value of less than 0.05 was considered statistically significant.

RESULTS

Characterization of Cultured HOS 58 Cells

To verify the in vitro differentiation of HOS 58 cells, the mRNA expression of AP and OCN, markers of the osteoblast differentiation, as well as of COL-1 was studied by RT-PCR (Fig. 1). mRNA species for AP and OCN were detectable 2 days after plating of HOS 58 cells showing a continuous increase during the in vitro differentiation with a maximum at Day 18 and 25 of culture, respectively. In contrast, the expression of COL-1 mRNA was highest at Day 4 of culture followed by a continuous decline reaching lowest levels at Day 25 (Fig. 1).

Expression of IGF-I and IGF-II mRNA

IGFs have been shown to be one of the most abundant growth factors in the bone microenvironment. Therefore, total RNA was isolated from HOS 58 cells at Days 2, 4, 8, 12, 18, and 25 after plating and mRNA expression of IGF-I and -II was analyzed by RT-PCR in relation to the house-keeping gene L7 (Fig. 2). An IGF-II specific 486 bp fragment was amplified that increased continuously with time of culture (Fig. 2A). Steady state IGF-II mRNA levels increased approximately sevenfold until Day 25 of culture compared with baseline levels as estimated by densitometry analysis of three independent experiments. To exclude any regulatory effects of steroid hormones present in fetal calf serum (FCS), HOS 58 cells were cultivated in phenol red-free medium

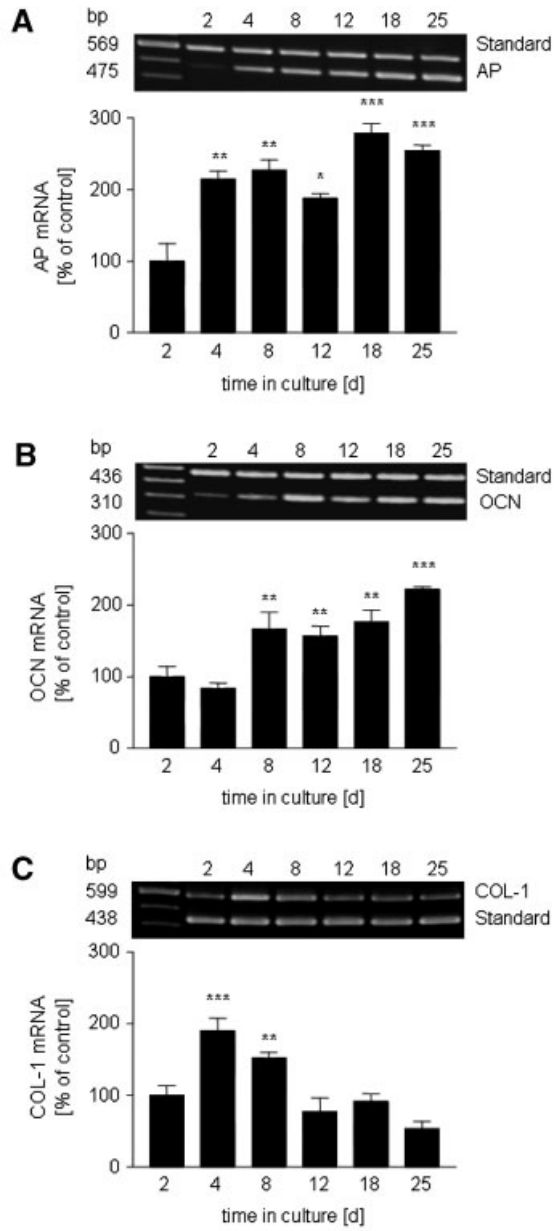


Fig. 1. mRNA expression of alkaline phosphatase (AP), osteocalcin (OCN), and of type I collagen (COL-1) during in vitro differentiation of HOS 58 cells. The osteoblastic differentiation markers AP (**A**), OCN (**B**), and COL-1 (**C**) were measured by RT-PCR isolated from HOS 58 cells cultured for 2–25 days as indicated. Representative results of PCR products and sizes are depicted. After densitometric analysis, the relative densities of bands were given as the mean ± SEM of five experiments with triplicate measurements compared with the respective values at Day 2. Statistics, $P < 0.0001$ by ANOVA for AP, OCN and COL-1. Post-test Newman-Keuls analysis: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for individual values compared with Day 2.

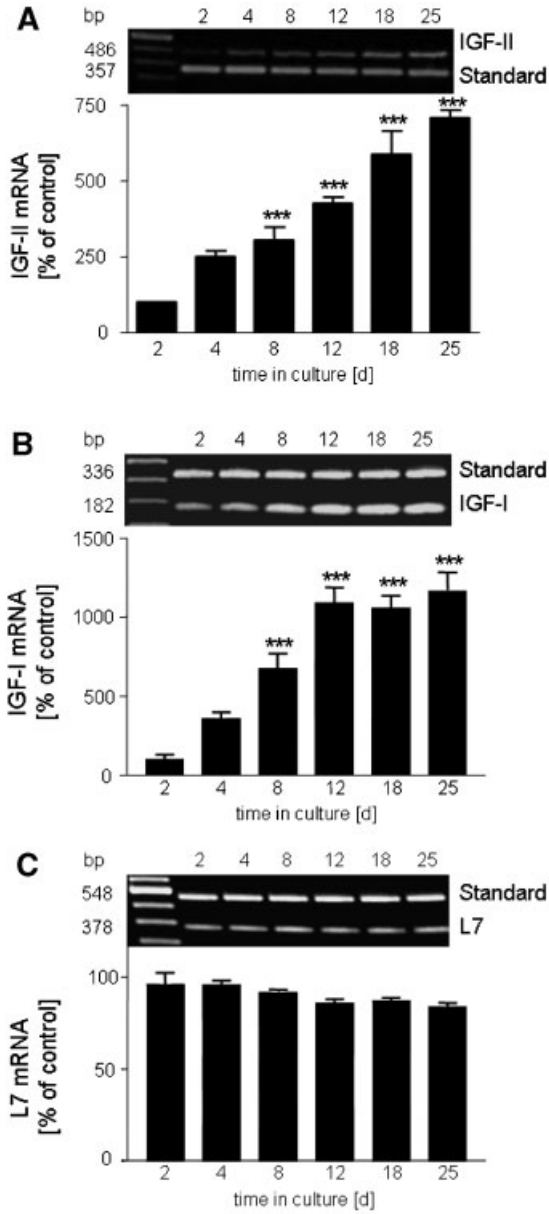


Fig. 2. mRNA expression of IGF-I and IGF-II during in vitro differentiation of HOS 58 cells. mRNA expression of IGF-II (**A**), IGF-I (**B**), and of the house-keeping gene L7 (**C**) was measured by RT-PCR isolated from HOS 58 cells cultured for the time indicated. Representative results of PCR products and sizes are shown. After densitometry, the relative densities of PCR bands are indicated as the mean ± SEM of five experiments with triplicate measurements normalized to respective values at Day 2. $P < 0.0001$ by ANOVA; post-test Newman-Keuls analysis: $^{***}P < 0.001$ for individual values compared with the respective control at Day 2.

supplemented with cs-FCS (data not shown). The increase in IGF-II mRNA expression under these conditions was similar to the pattern found in HOS 58 cells incubated in the presence of untreated FCS.

RT-PCR analysis for IGF-I specific mRNA transcripts revealed an amplified 182 bp fragment that increased with time of culture (Fig. 2B). In HOS 58 cells harvested at Day 25 of culture, the amount of the IGF-I specific fragment was 11.6-fold elevated as compared with HOS 58 cells at Day 2 after plating.

Expression of the IGF-IR and IGF-II/M6PR

When expression of the IGF-IR mRNA was analyzed by Northern blotting, an 11 kb IGF-IR mRNA species was detected in total RNA of HOS cells that was most abundant at Day 2, 4, and 8 of culture (Fig. 3A). During the cultivation period, the IGF-IR mRNA decreased continuously and dropped at Day 25 to approximately 20% of levels observed at Day 2. A comparable decrease of IGF-IR mRNA expression during the in vitro differentiation was observed when HOS cells were maintained in phenol red-free medium with cs-FCS (Fig. 3B). IGF-II/M6PR specific mRNA transcripts were detected at 9 kb showing high levels at Days 2–8 of culture declining with time of cultivation (Fig. 3). Densitometry analysis revealed that the amount of IGF-II/M6PR mRNA at Day 25 was approximately 60% of that at Day 2.

Secretion of IGFBPs

Human serum and conditioned media (CM) from cultured HOS 58 cells were analyzed by [^{125}I]-IGF-I ligand blotting for the presence of IGFBPs. CM of HOS 58 cells contained a prominent IGFBP with the apparent molecular mass of 32 kDa (Fig. 4A). This 32-kDa IGFBP showed immunoreactivity with an antiserum raised against IGFBP-2 (Fig. 4B). Additionally, faint IGF binding bands were detected at 24, 28, and 41–45 kDa identified as IGFBP-4, IGFBP-5, and IGFBP-3, respectively, by Western blotting (Fig. 4C).

Northern blot analysis demonstrated IGFBP-2 (at 1.4 kb), IGFBP-3 (at 2.5 kb), IGFBP-4 (at 2.5 kb), and IGFBP-5 (at 6.0 kb) coding transcripts in HOS 58 cells (Fig. 5). Steady-state mRNA levels of IGFBPs were low in HOS 58 cells at Day 2 of culture, but increased with time of culture. Densitometry analysis revealed that this increase was about threefold for IGFBP-2, threefold for IGFBP-3, fivefold for IGFBP-4, and sevenfold for IGFBP-5 mRNA levels in HOS 58 cells at Day 25 of culture

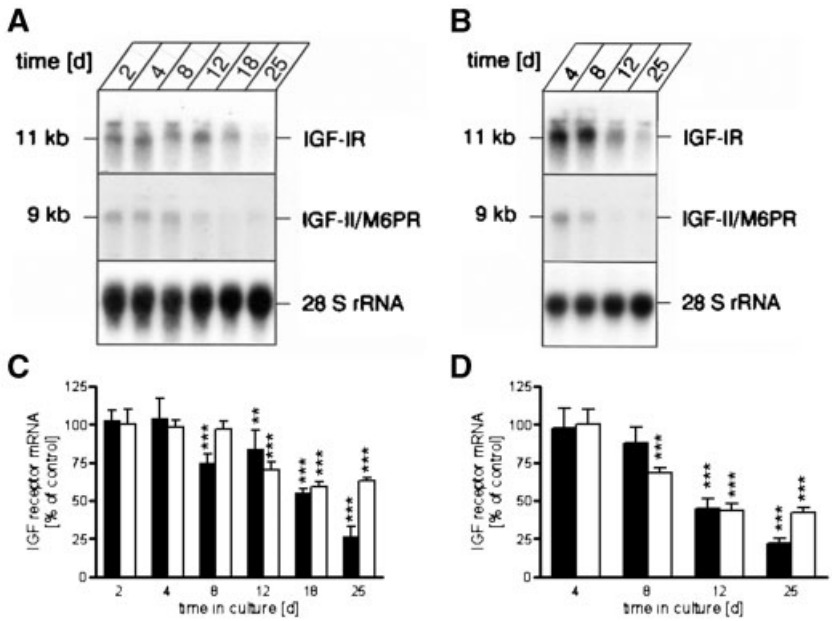


Fig. 3. IGF receptor mRNA expression during in vitro differentiation of HOS 58 cells. (**A**, **B**) **Upper panels:** 10 μg of total RNA were obtained from HOS 58 cells at different days of culture in the presence of untreated FCS (A) or in phenol red-free medium supplemented with charcoal-stripped FCS (B). Total RNA was separated by 2% agarose gel electrophoresis, blotted, and hybridized with cDNAs specific for human IGF-IR and IGF-II/M6PR, respectively. The sizes of the hybridization bands are indicated on the left. **Lower panel:** equal loading of RNA was demonstrated after stripping and rehybridization of membranes with an oligonucleotide complementary to 28 S ribosomal RNAs. (**C**, **D**) Densitometry analysis of IGF-IR and IGF-II/M6PR mRNA expression in HOS 58 cells. The relative densities of bands were expressed as the percentual decrease of IGF-IR mRNA (closed bar) and IGF-II/M6PR (open bar) compared with the respective mRNA level at Day 2 of culture ($n = 5$). Statistics, $P < 0.0001$ by ANOVA for IGF-IR and IGF-II/M6PR. Post-test Newman-Keuls analysis: $^{**}P < 0.01$, $^{***}P < 0.001$ for individual values compared with Day 2.

compared with Day 2, respectively. Similar results were observed in HOS 58 cells cultivated with phenol red-free medium with cs-FCS (Fig. 5B).

IGFBP Proteolysis

To determine whether IGFBP-proteolytic activities are present in CM of HOS 58 cells, [^{125}I]-labeled recombinant human IGFBP-2, -3, and -4 were incubated in the presence and the absence of CM from HOS 58 cells at various pH followed by SDS–PAGE and autoradiography. At neutral pH, the non-glycosylated 30 kDa [^{125}I]-IGFBP-3 was cleaved to a major fragment of 23 kDa (data not shown). No proteolytic activities against radiolabeled rhIGFBP-2 and -4 were observed (data not shown). At pH 4.0, radiolabeled rhIGFBP-2, -3 as well as rhIGFBP-4 were degraded to smaller fragments (data not shown). However, proteolytic activities both at neutral and acidic pH were too low to contribute to the regulation of IGFBP concentrations in CM of HOS 58 cells.

Proliferation of HOS 58 Osteosarcoma Cells

To study whether the observed alterations of the components of the IGF axis during in vitro differentiation of HOS 58 cells might affect DNA synthesis of HOS 58 cells, basal and IGF-I stimulated HOS 58 cell proliferation was examined at Days 2, 4, 8, 12, 18, and 25 of culture by means of [^{3}H]-thymidine incorporation (Fig. 6). Cells were kept under serum-free conditions for 1 h, before [^{3}H]-thymidine were added for 24 h. Under these conditions, the highest rate of [^{3}H]-thymidine incorporation was observed in HOS 58 cells at Day 4 of culture, thereafter declining continuously. Incubation of HOS 58 cells at Days 2 and 4 with IGF-I (100 nmol/L) for 24 h stimulated the DNA synthesis in HOS 58 cells approximately 1.9- and 1.4-fold, respectively, compared with the respective untreated control.

When HOS 58 cells at Day 4 of culture were coincubated with 100 nmol/L IGF-I and increasing concentrations of rhIGFBP-2, the rate of DNA synthesis was lower as compared with

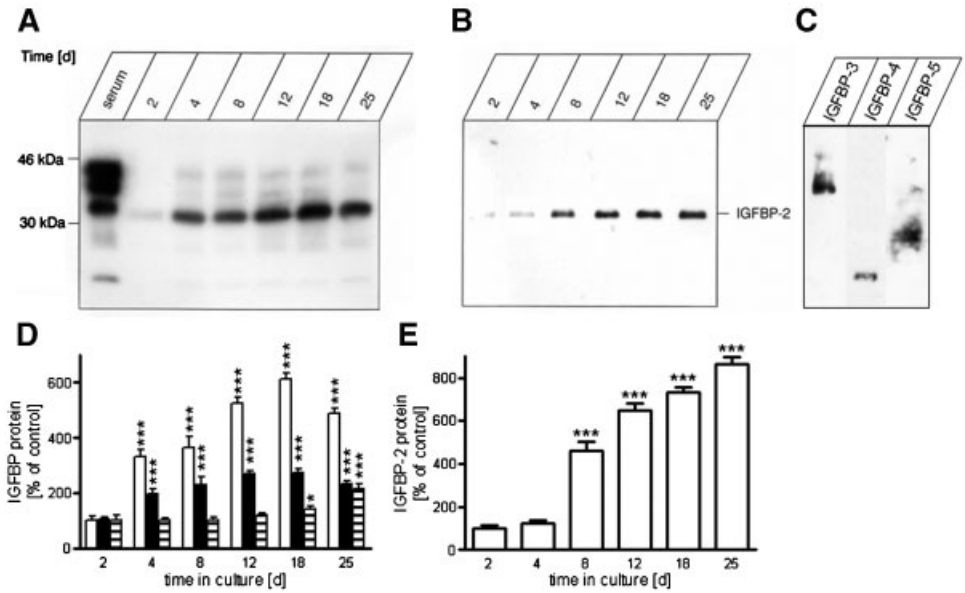


Fig. 4. IGFBP secretion during in vitro differentiation of HOS 58 cells. **A:** [^{125}I]-IGF-I ligand blot of human serum (serum) and of conditioned media (CM) from HOS 58 cells at different days of culture as indicated. Cultures of HOS 58 cells were kept under serum-reduced conditions for 24 h prior to further analysis. **B:** IGFBP-2 immunoblot of CM from HOS 58 cells at different days of culture. **C:** Immunoblots against IGFBP-3, -4, and -5 of CM from HOS 58 cells at Day 25 of culture. **D:** Densitometry analysis of IGFBP secretion by HOS 58 cells at different time points of culture. The relative densities of bands were expressed as the percentage of increase of IGFBP-2 (open bar), IGFBP-3 (closed bar), and IGFBP-4 (hatched bar) compared with the respective IGFBP at Day 2 of culture (n = 5). **E:** Densitometry analysis of IGFBP-2 secretion by HOS 58 cells at different days of culture as determined by Western blotting. The relative densities of IGFBP-2 were expressed as the percentage of increase compared with IGFBP-2 at Day 2 of culture. Statistics, $P < 0.0001$ by ANOVA for IGFBP-2, -3, and -4. Post-test Newman-Keuls analysis: $^{*}P < 0.05$, $^{***}P < 0.001$ for individual values compared with Day 2.

HOS 58 cells incubated with 100 nmol/L IGF-I alone (Fig. 7). The most pronounced inhibition of DNA synthesis was observed when 100 nmol/L IGF-I was coincubated with 150 nmol/L rhIGFBP-2. Preincubation of HOS 58 cells with rhIGFBP-2 followed by addition of IGF-I versus simultaneous addition of rhIGFBP-2 with IGF-I did not result in significant differences of inhibition of DNA synthesis (Fig. 7).

DISCUSSION

Recent studies demonstrated that the process of differentiation in HOS 58 cells comprises two distinct phases of cellular activity: (i) a rapid cell proliferation in low-density HOS 58 cells at early time points after plating that decreased continuously with time of culture and (ii) the secretion of matrix proteins that was low in low-density HOS 58 cells and that increased during their in vitro differentiation [Siggelkow et al., 1998; Siggelkow et al., 2002]. In the present paper we have studied the expression of individual components of the IGF axis in HOS 58 cells during their in vitro differentiation. Low-density HOS 58 cells at early time points of the in vitro differentiation (i) displayed a low expression of IGF-I, IGF-II; (ii) produced low levels of IGFBP-2, -3, -4, and -5; but (iii) showed high expression levels of both the type I and II IGF receptors. During the in vitro differentiation of HOS 58 cells, IGF-I and -II expression increased continuously, and an upregulation of IGFBP-2, -3, -4, and -5 was observed. At the same time, expression of the IGF-IR and IGF-II/M6PR was downregulated. From these data, the different phases of the in vitro differentiation observed in HOS 58 cells strongly correlated with alterations of the IGF axis as outlined in the present study. The high proliferative activity in low-density HOS 58 cells at early time points of the differentiation process was associated with high steady-state mRNA levels of the IGF-IR. The HOS 58 cell proliferation continuously decreased during cultivation in parallel with a decline in IGF-IR expression. Additionally, the presence of the most abundant IGFBP in CM of HOS 58 cells, IGFBP-2,

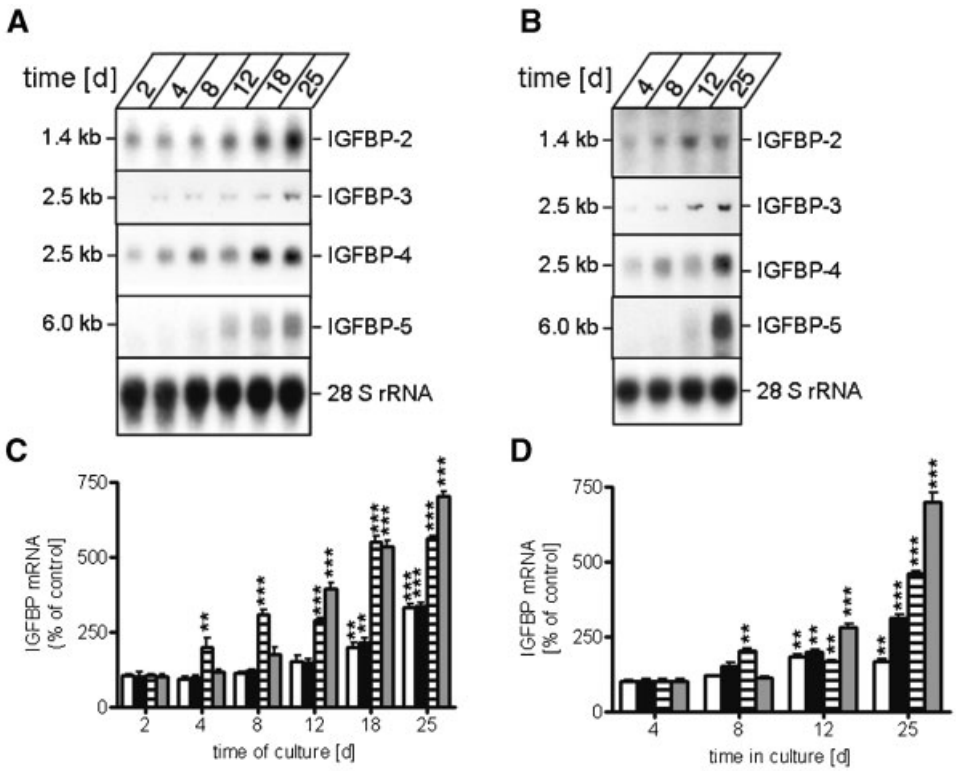


Fig. 5. IGFBP mRNA expression during in vitro differentiation of HOS 58 cells. (**A**, **B**) **Upper panels:** total RNA (10 μg) isolated from HOS 58 cells at different time points after plating of days were separated by 2% agarose gel electrophoresis, blotted and probed for IGFBP-2, -3, -4, and -5, respectively. **Lower panels:** equal loading of RNA was demonstrated after stripping and rehybridization of membranes with an oligonucleotide complementary to 28 S ribosomal RNAs. (**C**, **D**) Densitometry analysis of IGFBP expression. The relative densities of bands were expressed as the percentage of increase or decrease for IGFBP-2 (open bar), IGFBP-3 (closed bar), IGFBP-4 (hatched bar), and IGFBP-5 (gray bar) compared with the respective mRNA expression at Day 2 of culture (n = 5). Statistics, $P < 0.0001$ by ANOVA for IGFBP-2, -3, -4, and -5. Post-test Newman-Keuls analysis: $^{**}P < 0.01$, $^{***}P < 0.001$ for individual values compared with Day 2.

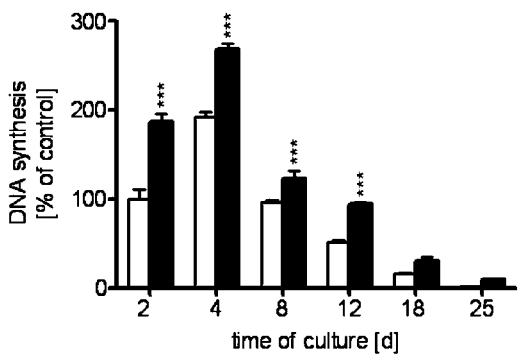
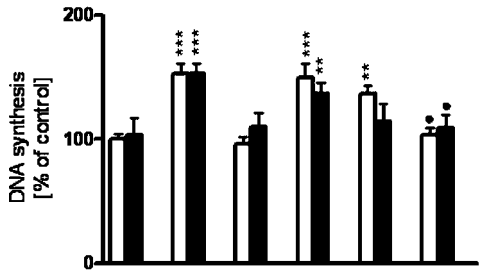


Fig. 6. DNA synthesis of HOS 58 cells during in vitro differentiation. After starvation in serum-reduced DMEM for 1 h, HOS 58 cells at different days of culture were pulse labeled with [^{3}H]-thymidine for 24 h (open bars). The effect of IGF-I on DNA synthesis of HOS 58 cells was evaluated by simultaneous addition of IGF-I at a concentration of 100 nmol/L (closed bars). Results are expressed as percent increase or decrease in DNA synthesis compared with untreated HOS 58 cells at Day 2 of culture (mean ± SEM; n = 3). Statistics, $P < 0.0001$ by ANOVA for DNA synthesis in control cells versus IGF-I treated HOS 58 cells. Post-test Newman-Keuls analysis: $^{***}P < 0.001$ for individual values compared with the untreated control.

increased with time of cultivation and was negatively correlated with the rate of cell proliferation.

IGF-I, IGF-II

Cells of the osteoblast lineage in culture express both IGF-I and IGF-II with important qualitative and quantitative differences among the various cell models. Data of the present study confirm previous investigations showing distinct species differences with rat osteoblasts primarily expressing IGF-I [Canalis et al., 1989] and human osteoblasts primarily expressing IGF-II [Okazaki et al., 1995]. In humans, IGF-II is not only more abundant than IGF-I in bone matrix but also the most abundant of all growth factors in bone [Seck et al., 1998]. In the present study, the pattern of both IGF-I and -II expressions in HOS 58 cells apparently correlated with the stage of their in vitro differentiation. In rodents, the stage of osteoblast differentiation apparently influenced the



IGF-I [nmol/L]	-	100	-	100	100	100
IGFBP-2 [nmol/L]	-	-	50	50	100	150

Fig. 7. Effect on rhIGFBP-2 on DNA synthesis of HOS 58 cells. After starvation in serum-reduced DMEM for 1 h, HOS 58 cells were preincubated with rhIGFBP-2 at concentrations of 50, 100, and 150 nmol/L (open bars) for 1 h followed by the addition of 100 nmol/L IGF-I. In the second approach, the effect of IGFBP-2 on IGF stimulated DNA synthesis was evaluated by simultaneous addition of rhIGFBP-2 at concentrations of 50, 100, and 150 nmol/L (solid bars) with 100 nmol/L IGF-I. Control cells were incubated for 24 h with either serum-reduced DMEM alone or 100 nmol/L IGF-I. Cells were labeled with [^{3}H]-thymidine for 24 h. Results are expressed as percent increase or decrease compared with untreated HOS 58 cells at Day 4 of culture (mean ± SEM; n = 3). Statistics, $P < 0.001$ by ANOVA for DNA synthesis in control cells versus IGF-I treated HOS 58 cells (*) or in IGF-I treated versus IGF-I/rhIGFBP-2 treated HOS 58 cells (●). Post-test Newman-Keuls analysis: $^{**}P < 0.01$, $^{***}P < 0.001$.

pattern of IGF expression. For example, in fetal rat calvarial cultures, IGF-I secretion was shown to be biphasic. Early-stage IGF-I expression was associated with cell proliferation. As preosteoblasts differentiated, IGF-I secretion decreased with a second increase late during mineralization [Birnbaum et al., 1995]. Again, these data point to species differences not only in respect to the predominant IGF but also to the expression pattern of these growth factors during differentiation. However, so far it remains unclear whether IGF-I and IGF-II actually serve specific functions which differ in the different species or whether the functions are the same and the bone physiology differs among species [Aerssens et al., 1998].

IGF Receptors

Similar to IGFs, a stage-dependent expression of the IGF-IR receptor was detected during the in vitro differentiation of HOS 58 cells. The high proliferation of HOS 58 cells at early time points of culture was associated with high levels of IGF-IR while the level of IGF-IR expression declined with the rate of cell proliferation during the course of differentiation. Accordingly, HOS 58 cells at Days 2 and 4 of culture were susceptible to IGF showing a stimulation of thymidine incorporation after treatment with IGF-I. Similarily, IGFs have been found to increase DNA synthesis and replication of cells of the osteoblast lineage and played a major role in stimulating the differentiated function of the mature osteoblast. In vitro, human and rodent osteoblast and osteosarcoma cells were responsive to ligand-activated type I IGF receptor stimulation with resulting increase in DNA and protein synthesis [Wergedal et al., 1990; Jonsson et al., 1993; Kanzaki et al., 1994; Raile et al., 1994]. Of note, IGF-I and IGF-II not only act to stimulate proliferation of osteoblast precursors and early-stage osteoblasts but they also promote bone matrix formation by the fully differentiated osteoblast [Mohan and Baylink, 1996; Rosen and Donahue, 1998].

The central role of the IGF-IR for bone formation has been demonstrated in IGF-IR (−/−) mice showing delayed skeletal calcification and severe growth retardation [Liu et al., 1993]. Severe disturbances in organ development and frequent lethality prohibited the use of these models for examination of the skeletal role of IGF-I in the mature animal. However, the targeted disruption of the IGF-IR gene in osteoblasts via the osteocalcin promoter and *Cre*-mediated recombination resulted in a reduction of trabecular bone formation, of cancellous bone volume, and of trabecular connectivity [Zhang et al., 2002].

IGFBPs

IGF bioavailability in bone is determined not only by bone cell expression and by release of the peptides from the matrix, but also by the presence of six high affinity IGF binding proteins (IGFBPs). All six IGFBPs have been detected in bone cells, however, like IGF peptides, IGFBP expression varied depending on different species, bone cell model, and culture conditions. In HOS 58 cells, expression of IGFBP-2, -3, -4, and -5 with IGFBP-2 as predominant IGFBP was detected. Similar to IGF peptides and IGF receptor, the pattern of IGFBP expression changed during the in vitro differentiation of HOS 58 cells: low levels of expression were detected for IGFBP-2, -3, -4, and -5 in HOS 58 cells at Days 2 and 4 of culture showing a continuous increase during the differentiation of HOS 58 cells.

In line with the present paper, previous studies have demonstrated the production of IGFBP-2 in rat and human osteoblasts as well as the human osteosarcoma lines MG-63 [Lalou et al., 1994] and TE-85 [Durham et al., 1995]. Similar to the data presented herein, IGFBP-2 primarily inhibited the action of IGFs in bone. For instance, addition of recombinant IGFBP-2 at excess concentrations inhibited the actions of IGF-I on fetal rat calvarial osteoblast replication and matrix synthesis [Feyen et al., 1991]. In vivo, overexpression of IGFBP-2 in transgenic mice in the absence and the presence of GH excess reduced bone length and bone mass, but not the bone density [Eckstein et al., 2002]. In contrast, in vitro data indicated that IGFBP-2 in complex with IGF-II has high affinity for bone cell-derived extracellular matrix [Khosla et al., 1998] and is able to stimulate osteoblast proliferation under these conditions [Conover and Khosla, 2003]. In line with these results, subcutaneous administration of IGF-II/IGFBP-2 complexes stimulated bone formation and prevented loss of bone mineral density in a rat model of disuse osteoporosis pointing to a novel anabolic approach to increase bone mass in humans with osteoporosis [Conover et al., 2002].

In contrast to IGFBP-2, only trace amounts of IGFBP-3, -4, and -5 were detected in CM of HOS 58 cells. In a number of different bone cell models, IGFBP-4 was a major IGFBP produced by bone cells that has been consistently shown to inhibit IGF-stimulated effects [Mohan et al., 1989; LaTour et al., 1990; Kiefer et al., 1992; Kassem et al., 1996]. Similarly, in transgenic mice overexpressing IGFBP-4 in osteoblasts revealed a significant decrease in bone volume, cortical bone density, osteoblast number, and bone formation [Zhang et al., 2003]. IGFBP-3 and -5 have been shown to exert both inhibitory and stimulatory effects on bone. Overexpression of IGFBP-3 in mice resulted in an increase of the osteoclast number, bone resorption, and consequently in reduced bone mineral density most likely due to sequestering of IGFs in the circulation in a ternary IGF transport complex of 150 kDa [Silha et al., 2003]. In contrast, IGFBP-3 added to osteoblasts in vitro stimulated IGF actions [Ernst and Rodan, 1990] pointing to different effects of local versus systemic IGFBP-3 in bone.

Intact and soluble IGFBP-5 inhibited IGF-stimulated bone cell growth [Kiefer et al., 1992]. However, IGFBP-5 is normally not intact or in solution in the bone cell environment due to an IGFBP-5 protease secreted by bone cells [Conover, 1995] and to its strong affinity to bind to hydroxyapatite, which preferentially anchors IGFBP-5 in the extracellular matrix [Bautista et al., 1991]. IGFBP-5 bound to the extracellular matrix acts as IGF-II storage [Bautista et al., 1991] and is associated with potentiation of IGF action [Jones et al., 1993].

On the basis of known biological effects of IGFBPs, we suggest that the observed changes in IGFBPs—especially the increase of mitoinhibitory IGFBP-2 during the in vitro differentiation of HOS 58 cells—may lead to inhibition of proliferation of HOS 58 cells and to induction of their differentiation.

In summary, the human osteosarcoma cell line HOS 58 demonstrated a characteristic expression pattern of IGFs, IGF receptors as well as of IGFBPs that changed dramatically during the in vitro differentiation of this cell line. The high proliferative activity in low cell density HOS 58 cells was associated with high mRNA levels of the IGF-IR, but low concentrations of the most abundant IGFBP secreted, IGFBP-2. The rate of proliferation of HOS 58 cells continuously decreased during cultivation in parallel with a decline in IGF-IR expression, but increase of IGFBP-2. These data are indicative for a role of the IGF axis during the in vitro differentiation of HOS 58 cells.

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