Inhibition of IGF-I-related intracellular signaling pathways by proinflammatory cytokines in growth plate chondrocytes

Daniela Choukair¹, Ulrike Hügél¹, Anja Sander², Lorenz Uhlmann² and Burkhard Tönshoff¹

BACKGROUND: Children with chronic inflammatory diseases suffer from severe growth failure associated with resistance toward the anabolic action of insulin-like growth factor I (IGF-I). We hypothesized that proinflammatory cytokines interfere with IGF-I signaling.

METHODS: We used the mesenchymal chondrogenic cell line RCJ3.1C5.18 as a model of the growth plate. Cell proliferation was assessed by [³H]-thymidine-uptake and differentiation by gene expression (quantitative reverse-transcriptase PCR) of specific differentiation markers. Key signaling molecules of the respective IGF-I-related intracellular pathways were determined by western immunoblotting.

RESULTS: Coincubation of the proinflammatory cytokines interleukin (IL)-1 β (10 ng/ml), IL-6 (100 ng/ml), or tumor necrosis factor- α (50 ng/ml) with IGF-I inhibited IGF-I-driven cell proliferation by 50%, while baseline cell proliferation was not altered. These cytokines attenuated the IGF-I-induced phosphorylation of AKT as a key signaling molecule of the phosphatidylinositol-3 kinase pathway by 30–50% and the phosphorylation of ERK as a key signaling molecule of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by 50–75%. Also, IGF-I-enhanced chondrocyte differentiation was inhibited by these proinflammatory cytokines.

CONCLUSION: The insensitivity toward the anabolic action of IGF-I in the growth plate in conditions of chronic inflammation is partially due to inhibition of IGF-I-specific signaling pathways by proinflammatory cytokines, which affect both IGF-I-driven chondrocyte proliferation and differentiation.

The growth plate is a dynamic tissue, in which cells undergo a developmental program from resting cells to proliferation, maturation, and hypertrophy, until they reach terminal differentiation and produce the mineralized matrix that supports endochondral bone formation (1). Insulin-like growth factor (IGF)-I is a potent growth factor exerting its actions by both endocrine and paracrine/autocrine mechanisms. It is known from cell culture (2,3) and gene knockout experiments (4) that IGF-I stimulates both proliferation and differentiation of growth plate chondrocytes *in vitro* and *in vivo*. IGF-I exerts its biological effect by binding to the transmembrane type 1

IGF receptor, whose activation leads to the extensive tyrosyl-phosphorylation of insulin receptor substrate-1, which acts as a docking protein for the downstream signal transduction pathways (5,6). Previously, we could demonstrate in a cell culture model of the growth plate, the mesenchymal chondrogenic cell line RCJ3.1C5.18, that two canonical pathways, the phosphatidylinositol-3 (PI-3)-kinase and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK)1/2 pathway, mediate the mitogenic response to IGF-I. When chondrocytes progress from proliferating cells to early and terminal differentiating cells, they progressively inactivate IGF-I-related intracellular signaling pathways, and only the PI-3 kinase pathway remains operative for IGF-I signaling (7).

An inhibition of longitudinal growth is commonly observed in children with chronic inflammatory diseases, such as juvenile idiopathic arthritis (8), chronic kidney disease (9), or chronic inflammatory bowel disease (10). In these patients, serum concentrations of proinflammatory cytokines such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α are elevated (11,12). Furthermore, overexpression of IL-6 in transgenic mice is associated with a 50–70% reduction of postnatal growth as compared with control animals (13). These proinflammatory cytokines appear to disturb longitudinal growth by a direct effect on the growth plate and by modulation of the systemic growth hormone (GH)/IGF axis. The constellation of inappropriately low IGF-I serum concentrations in the presence of normal circulating GH concentrations has been interpreted as a resistance to the action of GH for hepatic IGF-I production (14–16). On the other hand, suppression of chronic inflammation in children with juvenile idiopathic arthritis with etanercept, a TNF- α antagonist, is associated with a rise in IGF-I serum concentrations and catch-up growth (17).

In addition, the morphology of the growth plate in experimental animals with chronic inflammation is disturbed. For example, growth plates of rats with experimental colitis show a widening of the reserve zone and a reduced proliferative zone in comparison with pair-fed control animals (18). Currently, little is known about the effects of inflammatory cytokines on the activity of growth factors in the growth plate. As a step toward understanding the underlying mechanisms by which proinflammatory cytokines inhibit growth, we studied

¹Department of Pediatrics I, University Children's Hospital Heidelberg, Heidelberg, Germany; ²Institute of Medical Biometry and Informatics, University of Heidelberg, Heidelberg, Germany. Correspondence: Daniela Choukair (daniela.choukair@med.uni-heidelberg.de)

Received 28 August 2013; accepted 27 February 2014; advance online publication 16 July 2014. doi:10.1038/pr.2014.84

the interference of proinflammatory cytokines with IGF-I signaling pathways in the mesenchymal chondrogenic cell line RCJ3.1C5.18 as a cell culture model of the growth plate. RCJ cells progress in culture without biochemical or oncogenic transformation from mesenchymal chondroprogenitor cells to differentiated chondrocytes in a sequence that mimics the phenotype of chondrocytes of the growth plate (19–21). Furthermore, these cells do not express IGF-I or IGF-II; therefore, the action of these hormones can be studied without interference from endogenous IGF (20). We report here that the resistance toward the anabolic action of IGF-I in the growth plate in conditions of chronic inflammation is partially due to the inhibition of IGF-I-specific signaling pathways by proinflammatory cytokines, which affect both IGF-I-driven chondrocyte proliferation and differentiation.

RESULTS

Proinflammatory Cytokines Inhibit IGF-I-Stimulated Cell Proliferation

To evaluate the effect of proinflammatory cytokines on cell proliferation, chondrocytes were incubated with IL-1 β (10 ng/ml), IL-6 (100 ng/ml), and TNF- α (50 ng/ml) in the presence or absence of IGF-I. IGF-I (60 ng/ml) stimulated proliferation of RCJ cells fivefold, as assessed by [3 H]-thymidine incorporation (Figure 1). Coincubation with IL-1 β , IL-6, or TNF- α resulted in a 50% reduction of the mitogenic effect of IGF-I, while cell proliferation under baseline conditions was not affected by these proinflammatory cytokines (Figure 1). Proinflammatory cytokines without or with coincubation with IGF-I did not induce apoptosis measured by the MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) cell viability assay (Table 1).

Proinflammatory Cytokines Do Not Alter the Type 1 IGF Receptor Expression

Reduced IGF-I-induced cell proliferation in the presence of proinflammatory cytokines could be due to downregulation of the type 1 IGF receptor. However, neither IGF-I nor the investigated proinflammatory cytokines altered the mRNA abundance of the type 1 IGF receptor in cultured chondrocytes (Figure 2). Hence, the observed effects of proinflammatory cytokines on IGF-I-stimulated chondrocyte proliferation cannot be ascribed to reduced expression of the type 1 IGF receptor.

Proinflammatory Cytokines Attenuate the IGF-I-Activated Signaling Pathways in Proliferating Cells

IGF-I exerts its mitogenic effect in growth plate chondrocytes via two canonical signaling pathways, the PI-3 kinase and the MAPK/ERK 1/2 pathways. We therefore investigated whether the proinflammatory cytokines IL-1 β , IL-6, and TNF- α inhibit IGF-I-activated key signaling molecules of these respective pathways in proliferating chondrocytes. IGF-I increased phosphorylation of ERK as a member of the MAPK/ERK1/2 signaling pathway fivefold (Figure 3). All three proinflammatory cytokines also tended to decrease the phosphorylation of ERK under baseline conditions. Coincubation of IGF-I with

Figure 1. IGF-I-induced cell proliferation is reduced by coincubation with proinflammatory cytokines. RCJ cells were grown to 80–90% of confluence (day 4 of culture defined as baseline, corresponding to proliferating chondrocytes) and were cultured in serum-free medium for 12 h. Medium was changed to α -MEM, and IGF-I (60 ng/ml), proinflammatory cytokines (TNF- α (50 ng/ml), IL-1 β (10 ng/ml), IL-6 (100 ng/ml)), or vehicle were added for 48 h as indicated. [3 H]-thymidine incorporation was determined as described in the Methods section. Data are the mean $\pm$ SE of three independent experiments with 9–12 parallel dishes per treatment combination expressed as the percentage of control values. Statistics by ANOVA: $F_{1,3} = 150.63$ and $P < 0.0001$; $F_{2,1} = 1,632.93$ and $P < 0.0001$; $F_{12,3} = 157.85$ and $P < 0.0001$. * $P < 0.05$ vs. control; § $P < 0.05$ vs. IGF-I. IGF, insulin-like growth factor.

Table 1. Proinflammatory cytokines with or without coincubation with IGF-I do not induce apoptosis in RCJ3.1C5.18 cells

	Control	TNF- α	IL-1 β	IL-6
	100 $\pm$ 15	101 $\pm$ 18	100 $\pm$ 26	105 $\pm$ 15
IGF-I added	102 $\pm$ 6	107 $\pm$ 5	105 $\pm$ 22	102 $\pm$ 10

Cell viability was assessed by the MTT assay as described in the Methods section. Data are mean $\pm$ SE expressed as the percentage of control values. Statistics by ANOVA with 11 parallel dishes per group: $F_{1,3} = 0.08$ and $P = 0.968$; $F_{2,1} = 0.35$ and $P = 0.557$; $F_{12,3} = 0.27$ and $P = 0.846$.

IL-6 decreased IGF-I-induced phosphorylation of ERK by 50%, while coincubation with IL-1 β or TNF- α inhibited IGF-I-induced phosphorylation of ERK by 70%.

IGF-I increased sixfold the phosphorylation of AKT, a key signaling molecule of the PI-3 kinase pathway (Figure 4). Under baseline conditions, all investigated proinflammatory cytokines numerically decreased phosphorylation of AKT. Coincubation of IGF-I with IL-1 β , IL-6, or TNF- α reduced AKT phosphorylation by at least 30% as compared with IGF-I-activated phosphorylation of AKT (Figure 4). These results indicate that proinflammatory cytokines diminish the IGF-I-mediated cell proliferation by reducing the activation of the PI-3 kinase and MAPK/ERK1/2 signaling pathways.

Proinflammatory Cytokines Inhibit IGF-I-Stimulated Cell Differentiation

The mesenchymal RCJ cell line spontaneously differentiates from displayed polygonal-shaped isolated chondrocytes to

Figure 2. Type 1 IGF receptor expression is not altered by proinflammatory cytokines. RCJ cells at day 4 of culture were grown until confluence, serum starved for 12 h, and IGF-I, proinflammatory cytokines, or vehicle were added as indicated above for 12 h. Total RNA was extracted, and the gene expression of the type 1 IGF receptor was determined by real-time RT-PCR. The columns represent the mean $\pm$ SE of three independent experiments with three parallel dishes per treatment combination. Statistics by ANOVA: $F_{1,3} = 1.22$ and $P = 0.316$; $F_{2,1} = 5.96$ and $P = 0.019$; $F_{12,3} = 1.69$ and $P = 0.184$. IGF, insulin-like growth factor; RT-PCR, reverse-transcriptase PCR.

Figure 3. Proinflammatory cytokines diminish phosphorylation of the IGF-I-activated signaling pathway MAPK/ERK1/2 in proliferating chondrocytes. RCJ cells at day 4 of culture were grown until confluence, serum starved for 12 h, and IGF-I, proinflammatory cytokines, or vehicle were added as indicated above for an additional hour. Cell lysates were subjected to western immunoblot analysis, and the respective membranes were probed with specific antibodies against p-ERK1/2 and total ERK 1/2. (a) The columns represent the mean $\pm$ SE from five independent experiments with one dish per treatment combination. Statistics by ANOVA: $F_{1,3} = 30.88$ and $P < 0.0001$; $F_{2,1} = 145.07$ and $P < 0.0001$; $F_{12,3} = 13.62$ and $P < 0.0001$. * $P < 0.05$ vs. control; \$ $P < 0.05$ vs. IGF-I. (b) A representative western immunoblot. IGF, insulin-like growth factor.

Figure 4. Proinflammatory cytokines diminish phosphorylation of the IGF-I-activated signaling pathway PI-3 kinase in proliferating chondrocytes. RCJ cells at day 4 of culture were grown until confluence, serum starved for 12 h, and IGF-I, proinflammatory cytokines, or vehicle were added as indicated above for an additional hour. Cell lysates were subjected to western immunoblot analysis, and the respective membranes were probed with specific antibodies against p-AKT and total AKT. (a) The columns represent the mean $\pm$ SE from four independent experiments with one dish per treatment combination. Statistics by ANOVA: $F_{1,3} = 13.66$ and $P < 0.0001$; $F_{2,1} = 215.76$ and $P < 0.0001$; $F_{12,3} = 6.31$ and $P = 0.003$. * $P < 0.05$ vs. control; \$ $P < 0.05$ vs. IGF-I. (b) A representative western immunoblot. IGF, insulin-like growth factor.

cartilage nodules over 4 to 14 d of culture in differentiating medium in the presence of dexamethasone (21). Differentiation of RCJ cells was promoted by incubating the cells with β -glycerolphosphate and ascorbic acid from day 4 of culture, as described in the Methods section. To evaluate the effect of proinflammatory cytokines on IGF-I-induced chondrocyte differentiation, RCJ cells were cultured in differentiating medium from day 4 of culture until day 14, followed by serum deprivation for 12 h and treatment with IGF-I (60 ng/ml) and the indicated proinflammatory cytokines for additional 24 h. Exogenous IGF-I enhanced the expression of Ihh and collagen type X, two markers of terminally differentiated chondrocytes, four to sevenfold (Figure 5), consistent with our previous observations (7). While IL-6 and IL-1 β reduced IGF-I-induced Ihh expression by $\sim$ 60%, TNF- α completely abolished IGF-I-induced Ihh expression (Figure 5a). In addition, proinflammatory cytokines reduced IGF-I-induced collagen type X expression by $\sim$ 50% (Figure 5b). These cytokines had no effect on Ihh or collagen type X expression in the absence of IGF-I (Figure 5). Furthermore, we measured the effect of IGF-I and proinflammatory cytokines on proteoglycan synthesis as an additional marker of chondrocyte differentiation. Exogenous IGF-I enhanced proteoglycan synthesis threefold

Figure 5. Proinflammatory cytokines inhibit IGF-I-stimulated cell differentiation. RCJ cells were cultured as described in the Methods section. At day 14 of culture, cells were serum starved for 12 h, and IGF-I, proinflammatory cytokines, or vehicle were added as indicated above for 24 h. Total RNA was extracted, and the gene expression of the differentiation markers (a) *Ihh* and (b) collagen type X were determined by real-time RT-PCR. The columns represent the mean $\pm$ SE of four independent experiments with four parallel dishes per treatment combination. Statistics by ANOVA: (a) $F_{1,3} = 18.53$ and $P < 0.0001$; $F_{2,1} = 108.16$ and $P < 0.0001$; $F_{12,3} = 22.31$ and $P < 0.0001$. (b) $F_{1,3} = 15.97$ and $P < 0.0001$; $F_{2,1} = 139.62$ and $P < 0.0001$; $F_{12,3} = 19.87$ and $P < 0.0001$. * $P < 0.05$ vs. control; $^{\$}P < 0.05$ vs. IGF-I. IGF, insulin-like growth factor; RT-PCR, reverse-transcriptase PCR.

Figure 6. Proinflammatory cytokines inhibit IGF-I-stimulated proteoglycan synthesis. Accumulation of proteoglycans was estimated by the Alcian blue assay as described in the Methods section. Each experiment was performed three times with 10 parallel wells per treatment combination. Bars represent means $\pm$ SE. Statistics by ANOVA: $F_{1,3} = 48.56$ and $P < 0.0001$; $F_{2,1} = 139.73$ and $P < 0.0001$; $F_{12,3} = 43.95$ and $P < 0.0001$. * $P < 0.05$ vs. control; $^{\$}P < 0.05$ vs. IGF-I. IGF, insulin-like growth factor.

(Figure 6), consistent with our previous observations (7), while proinflammatory cytokines reduced IGF-I-induced proteoglycan synthesis by ~50% (Figure 6).

Proinflammatory Cytokines Attenuate the IGF-I-Activated Signaling Pathway in Differentiating Cells

We previously observed that in RCJ cells, only the PI-3 kinase pathway is required for early and terminal differentiation in response to IGF-I (7). To determine the effect of proinflammatory cytokines on the IGF-I-mediated activation of AKT as a key signaling molecule of the PI-3 kinase pathways,

differentiated RCJ cells were incubated at day 14 with IGF-I, proinflammatory cytokines, or vehicle. IGF-I increased the phosphorylation of AKT 12-fold. Also, under baseline conditions, all investigated cytokines tended to decrease phosphorylation of AKT (Figure 7). Coincubation of these cytokines reduced IGF-I-stimulated phosphorylation of AKT by 50% (IL-6 and IL-1β) to 70% (TNF-α) (Figure 7). These results indicate that proinflammatory cytokines diminish the IGF-I-mediated cell differentiation by reducing the activation of the PI-3 kinase signaling pathway.

DISCUSSION

The main finding of this study is that the proinflammatory cytokines IL-1β, IL-6, and TNF-α attenuate the mitogenic and differentiation-enhancing effect of IGF-I in growth plate chondrocytes and that these inhibitory effects are associated with a reduced phosphorylation of key signaling molecules of the PI-3 kinase and MAPK/ERK1/2 signaling pathways, which are critical for IGF-I signaling in these cells (7). Our data give a mechanistic explanation for the reduced biological activity of IGF-I in the presence of proinflammatory cytokines. According to our data, chronic inflammation negatively impacts longitudinal growth not only by interference with circulating components of the GH/IGF axis but also by interference with the activity of IGF-I in the growth plate as the important target tissue for longitudinal growth.

An advantage of RCJ cells as a growth plate model is that these cells do not express IGF-I or IGF-II (20). Hence, the action of these hormones can be studied without interference from endogenous IGFs. According to our data, proinflammatory cytokines do not have a direct, IGF-I-independent impact on proliferation and differentiation of growth plate chondrocyte but exert their inhibitory activity through the attenuation of IGF-I-related signaling pathways. We could exclude

Figure 7. Proinflammatory cytokines diminish phosphorylation of the IGF-I-activated signaling pathway PI-3 kinase in differentiating chondrocytes. RCJ cells at day 14 of culture were grown until confluence, serum starved for 12 h, and IGF-I, proinflammatory cytokines, or vehicle were added as indicated above for an additional hour. Cell lysates were subjected to western immunoblot analysis, and the respective membranes were probed with specific antibodies against p-AKT and total AKT. **(a)** The columns represent the mean $\pm$ SE from four independent experiments with one dish per treatment combination. Statistics by ANOVA: $F_{1,3} = 195.90$ and $P < 0.0001$; $F_{2,1} = 2,073.07$ and $P < 0.0001$; $F_{12,3} = 142.77$ and $P < 0.0001$. * $P < 0.05$ vs. control; § $P < 0.05$ vs. IGF-I. **(b)** A representative western immunoblot. IGF, insulin-like growth factor.

that the observed effects are due to apoptosis. Previous studies on this topic have produced conflicting results. In most of the previous studies, IL-6 had no direct effect on growth plate chondrocyte dynamics (22–26). However, in a study using cultured metatarsal bones of fetal rat, IL-1 β and IL-6 in high concentrations inhibited cell proliferation in the absence of exogenous IGF-I and had a synergistic inhibitory effect in lower concentrations (22). This inhibitory effect was partially reversible by IGF-I (22). Also, in the ATDC5 chondrocyte cell line, IL-1 β and TNF- α reduced cell proliferation in the absence of exogenous IGF-I (25). The most likely explanation for these discrepant findings is that in the latter two models of the growth plate proinflammatory cytokines interfere with the activity of endogenous IGF-I, which is produced by cultured metatarsal bones of fetal rat and ATDC5 cells (27). Hence, it appears that proinflammatory cytokines inhibit chondrocyte proliferation and differentiation by interference with IGF-I signaling rather than by a direct IGF-independent effect on chondrocyte dynamics.

IGF-I not only is an important endocrine and paracrine/autocrine regulator of chondrocyte proliferation but also enhances chondrocyte differentiation both *in vivo* (4) and *in vitro* (2,3). We have demonstrated previously that IGF-I

also enhances differentiation of RCJ cells (28). In this study, we extend this observation and describe that proinflammatory cytokines also attenuate the IGF-I-enhancing effect on cell differentiation. This finding is consistent with data in cartilage from hypophysectomized rats, in which IL-6 diminishes the enhancing effect of IGF-I on proteoglycan synthesis (29). Our observation that the inhibitory effect of inflammatory cytokines on IGF-I-enhanced cell differentiation is associated with a reduced activity of the PI-3 kinase pathway gives a likely mechanistic explanation.

The precise mechanism of how inflammatory cytokines interfere with the PI-3 kinase and the MAP/EK1/2 kinase pathways remains to be determined. In our experimental model, we also observed in the absence of IGF-I a slightly reduced phosphorylation of AKT as a key signaling molecule of the PI-3 kinase pathway and of ERK as a key signaling molecule of the MAP/EK1/2 kinase pathway. Also, data from other investigators indicate that IL-6, IL-1 β , or TNF- α interfere directly with the PI-3 kinase and MAP/EK1/2 kinase signaling pathways (30–32).

Theoretically, the reduced biological activity of IGF-I in growth plate chondrocytes in the presence of proinflammatory cytokines could also be due to downregulation of the type 1 IGF receptor. However, we did not observe an effect of proinflammatory cytokines on the gene expression of the type 1 IGF receptor, consistent with findings in breast cancer cells or myoblasts (33,34). Whether proinflammatory cytokines inhibit IGF-I activity in the growth plate also by induction of inhibitory IGF-binding proteins will be the focus of future investigations.

From animal studies, it is known that dexamethasone reduces the number of chondrocytes expressing IGF-I mainly in the proliferative and prehypertrophic zones (35). On the other hand, glucocorticoids are an established treatment option for juvenile idiopathic arthritis to reduce inflammation (8). It will therefore be interesting to investigate the impact of dexamethasone on the interaction of proinflammatory cytokines with IGF-I in RCJ cells. This issue will be addressed in future experiments.

In conclusion, our data indicate that the proinflammatory cytokines IL-1 β , IL-6, and TNF- α attenuate the mitogenic and differentiation-enhancing effects of IGF-I in growth plate chondrocytes, most likely by interference with the IGF-I-related PI-3 kinase and MAP/EK1/2 kinase signaling pathways. This mechanism likely contributes to the complex pathophysiology of growth failure in chronic inflammation.

METHODS

Reagents

Phosphate-buffered saline, alanyl-L-glutamine, and penicillin-streptomycin were obtained from Seromed Biochrom KG (Berlin, Germany). Bovine serum albumin was purchased from Sigma-Aldrich Chemicals (Deisenhofen, Germany). Alpha-minimum essential medium (MEM) and fetal calf serum were obtained from Lonza (Verviers, Belgium). Ascorbic acid, β -glycerophosphate, and dexamethasone were obtained from Sigma (Taufkirchen, Germany). Recombinant human IGF-I, recombinant human GH, IL-1 β , IL-6, and TNF- α were supplied by Bachem (Weil am Rhein, Germany). Enhanced chemiluminescence reagents were obtained from

Amersham Pharmacia Biotech (Freiburg, Germany). The ERK1/2, p-ERK1/2, AKT, p-AKT, and the horseradish peroxidase-conjugated (antirabbit and antimouse) antibodies were obtained from Cell Signaling Technology (Frankfurt a. M., Germany).

Cell Culture

RCJ3.1C5.18 cells (kindly provided by Dr Anna Spagnoli, Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN) derived from fetal rat calvaria were grown without biochemical or oncogenic transformation at 37 °C in humidified 5% CO₂ atmosphere in α -MEM (with Earle's salts) supplemented with 1 mmol/l L-alanyl-L-glutamine, 100 U/ml penicillin-streptomycin, 15% heat-inactivated fetal bovine serum, and 10⁻⁷ mmol/l dexamethasone and studied within 25 passages. Cells were plated at a density of 6 × 10⁴ cells/well in six-well dishes for RNA extraction and protein isolation. After reaching confluence (day 4), the differentiation of cartilage nodules was initiated by the additional supplement of 10 mmol/l β -glycerophosphate and 50 μ g/ml ascorbic acid to the medium, as previously reported (20). Cells were fed at days 7 and 11 of culture and monitored over a total period of 14 d. RCJ cells are capable of differentiating spontaneously from displayed cuboidal-shaped isolated chondrocytes to cartilage nodules over 14 d of culture in differentiating medium in the presence of dexamethasone (20). At day 4 of culture, the cells represent proliferating chondrocytes (7). Prior to incubating the cells with cytokines and/or IGF-I, RCJ3.1C5.18 cells were cultured in serum-free medium for 12 h.

[³H]-Thymidine Assay

The extent of thymidine incorporation into DNA was determined as uptake of radioactivity in precipitated material, as described previously (36).

Briefly, chondrocytes were grown in 96-well plates to confluence for 3 d; thereafter, the cultures were changed to serum-free medium. After 12 h, proinflammatory cytokines with or without IGF-I were added to the medium, and the incubation was continued for further 48 h. The rate of chondrocyte proliferation was assessed by incubating the cells with 3 mCi/ml [³H]-thymidine for the final 4 h. Subsequently, cells were rinsed twice with phosphate-buffered saline. The radiolabeled DNA was precipitated by acetic acid and dissolved in 1 mol/l NaOH. [³H]-thymidine incorporation into the acid-extractable pool as a measure of DNA synthesis was determined by scintillation counting.

MTT Cell Viability Assay

The MTT assay was carried out according to the method by Mosmann (37). Briefly, chondrocytes were grown in 96-well plates to confluence for 3 d; thereafter, the cultures were changed to serum-free medium. After 12 h, proinflammatory cytokines with or without IGF-I were added to the medium and incubated for 48 h. Then, 100 μ l of MTT solution (Sigma-Aldrich) was added for 3 h. The formazan crystals were dissolved in 100 μ l of dimethyl sulfoxide for 24 h. Absorbance was measured at 570 nm using a microplate spectrophotometer (Multiskan Ascent Reader, Thermo Scientific, Schwerte, Germany). The assay was carried out with 11 replicates for each group.

Real-Time Reverse-Transcriptase PCR

For analysis of the expression pattern of the differentiation markers collagen type X and Ihh and the type 1 IGF receptor, RCJ cells were cultured in differentiating medium. At the indicated time points, RNA was isolated using the RNeasy mini columns (QIAGEN, Hilden, Germany). One microgram of RNA was reverse transcribed using MuLV reverse transcriptase and oligo(dt)/random hexamer primers (10:1) from Applied Biosystems (Darmstadt, Germany). For quantitative analysis, real-time reverse-transcriptase PCR was performed using the Abi 7000 system (Applied Biosystems) according to the manufacturer's protocol. The following set of primers was chosen: for Ihh (forward: 5'-CGACCGAAATAAGTACGGACTACTG-3'; reverse: 5'-TCAGACTTGACAGAGCAATGAACG-3'), for collagen type X (forward: 5'-GGTAAAGAGATTTCAGTAAGAGGAGAGAACA-3'; reverse: 5'-ACTTCCATGCCTGGCTTTCC-3'), for the type 1 IGF receptor (forward: 5'-AGCCCATGTGTGAGAAGACC-3'; reverse: 5'-CGCACACGCCTTTGTAGTAG-3'), and for rat 18S by the Primer Express program (forward: 5'-AGTTGGTGGAGCGATTTGTC-3'; reverse: 5'-GCTGAGCCAGTTCAGTGTAGC-3'). The software

provided from the company allowed the quantitative detection of fluorescence by the incorporation of the substance SYBR green into the amplification products. Amplification was performed in the presence of Universal Mastermix (PE Applied Biosystems) with SYBR green to detect PCR products at the end of each amplification step, and results were analyzed, as described previously (7).

Alcian Blue Assay

Cartilage matrix deposition in cells was quantified by Alcian blue staining, which detects the glycosaminoglycans of proteoglycans. Cells were seeded at a density of 6 × 10⁴/well in six-well dishes. After cells reached 80–90% confluence (corresponding to day 4 of culture), differentiation was initiated by adding 10 mmol/l β -glycerophosphate and 50 μ g/ml ascorbic acid to the medium, as described previously (20). Beginning at day 4, proinflammatory cytokines, IGF-I, or vehicle were added to the medium every second day. At day 14, cells were washed in phosphate-buffered saline and stained with 500 μ l of 1% Alcian blue (Serva, Heidelberg, Germany) in 3% acetic acid for 30 min at room temperature, washed three times for 2 min in 3% acetic acid, and rinsed with distilled water. Alcian blue bound to the cells was dissolved in 1 ml of 1% sodium dodecyl sulfate, and the absorbance was measured by spectrophotometry at 605 nm.

Western Immunoblotting

For western immunoblot analysis, cells were dissolved in lysis buffer, and the cell extracts were treated, as previously described (7). The membranes were blocked, either in 5% bovine serum albumin, or in 3% milk for 1 h at room temperature, incubated overnight with the first antibody (dilution 1:4,000 for total Akt in 3% milk, dilution 1:2,000 for p-ERK1/2 and ERK1/2 in 5% bovine serum albumin, dilution 1:1,000 for p-Akt in 5% bovine serum albumin), washed extensively over a period of 30 min with Tris-buffered saline with Tween 20 0.05%, and then incubated for 1 h with the secondary antibody (dilution 1:2,000 in 3% milk), followed by further washing over a period of 30 min. Immune complexes were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany). Blots were exposed to x-ray films (Kodak, Stuttgart, Germany).

Statistical Analysis

All experiments were performed at least three times. For measurements with the ABI 7000 system, samples were run in duplicate to account for technical and biological variability within and between experiments. Data are given as mean $\pm$ SE. All data were examined for normal and non-Gaussian distribution by the Kolmogorov-Smirnov test. For comparison among normally distributed groups, two-way ANOVA was used, including group and IGF-I as factors and their interaction. Results are given in the format $F_{1,3}$ (F value of group effect), $F_{2,1}$ (F value of IGF effect), and $F_{12,3}$ (F value of interaction) with the respective P values. Depending on the results of the ANOVA, pairwise comparisons were conducted using Dunnett's method (comparing each IGF group against the control and each IGF + cytokine group against IGF only). $P < 0.05$ was considered as statistically significant.

STATEMENT OF FINANCIAL SUPPORT

This study was supported by a research grant from the Faculty of Medicine, University of Heidelberg.

Disclosure: The authors have nothing to disclose.

REFERENCES

1. Kronenberg HM. Developmental regulation of the growth plate. *Nature* 2003;423:332–6.
2. Olney RC, Wang J, Sylvester JE, Mougey EB. Growth factor regulation of human growth plate chondrocyte proliferation *in vitro*. *Biochem Biophys Res Commun* 2004;317:1171–82.
3. Reinecke M, Schmid AC, Heyberger-Meyer B, Hunziker EB, Zapf J. Effect of growth hormone and insulin-like growth factor I (IGF-I) on the expression of IGF-I messenger ribonucleic acid and peptide in rat tibial growth plate and articular chondrocytes *in vivo*. *Endocrinology* 2000;141:2847–53.
4. Wang J, Zhou J, Bondy CA. Igf1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. *FASEB J* 1999;13:1985–90.

5. Párrizas M, Saltiel AR, LeRoith D. Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3'-kinase and mitogen-activated protein kinase pathways. *J Biol Chem* 1997;272:154–61.
6. Tsakiridis T, Tsiani E, Lekas P, et al. Insulin, insulin-like growth factor-I, and platelet-derived growth factor activate extracellular signal-regulated kinase by distinct pathways in muscle cells. *Biochem Biophys Res Commun* 2001;288:205–11.
7. Ciarmatori S, Kiepe D, Haarmann A, Huegel U, Tönshoff B. Signaling mechanisms leading to regulation of proliferation and differentiation of the mesenchymal chondrogenic cell line RCJ3.1C5.18 in response to IGF-I. *J Mol Endocrinol* 2007;38:493–508.
8. Simon D, Lucidarme N, Prieur AM, Ruiz JC, Czernichow P. Treatment of growth failure in juvenile chronic arthritis. *Horm Res* 2002;58:Suppl 1:28–32.
9. Tönshoff B, Kiepe D, Ciarmatori S. Growth hormone/insulin-like growth factor system in children with chronic renal failure. *Pediatr Nephrol* 2005;20:279–89.
10. Cezard JP, Touati G, Alberti C, Hugot JP, Brinon C, Czernichow P. Growth in paediatric Crohn's disease. *Horm Res* 2002;58:Suppl 1:11–5.
11. Kutukculer N, Caglayan S, Aydogdu F. Study of pro-inflammatory (TNF-alpha, IL-1alpha, IL-6) and T-cell-derived (IL-2, IL-4) cytokines in plasma and synovial fluid of patients with juvenile chronic arthritis: correlations with clinical and laboratory parameters. *Clin Rheumatol* 1998;17:288–92.
12. Ji H, Pettit A, Ohmura K, et al. Critical roles for interleukin 1 and tumor necrosis factor alpha in antibody-induced arthritis. *J Exp Med* 2002;196:77–85.
13. De Benedetti F, Alonzi T, Moretta A, et al. Interleukin 6 causes growth impairment in transgenic mice through a decrease in insulin-like growth factor-I. A model for stunted growth in children with chronic inflammation. *J Clin Invest* 1997;99:643–50.
14. Tönshoff B, Blum WF, Mehls O. Serum insulin-like growth factors and their binding proteins in children with end-stage renal disease. *Pediatr Nephrol* 1996;10:269–74.
15. Ballinger A. Fundamental mechanisms of growth failure in inflammatory bowel disease. *Horm Res* 2002;58:Suppl 1:7–10.
16. Allen RC, Jimenez M, Cowell CT. Insulin-like growth factor and growth hormone secretion in juvenile chronic arthritis. *Ann Rheum Dis* 1991;50:602–6.
17. Schmeling H, Seliger E, Horneff G. Growth reconstitution in juvenile idiopathic arthritis treated with etanercept. *Clin Exp Rheumatol* 2003;21:779–84.
18. Koniaris SG, Fisher SE, Rubin CT, Chawla A. Experimental colitis impairs linear bone growth independent of nutritional factors. *J Pediatr Gastroenterol Nutr* 1997;25:137–41.
19. Lunstrum GP, Keene DR, Weksler NB, Cho YJ, Cornwall M, Horton WA. Chondrocyte differentiation in a rat mesenchymal cell line. *J Histochem Cytochem* 1999;47:1–6.
20. Spagnoli A, Hwa V, Horton WA, et al. Antiproliferative effects of insulin-like growth factor-binding protein-3 in mesenchymal chondrogenic cell line RCJ3.1C5.18. relationship to differentiation stage. *J Biol Chem* 2001;276:5533–40.
21. Grigoriadis AE, Heersche JN, Aubin JE. Analysis of chondroprogenitor frequency and cartilage differentiation in a novel family of clonal chondrogenic rat cell lines. *Differentiation* 1996;60:299–307.
22. Mårtensson K, Chrysis D, Sävdahl L. Interleukin-1beta and TNF-alpha act in synergy to inhibit longitudinal growth in fetal rat metatarsal bones. *J Bone Miner Res* 2004;19:1805–12.
23. Ye W, Ma RF, Ding Y, et al. [Effect of interleukin-6 on the chondrocytes in the cartilage endplate of rabbits *in vitro*]. *Nan Fang Yi Ke Da Xue Xue Bao* 2007;27:1187–9.
24. Horan J, Dean DD, Kieswetter K, Schwartz Z, Boyan BD. Evidence that interleukin-1, but not interleukin-6, affects costochondral chondrocyte proliferation, differentiation, and matrix synthesis through an autocrine pathway. *J Bone Miner Res* 1996;11:1119–29.
25. MacRae VE, Farquharson C, Ahmed SF. The restricted potential for recovery of growth plate chondrogenesis and longitudinal bone growth following exposure to pro-inflammatory cytokines. *J Endocrinol* 2006;189:319–28.
26. Nakajima S, Naruto T, Miyamae T, et al. Interleukin-6 inhibits early differentiation of ATDC5 chondrogenic progenitor cells. *Cytokine* 2009;47:91–7.
27. Akiyama H, Fukumoto A, Shigeno C, et al. TAK-778, a novel synthetic 3-benzothiepin derivative, promotes chondrogenesis *in vitro* and *in vivo*. *Biochem Biophys Res Commun* 1999;261:131–8.
28. Kiepe D, Ciarmatori S, Haarmann A, Tönshoff B. Differential expression of IGF system components in proliferating vs. differentiating growth plate chondrocytes: the functional role of IGFBP-5. *Am J Physiol Endocrinol Metab* 2006;290:E363–71.
29. Lazarus DD, Moldawer LL, Lowry SF. Insulin-like growth factor-1 activity is inhibited by interleukin-1 alpha, tumor necrosis factor-alpha, and interleukin-6. *Lymphokine Cytokine Res* 1993;12:219–23.
30. Legendre F, Bogdanowicz P, Boumediene K, Pujol JP. Role of interleukin 6 (IL-6)/IL-6R-induced signal transducers and activators of transcription and mitogen-activated protein kinase/extracellular. *J Rheumatol* 2005;32:1307–16.
31. Farquharson C, Ahmed SF. Inflammation and linear bone growth: the inhibitory role of SOCS2 on GH/IGF-1 signaling. *Pediatr Nephrol* 2013;28:547–56.
32. Macrae VE, Ahmed SF, Mushtaq T, Farquharson C. IGF-I signalling in bone growth: inhibitory actions of dexamethasone and IL-1beta. *Growth Horm IGF Res* 2007;17:435–9.
33. Shen WH, Zhou JH, Broussard SR, Freund GG, Dantzer R, Kelley KW. Proinflammatory cytokines block growth of breast cancer cells by impairing signals from a growth factor receptor. *Cancer Res* 2002;62:4746–56.
34. Broussard SR, McCusker RH, Novakofski JE, et al. IL-1beta impairs insulin-like growth factor i-induced differentiation and downstream activation signals of the insulin-like growth factor i receptor in myoblasts. *J Immunol* 2004;172:7713–20.
35. Smink JJ, Gresnigt MG, Hamers N, Koedam JA, Berger R, Van Buul-Offers SC. Short-term glucocorticoid treatment of prepubertal mice decreases growth and IGF-I expression in the growth plate. *J Endocrinol* 2003;177:381–8.
36. Kiepe D, Andress DL, Mohan S, et al. Intact IGF-binding protein-4 and -5 and their respective fragments isolated from chronic renal failure serum differentially modulate IGF-I actions in cultured growth plate chondrocytes. *J Am Soc Nephrol* 2001;12:2400–10.
37. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63.