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Interleukin-1β and TNF-α Act in Synergy to Inhibit Longitudinal Growth in Fetal Rat Metatarsal Bones

Katarina Mårtensson,[1,2] Dionisios Chrysis,[1,2] and Lars Sävendahl[1]

ABSTRACT: We hypothesized that pro-inflammatory cytokines can act locally in the growth plate to impair longitudinal growth. In a model of cultured fetal rat metatarsal bones, we found that IL-1β and TNF-α act in synergy to inhibit longitudinal growth, an effect linked to decreased proliferation and increased apoptosis of growth plate chondrocytes. IGF-I could partially reverse all these effects.

Introduction: Children with chronic inflammatory conditions, such as Crohn's disease or rheumatoid arthritis, experience impaired longitudinal growth. The inflammatory process itself, which includes upregulation of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and TNF-α, is believed to be at least partly responsible for the poor growth in these patients. This study aimed to clarify whether these cytokines can act locally in the growth plate to suppress longitudinal growth and whether any negative effects can be reversed by insulin-like growth factor-I (IGF-I).
Materials and Methods: The effects of cytokines on longitudinal bone growth were studied in fetal (day E20) rat metatarsal bones kept in culture. After a 7-day culture, the bones were sectioned, and chondrocyte proliferation was assessed by bromodeoxyuridine (BrdU) incorporation and apoptosis by TUNEL.
Results: When added separately, IL-1β and TNF-α impaired longitudinal bone growth only at a high concentration (100 ng/ml each; $p < 0.05$ versus control). In contrast, when added in combination, IL-1β and TNF-α potently inhibited growth at far lower concentrations (from 3 ng/ml each; $p < 0.001$ versus control) and also decreased chondrocyte proliferation and increased apoptosis. Growth failure induced by the combination of IL-1β and TNF-α (10 ng/ml each) could be counteracted by anti-IL-1β (100 ng/ml; $p < 0.001$), anti-TNF-α (100 ng/ml; $p < 0.001$), or IGF-I (100 ng/ml; $p < 0.01$). IL-6 did not affect longitudinal growth even when added in combination with IL-1β or TNF-α (10 ng/ml each).
Conclusions: We show that IL-1β and TNF-α act in synergy to locally suppress longitudinal growth, an effect that can be partially reversed by IGF-I. Although growth hormone (GH)/IGF-I may improve longitudinal growth in children with chronic inflammatory diseases, our results suggest that the inflammatory process itself must be targeted to achieve normal growth.
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Key words: interleukin-1, TNF, growth plate, chondrocytes, insulin-like growth factor I

INTRODUCTION

Children with Crohn's disease,[1,2] rheumatoid arthritis,[3] and other chronic inflammatory diseases often experience impaired longitudinal growth rate compared with healthy children. Early growth delay in patients with chronic inflammation also has been associated with permanent stunting.[4,5] Chronic inflammation may lead to malnutrition, which can contribute to the impaired growth. However, some children fail to improve their growth and achieve their growth potential despite nutritional intervention, suggesting that other factors play important etiological roles. Data retrieved from rat models of experimental colitis including pair-fed controls show that the impaired longitudinal growth in colitis cannot only be caused by malnutrition.[6,7] This indicates that inflammation, by itself, inhibits longitudinal growth. In inflammatory bowel disease[8,9] and rheumatoid arthritis,[10] the major upregulated cytokines are interleukin (IL)-1β, IL-6, and TNF-α. These cytokines could affect growth through a systemic effect[11] and/or through a local effect on the growth plate. A very recent report[12] shows catch-up growth in children with refractory juvenile idiopathic arthritis treated with the TNF antagonist etanercept. The improved growth was associated with increased serum insulin-like growth factor (IGF)-I levels, suggesting that systemic effects facilitating growth is achieved by antagonizing TNF action in these patients. Whereas the effects of cytokines on the growth hormone

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[1]Pediatric Endocrinology Unit, Department of Woman and Child Health, Karolinska Institutet, Stockholm, Sweden; [2]These authors contributed equally.

(GH)/IGF-I axis have been studied, little is known about their local effects on the growth plate. As an example, in rats with experimental colitis, growth plate morphology is affected with a larger resting zone and a smaller proliferative zone compared with pair-fed animals.[7] It is unknown if these changes are secondary to systemic effects of altered serum levels of growth factors or because of direct effects of inflammatory cytokines in the growth plate. Some evidence that cytokines could directly affect the growth plate come from in vitro experiments in articular chondrocytes. IL-1β and TNF-α increase the expression of metalloproteases[13] and downregulate genes encoding cartilage matrix proteins.[14] In addition, a recent report show that human articular chondrocytes stimulated with the combination of IL-1β and TNF-α turn on a specific gene program, which involves production of proteins engaged in remodeling and destruction of cartilage matrix.[15]

Based on knowledge from studies using articular cartilage cells, we hypothesized that pro-inflammatory cytokines may have direct effects on growth plate chondrocytes. Our study was designed to investigate any local effects of IL-1β, IL-6, and TNF-α on the epiphyseal growth plate. We chose an in vitro system based on organ cultures of fetal rat metatarsal bones, which is a well-established model, to study direct effects on the growth plate without confounding systemic effects, while still mimicking the in vivo situation.[16]

MATERIALS AND METHODS

Metatarsal organ culture

The three middle metatarsal bones were dissected from the paws of fetal Sprague-Dawley rats (19–20 days p.c.). The dissection media was PBS with Fungizone (2.5 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml; all from Invitrogen, Carlsbad, CA, USA). The bones were cultured in 24-well plates with 0.5 ml/well of MEM with L-glutamine (Invitrogen), supplemented with 0.05 mg/ml ascorbic acid (Apoteksbolaget, Göteborg, Sweden), 1 mM sodium glycerophosphate (Sigma), 0.2% bovine serum albumin (BSA; Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. The metatarsals were cultured for 7 days at 37°C with 5% CO_2. Medium was changed, on average, every 2 days (1–3 days). The metatarsals were cultured with the recombinant rat cytokines IL-6, IL-1β, and/or TNF-α. Blocking experiments were made with specific cytokine antibodies. All cytokines and cytokine antibodies were from R&D Systems, and lipopolysaccharide (LPS) was from Sigma. Recombinant IGF-I was kindly provided by Pharmacia.

RT-PCR analyses of IL-1 and TNF receptor mRNA expression

Total RNA was extracted from five fetal rat metatarsal bones (19–20 days p.c.) using the Ultraspec II kit (BIOTECX Laboratories, Houston, TX, USA). Two RT-PCR analyses were performed. Total RNA from inflammation-induced peritoneal exudates cells serving as a crude source of activated rat macrophages was used as a positive control.[17] Primer pairs specific for rat IL-1 receptor type I (5′primer 5′-CACGGAGTATCCAAATGAGG-3′; 3′primer 5′-CCATTCCACTTCCAGTAGAC-3′; product size 725 bp), rat IL-1 receptor type II (5′primer 5′-CATCAGAGAAG-CACCCAGTTC-3′; 3′primer 5′-CATTTGTTCACAGT-GGGACGC-3′; product size 301 bp), and TNF receptor type I (5′primer 5′-ACCAAGTGCCACAAAGGAAC-3′, 3′primer 5′-CTGGAGGTAGGCACAGCTTC-3′; product size 418 bp) were used. The housekeeping gene *GA3PDH* was used as normalization control. The recovered RNA was further processed using Superscript RNase Reversed Transcriptase kit (Life Technologies, Gaithersburg, MD, USA) to produce cDNA. One microgram of total RNA and 0.5 μg of the oligo(dT) primer (Invitrogen) were incubated for 10 minutes at 70°C, followed by incubation for 60 minutes at 37°C in the presence of 300 U Superscript reverse transcriptase (Life Technologies) and 60 U of RNase Inhibitor (Roche Diagnostics) in a total 20-μl reaction. The cDNA products were directly used for PCR or stored at −80°C for later analysis. The reaction (50 μl total volume) was performed using a Perkin-Elmer GeneAmp PCR System 2400 in the presence of 25 pmol primers, 10 pmol dNTP, 75 pmol MgCl2 (Expand High Fidelity PCR buffer; Boehringer Mannheim, Mannheim, Germany) and 1.75 U Expand High Fidelity DNA polymerase (Boehringer Mannheim). The conditions for amplification were 5 minutes at 96°C, followed by 30 cycles (35 cycles for IL-1RI) of denaturation for 30 s at 96°C, annealing for 30 s at 56°C (1 minute at 52°C for IL-1RI), extension for 1 minute (2 minutes for IL-1RI) at 72°C, and finally, extension for 5 minutes at 72°C. Reaction products were separated by electrophoresis in a 2% agarose gel with ethidium bromide (15 μg/10 ml).

Measurement of longitudinal growth

Digital pictures were taken of each metatarsal bone at days 0, 1, 2, 5, and 7 of culture, using a Hamamatsu C4742–95 digital camera attached to a Nikon SMZ-U microscope. The longitudinal growth of the metatarsals was measured by using the Image-Pro image analysis system (Media Cybernetics). The increase in growth is expressed as a percentage of the length at the day of dissection (day 0 = baseline).

Cell proliferation study

For cell proliferation studies, bromodeoxyuridine (BrdU; 1:200 dilution of labeling reagent, Cell Proliferation Kit RPN20; Amersham Biosciences, Buckinghamshire, UK) was added into the culture medium during the last 4 h of incubation. The metatarsal bones were fixed in 4% formalin (2.5 h), decalcified (2 h; 10% EDTA), and stored in 70% ethanol until embedded in paraffin. Five-micrometer-thick sections were cut longitudinally and mounted on objective slides. On each slide, one section from each experimental group was mounted to ensure similar experimental conditions during immunohistochemistry. The degree of proliferation was assessed with immunohistochemistry according to the protocol for the cell proliferation kit. Positive cells (proliferative) were counted in each section and expressed as number of positive cells per square millimeter of surface area. The number of chondrocytes per surface area was the same in all groups (data not shown). Surface area was measured by the Image-Pro image analysis system.

Detection of apoptosis

Apoptotic cells were identified by TUNEL immunohistochemistry according to instructions for the TdT-FragEL DNA fragmentation kit (Oncogene Research), with the modification that sections were treated with 5 μg/ml proteinase K at 37°C for 10 minutes. Sections were counterstained with methyl green, dehydrated, and mounted. Five metatarsals were used to determine the number of apoptotic cell, and at least two sections from each metatarsal were assessed. The number of apoptotic chondrocytes was counted and expressed per square millimeter of surface area. After pretreatment of sections with distilled water instead of TdT, all cells were negative, whereas treatment with DNase labeled all cells.

Immunohistochemistry

Immunohistochemistry was performed as previously reported.[18] Briefly, antigen retrieval was performed in 10 mM sodium citrate buffer (pH = 6.0) at 98–100°C for 10 minutes (only for Lamin A), and endogenous peroxidase activity was quenched, blocked in 5% horse serum in TBS for 1 h at room temperature. Sections were incubated overnight at 4°C with primary antibody diluted 1:100 (Lamin A), 1:50 (IL-1RI), and 1:100 (TNF-RI), and with secondary biotinylated antibody and avidin-conjugated peroxidase (Vector). Peroxidase activity was detected using a DAB kit that generates a brown color (Vector, Burlingame, CA, USA). The primary antibody for Lamin A was purchased from Cell Signaling Technology (2035) and recognizes only the small subunit of cleaved Lamin A. During apoptosis, Lamin A (70 kDa) is cleaved by activated caspases to a large (40–45 kDa) and a small (28 kDa) fragment.[19] Rabbit polyclonal antibodies for IL-1RI (M-20; sc689) and TNF-RI (H-271; sc7895) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis

Results are presented as means ± SE. Differences between the groups were tested by one-way ANOVA. All p values were calculated using the Newman-Keuls post-test. A value of $p < 0.05$ was considered significant.

RESULTS

Cytokine receptor expression in rat metatarsal bones

The presence of receptors for IL-1 and TNF-α was confirmed by RT-PCR using RNA extracted from whole metatarsal bones. Figure 1 shows that IL-1 receptor type I (IL-1RI; Fig. 1C) and TNF-receptor type I (TNF-RI; Fig. 1D) mRNA is expressed in metatarsal bones. We could not detect expression of IL-1 receptor type II mRNA (IL-1RII; Fig. 1D). To localize the expression of cytokine receptors, immunohistochemistry was performed. Most epiphyseal (resting), proliferative, and hypertrophic chondrocytes were immunopositive for IL-1RI (Fig. 1E) and TNF-RI (Fig. 1F). Immunostainings of serial sections of the same metatarsal bone strongly suggest that many chondrocytes co-express IL-1RI and TNF-RI (data not shown).

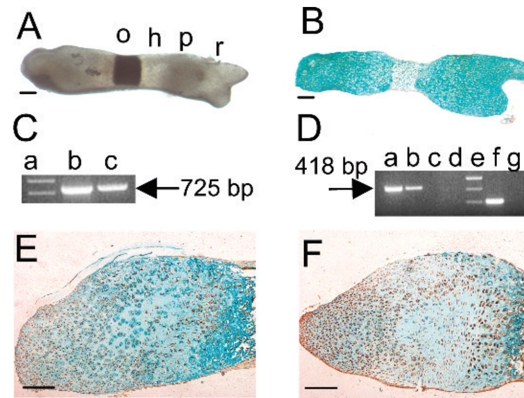

FIG. 1. (A) Image of a whole fetal rat metatarsal bone (day E20) as captured by a stereo microscope. o, ossification center; h, hypertrophic zone; p, proliferative zone; r, resting zone. Bar = 100 μm. (B) Metatarsal bone stained with Alzian blue/Van Gieson. Bar = 100 μm. (C) Expression of IL-1RI mRNA (RT-PCR) in metatarsal bones. a, marker; b, rat macrophage (positive control); c, metatarsal bones. (D) Expression of TNF-RI mRNA (RT-PCR) in rat metatarsal bones. a, rat macrophage (positive control); b, metatarsal bones; c, RT blank; d, PCR blank; e, marker. No expression of IL-1RII mRNA in metatarsals. f, rat macrophage (positive control); g, metatarsal bones. (E) Immunohistochemistry (IHC) for IL-1RI in a rat metatarsal bone. Bar = 100 μm. (F) IHC for TNF-RI. Bar = 100 μm.

Cytokine effects on metatarsal bone growth

Metatarsal bones treated for 7 days with IL-1β (10 ng/ml) had grown 40.1 ± 2.6%, a small but significant increase in length compared with the 33.9 ± 1.4% growth of control bones ($p < 0.05$). TNF-α, at 10 ng/ml, had no effect on growth (34.7 ± 2.3% versus 33.9 ± 1.4%). However, at a high concentration (100 ng/ml), IL-1β (19.5 ± 3.0%)- and TNF-α (22.3 ± 2.9%)-treated bones grew slower compared with control bones (33.9 ± 1.4%; $p < 0.05$ versus IL-1β or TNF-α; Figs. 2A and 2B).

Because normally more than one cytokine is upregulated during chronic inflammation in vivo, we also studied the effect of the combination of IL-1β and TNF-α (IL-1β/TNF-α) on metatarsal bone growth. We found that this combination severely inhibited metatarsal growth (Fig. 2C). At a relatively low concentration (3 ng/ml each), IL-1β/TNF-α significantly impaired metatarsal growth over the 7-day period of culture ($p < 0.001$ versus control). Moreover, at a moderate concentration (both at 10 ng/ml), they almost completely blocked metatarsal growth (4.1 ± 1.5% increase in length), a striking difference from growth of bones treated with IL-1β or TNF-α separately at the same concentration ($p < 0.001$ versus IL-1β or TNF-α alone). The growth arrest caused by the IL-1β/TNF-α combination could be counteracted by co-treatment with anti-IL-1β (100 ng/ml; $p < 0.001$) or anti-TNF-α (100 ng/ml; $p < 0.001$; Fig. 3).

IL-6 (100 ng/ml) had no effect on metatarsal growth compared with control bones (34.2 ± 2.0% and 36.1 ± 1.4%, respectively; $p > 0.05$). Interestingly, growth was also unaffected when IL-6 (100 ng/ml) was combined with IL-1β (10 ng/ml; 37.7 ± 3.4%) or TNF-α (10 ng/ml; 34.4 ± 2.6%). To indirectly rule out any local production of cyto-

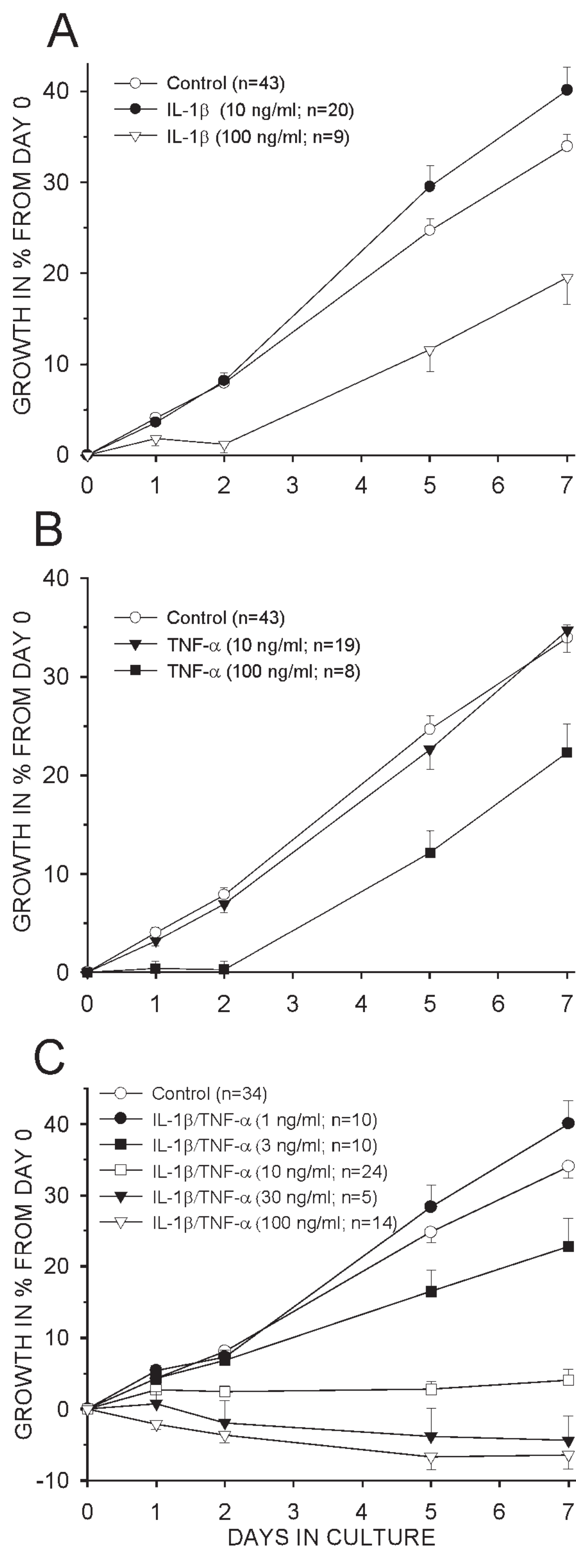

FIG. 2. Effects of IL-1β and/or TNF-α on longitudinal growth of fetal rat metatarsal bones cultured for 7 days. (A) Dose response to IL-1β. (B) Dose response to TNF-α. (C) Dose response to the combination of IL-1β and TNF-α (same concentrations for IL-1β and TNF-α). Growth is expressed as percent increase in metatarsal bone length from day 0 (mean $\pm$ SE). Substances were included in the culture medium from day 0. The numbers of studied metatarsals are indicated within parentheses.

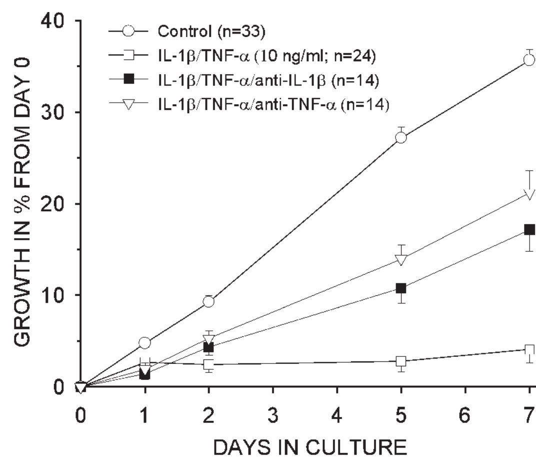

FIG. 3. Effects of blocking antibodies to IL-1β (anti-IL-1β; 100 ng/ml) or TNF-α (anti-TNF-α; 100 ng/ml) on cytokine-induced growth retardation in cultured rat metatarsal bones. IL-1β (10 ng/ml) and TNF-α (10 ng/ml) were added to all groups except the control group. Growth is expressed as percent increase in metatarsal bone length from day 0 (mean $\pm$ SE). Substances were included in the culture medium from day 0. The numbers of studied metatarsals are indicated within parentheses. Ten metatarsal bones in the control group and 10 metatarsals treated with IL-1β (10 ng/ml), and TNF-α (10 ng/ml) were also included in Fig. 2C.

kines of physiological relevance, metatarsal bones were treated with lipopolysaccharide (LPS; 100 μg/ml) for 7 days. LPS-treated metatarsals grew 29.7 $\pm$ 2.3%, which was not significantly different from control bones (30.8 $\pm$ 2.5%; $p > 0.05$).

Effect of IGF-I on IL-1β + TNF-α–induced growth retardation

In an attempt to overcome the growth inhibitory effect caused by the combination of IL-1β/TNF-α, the potential beneficial effect of IGF-I was studied. As seen in Fig. 4, IGF-I prevented growth retardation induced by IL-1β/TNF-α. Bones co-treated with IGF-I (100 ng/ml) + IL-1β/TNF-α (10 ng/ml each) grew 16.3 $\pm$ 6.1% over 7 days, which is significantly better than IL-1β/TNF-α bones alone (4.1 $\pm$ 1.5%; $p < 0.01$). However, longitudinal growth could not be completely rescued by adding IGF-I (up to 100 ng/ml) to bones cultured with IL-1β/TNF-α. Control bones grew significantly better than bones treated with IGF-I (100 ng/ml) + IL-1β/TNF-α (10 ng/ml each; 36.4 $\pm$ 1.3% versus 16.3 $\pm$ 6.1%, $p < 0.001$).

Cell proliferation (BrdU incorporation)

To study the mechanism by which IL-1β and/or TNF-α affect longitudinal growth, cell proliferation was assessed by BrdU staining after 7 days of culture (Fig. 5). BrdU$^+$ staining was identified in proliferative chondrocytes only (epiphyseal region and proliferative zone). Compared with the control group, bones treated with IL-1β (10 ng/ml) or TNF-α (10 ng/ml) had the same number of proliferating chondrocytes (Fig. 6A; Table 1). In contrast, combined treatment with IL-1β/TNF-α (10 ng/ml each) caused a significant decrease in chondrocyte proliferation compared

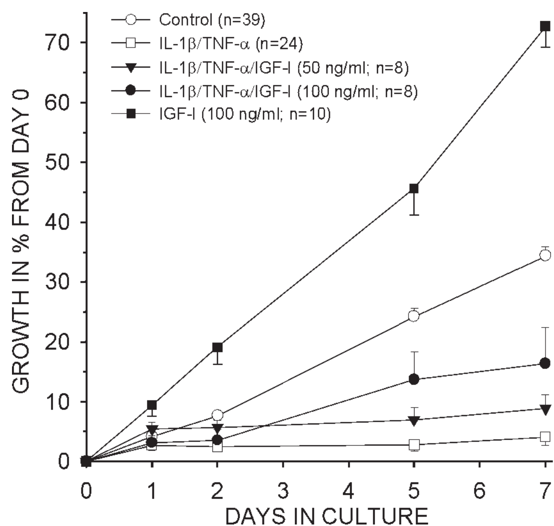

FIG. 4. Effect of IGF-1 (50 or 100 ng/ml) on cytokine-induced growth retardation in cultured rat metatarsal bones. The cytokines, IL-1β, and TNF-α (IL-1β/TNF-α; 10 ng/ml each), were added to all groups except the control and IGF-I–only groups. Growth is expressed as percent increase in metatarsal bone length from day 0 (mean ± SE). Substances were included in the culture medium from day 0. All 24 metatarsal bones treated with IL-1β (10 ng/ml) and TNF-α (10 ng/ml), and 24 of the control bones were also included in Figs. 2C and 3.

with control bones ($p < 0.001$). The addition of IGF-I (100 ng/ml) to IL-1β/TNF-α–treated metatarsals significantly increased proliferation compared with IL-1β/TNF alone ($p < 0.001$; Fig. 6A; Table 1).

Apoptosis (TUNEL, Lamin A)

In addition to impaired proliferation, we also investigated whether the observed growth retardation induced by IL-1β/TNF-α could be caused by increased apoptosis. TUNEL immunohistochemistry was performed in sections of 7-day cultured rat metatarsal bones. In all groups, the vast majority of apoptotic chondrocytes were proliferative cells in the epiphyseal region and the proliferative zone (Fig. 5). IL-1β (10 ng/ml) did not significantly alter apoptosis (Fig. 6B; Table 1). Treatment with TNF-α increased apoptosis almost three times, but this effect did not reach statistical significance compared with control bones (Fig. 6B; Table 1). Combined treatment with IL-1β + TNF-α (10 ng/ml each) significantly increased apoptosis compared with control bones or bones treated with IL-1β or TNF-α separately (Fig. 6B; Table 1). Co-administration of IGF-I (100 ng/ml) to bones treated with IL-1β + TNF-α significantly decreased apoptosis, reaching a level similar to that observed in bones treated with TNF-α alone, but remained higher compared with control and IL-1β alone (Fig. 6B; Table 1).

Immunoreactive chondrocytes for cleaved Lamin A were proliferative cells in the epiphyseal region and in the proliferative zone (data not shown). Control and IL-1β–treated metatarsals (10 ng/ml) were negative for cleaved Lamin A immunoreactivity (0.0 ± 0.0 cells/mm, respectively). TNF-α (10 ng/ml) increased the number of immunoreactive cells (0.5 ± 0.17 cells/mm), but it was not statistically different from the control or IL-1β groups ($p > 0.05$). IL-1β/TNF-α (10 ng/ml each) increased the number of Lamin A immunoreactive cells to 11.3 ± 1.5 cells/mm; $p <$

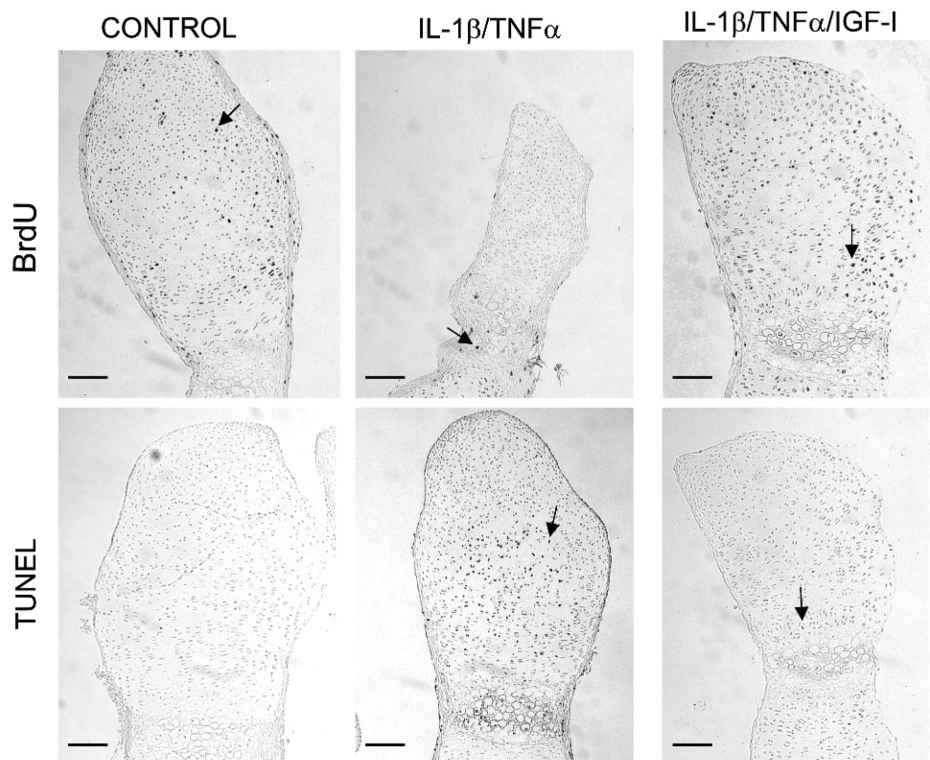

FIG. 5. The panels show BrdU and TUNEL immunohistochemistry in representative sections of rat metatarsal bones. Three groups are shown; the control group, a second group treated with the combination of IL-1β (10 ng/ml) plus TNF-α (10 ng/ml; IL-1β/TNF-α), and a third group also treated with IGF-I (100 ng/ml) in addition to IL-1β/TNF-α (IL-1β/TNF-α/IGF-I). All test substances were included in the culture medium from day 0. After 7 days of culture, the bones were fixed, decalcified, sectioned, and processed as detailed in the Materials and Methods section. The arrows indicate positive cells as determined by BrdU and TUNEL immunohistochemistry. The bars correspond to 100 μm.

0.001 versus control), and the addition of IGF-I (100 ng/ml) to IL-1β + TNF-α significantly decreased the number of Lamin A^+ cells to 2.3 ± 1.6 cells/mm ($p < 0.001$).

DISCUSSION

The primary objective of our study was to investigate whether cytokines affect longitudinal bone growth by a local effect in the growth plate. We focused on IL-1β, IL-6, and TNF-α because these cytokines are increased in most inflammatory diseases known to affect longitudinal growth in children. Our results clearly show that IL-1β and TNF-α can affect longitudinal growth through direct actions in growth plate cartilage, whereas IL-6 has no effect. In addition, we showed that IL-1β and TNF-α act in synergy to decrease longitudinal growth in organ cultures of fetal rat metatarsal bones, an effect that could be partly prevented by co-treatment with anti-IL-1β, anti-TNF-α, or IGF-I.

It has been proposed that, in inflammatory conditions, growth retardation could be at least partially a result of systemic effects of cytokines by affecting for example the GH/IGF-I axis.[1,12,20,21] Our results provide strong evidence that, in addition to possible systemic effects, cytokines impair longitudinal bone growth by directly affecting growth plate homeostasis. A direct effect on growth plate chondrocytes is supported by our demonstration of receptors for IL-1 and TNFα. However, indirect effects mediated through other cell lineages cannot be excluded.

TABLE 1. EFFECTS OF CYTOKINES ON CHONDROCYTE PROLIFERATION (BRDU) AND APOPTOSIS (TUNEL)

	BrdU	*TUNEL*	*Growth (%)*
Control	75.2 ± 10.0*†	13.3 ± 4.7*†	33.9 ± 1.4*§
IL-1β (10 ng/ml)	60.2 ± 5.4*‡	14.9 ± 2.4*†	40.1 ± 2.6*
TNF-α (10 ng/ml)	74.8 ± 4.6*†	32.1 ± 1.6*	34.7 ± 2.3*
IL-1β/TNF-α (10/10 ng/ml)	18.0 ± 4.2	112.0 ± 9.9	4.1 ± 1.5‡
IL-1β/TNF-α/ IGF-I (10/10/ 100 ng/ml)	105.5 ± 11.1*	41.0 ± 7.2*	16.3 ± 6.1

Chondrocyte proliferation (BrdU) is expressed as number of proliferative cells/mm^2 growth plate and apoptosis (TUNEL) as number of apoptotic cells/mm^2 growth plate. Metatarsal growth is expressed as percent increase in length between day 0 and day 7.

* $p < 0.001$ vs. IL-1β/TNF-α.
† $p < 0.05$ vs. IL-1β/TNF-α/IGF-I.
‡ $p < 0.01$ vs. IL-1β/TNF-α/IGF-I.
§ $p < 0.05$ vs. IL-1β.

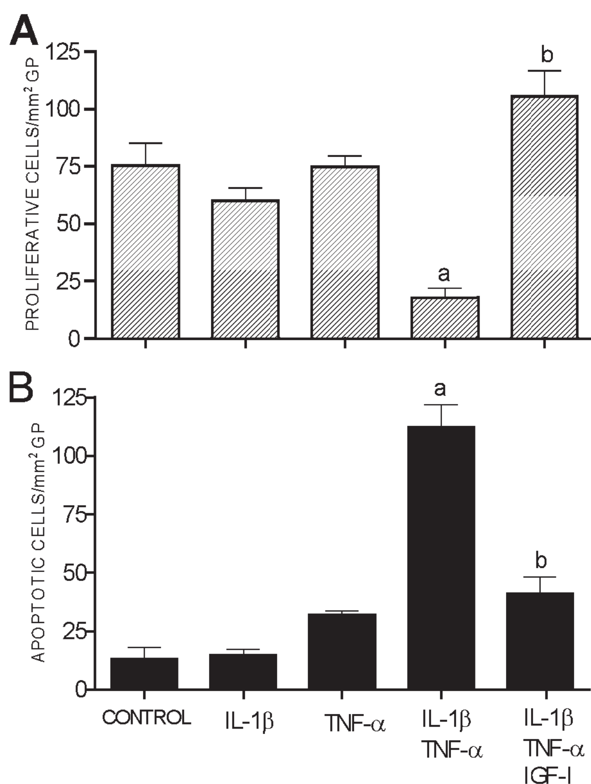

FIG. 6. Effect of IL-1β (10 ng/ml) and TNF-α (10 ng/ml) when added individually or in combination at the same concentrations to fetal rat metatarsal bones kept in culture for 7 days. One group of bones was also treated with IGF-I (100 ng/ml) in addition to IL-1β/TNF-α (10 ng/ml each). Results are expressed as number of positive cells per square millimeter growth plate surface area (mean ± SE). (A) Evaluation of cell proliferation with BrdU immuno-labeling. (B) Determination of apoptosis by TUNEL immunohistochemistry. $^a p < 0.001$ vs. control; $^b p < 0.001$ vs. IL-1β/TNF-α.

When added separately, we found that IL-1β and TNF-α impaired growth only at high concentrations. On the contrary, the combination of these cytokines had a strong inhibitory effect on growth at far lower concentrations showing a synergistic effect. These effective concentrations of IL-1β and TNF-α are supraphysiological compared with the serum levels reported in normal children but not far from those reported in children with juvenile rheumatoid arthritis.[22] Synergistic effects of cytokines have been described in other tissues (i.e., for IL-1β, TNF-α, and interferon-γ in pancreatic islets of Langerhans.[23] A possible explanation could be that these cytokines share some MAPK-signaling pathways and that both cytokines are needed to evoke effects.[23] In addition, in many inflammatory diseases associated with growth retardation, more than one cytokine is upregulated, suggesting that growth retardation could be a result of the synergistic effect of two or more cytokines. Therefore, we believe that our observation that IL-1β and TNF-α act in synergy to impair longitudinal bone growth reflects the in vivo situation in inflammatory diseases.

We also studied possible mechanisms through which the combination of IL-1β and TNF-α arrest metatarsal bone growth. Studies of BrdU incorporation revealed that these cytokines clearly decreased proliferation of growth plate chondrocytes. Our results are in line with a study in rats with experimental colitis where the proliferative zone of the growth plate was smaller in animals with colitis,[7] suggesting that pro-inflammatory cytokines may impair proliferation of growth plate chondrocytes. Furthermore, because growth is the balance between cell proliferation and cell death, we hypothesized that apoptosis is increased during cytokine-induced growth retardation. Indeed, we found a

significant increase in apoptosis after treatment with the combination of IL-1β and TNF-α. Apoptotic cells were almost exclusively proliferative chondrocytes with almost none of the hypertrophic cells affected. This cytokine-induced loss of early chondrocytes depletes the growth plate of cells that are programmed to give longitudinal growth. Therefore, it could be hypothesized that loss of proliferative or resting chondrocytes could impair final height in patients with inflammatory diseases, despite growth-promoting interventions or long periods of recession of the main disease. Thus, two mechanisms, decreased proliferation and increased apoptosis of proliferative chondrocytes, are involved in growth retardation induced by IL-1β and TNF-α. This combined effect on proliferative chondrocytes could explain the near total growth arrest observed in our experiments.

In an attempt to prevent cytokine-induced growth retardation, we studied the effect of a growth-promoting factor, IGF-I, which has both mitogenic and anti-apoptotic effects. Indeed, our data show that IGF-I is capable of, at least partially, rescuing the metatarsals from cytokine-induced growth arrest, although growth did not reach the same magnitude as in control bones. The growth promoting effect of IGF-I was associated with increased proliferation and survival of proliferative chondrocytes. Our data fit well with the observation that longitudinal growth can be only partly rescued by IGF-I administration to rats with experimental colitis.[6] Extrapolating these results to human disease suggests that IGF-I treatment is not sufficient to achieve normal growth in patients with chronic inflammatory diseases. The fact that IGF-I was able to only partially restore cytokine-induced growth retardation, despite a significant improvement in cell proliferation and decrease of chondrocyte apoptosis, suggests that additional mechanisms are involved. These may include decreased chondrocyte hypertrophy, decreased collagen production, or increased matrix degradation.

We showed that anti-IL-1β and anti-TNF-α are able to counteract growth retardation induced by the combination of IL-1β and TNF-α. It can therefore be hypothesized that anti-cytokine treatment could improve growth in children with chronic inflammatory diseases. However, the data in literature are limited,[12,24] and the efficacy of this treatment on growth and final height has to be established. A recent report showed increased serum IGF-I levels in children with idiopathic arthritis treated with a TNF-antagonist.[12] In contrast, treatment with antibodies against TNF-α improves growth in pair-fed colitic rats without affecting circulating levels of IGF-I.[2] Based on this article and other findings discussed in this paper, it is very likely that cytokines may hamper growth through effects other than on the IGF system. Therefore, IGF-I treatment, when available, may be combined with anti-cytokine treatment to improve growth and final height in patients with chronic inflammatory diseases.

In conclusion, we found that IL-1β and TNF-α act in synergy to greatly decrease longitudinal growth in rat metatarsal bones, an effect that could be partly reversed by anti-IL-1β, anti-TNF-α, or IGF-I. The fact that the two cytokines IL-1β and TNF-α are especially harmful in combination supports the use of either anti-TNF-α or anti-IL-1 in attempts to restore growth in patients with chronic inflammation. Our data also suggest that IGF-I treatment, when available, may be used in combination with more specific anti-cytokine treatment to optimize growth in patients with chronic inflammatory diseases.

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Address reprint requests to:
Lars Sävendahl, MD, PhD
Pediatric Endocrinology Unit Q2:08
Department of Woman and Child Health
Karolinska Hospital
Stockholm SE-171 76, Sweden
E-mail: lars.savendahl@kbh.ki.se

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