The restricted potential for recovery of growth plate chondrogenesis and longitudinal bone growth following exposure to pro-inflammatory cytokines

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Abstract

Childhood chronic inflammatory disease can be associated with transient and permanent growth retardation. This study examined the potential for spontaneous growth recovery following pro-inflammatory cytokine exposure. Murine ATDC5 chondrogenic cells and postnatal metatarsals were exposed to interleukin (IL)-1 β , IL-6 and tumour necrosis factor- α (TNF α), and their growth and proliferative capacity were determined following recovery. TNF α and IL-1 β reduced chondrocyte proliferation and aggrecan and collagen types II and X expression at minimum concentrations of 10 ng/ml and 0.1 ng/ml respectively. TNF α but not IL-1 β exposure led to increased caspase-3 activity and altered cellular morphology, consistent with reduced viability. Cytokine exposure particularly inhibited proteoglycan synthesis. This effect was dose and duration dependent. Compared with the control, IL-1 β and TNF α led to a 71% and 45% reduction in metatarsal growth after 8 days of exposure

respectively ($P < 0.05$). An additive effect of IL-1 β combined with TNF α was observed (110% decrease; $P < 0.05$). Metatarsals exposed to IL-1 β or TNF α individually for a 2-day period, and allowed to recover spontaneously in the absence of cytokines for a further 6 days, showed normal growth trajectories. In combination, growth was 59% lower ($P < 0.01$) compared with control metatarsals at the end of the recovery period. Exposure to the combination for 4 days followed by a 4-day recovery period resulted in 87% decrement compared with controls ($P < 0.05$). IL-6 did not alter any parameter studied. IL-1 β and TNF α exert diverse inhibitory effects on ATDC5 chondrocyte dynamics and metatarsal growth. The extent of recovery following cytokine exposure depends on the duration of exposure, and may be incomplete following longer periods of exposure.

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Introduction

Adverse longitudinal growth in children with chronic inflammatory diseases is common, and can be attributed to a combination of systemic factors that include poor nutrition, excess glucocorticoids, sex steroid deficiency and defects in growth hormone (GH) secretion and action (Farthing *et al.* 1981, Kirscher *et al.* 1981, Mainardi *et al.* 2002, Mushtaq & Ahmed 2002). Although this may be a transient phenomenon that is dependent on the resolution of the above factors (Kirscher *et al.* 1981), there are some concerns that it may also result in permanent growth restriction (Sawczenko *et al.* 2003). Although cytokines may affect growth through systemic mechanisms that alter GH secretion (Mainardi *et al.* 2002), growth disorders in chronic inflammatory disease may also be due to a direct effect of cytokines at the level of the growth plate (de Hooge *et al.* 2003, Martensson *et al.* 2004).

The inhibitory effects of the pro-inflammatory cytokines interleukin (IL)-1 β , tumour necrosis factor- α (TNF α) and IL-6 on articular chondrocyte growth dynamics are well recognised (Goldring 2003). However, the direct effects of these cytokines on growth plate chondrocytes are not clearly understood. Previous studies that have shown that IL-1 β and TNF α inhibit growth plate chondrocyte differentiation and induce cell death (Kato *et al.* 1993, Aizawa *et al.* 2001). However, these studies have examined the effect of pro-inflammatory cytokines on primary growth plate chondrocytes and were, therefore, unable to examine the effect of cytokines on the different stages of the chondrocyte life cycle (Robson *et al.* 1998, Koedam *et al.* 2000). The rate of longitudinal bone growth is determined by a complex interplay of proliferative kinetics, size of the proliferative pool matrix synthesis and hypertrophic chondrocyte enlargement (Atsumi *et al.* 1990, Breur *et al.* 1991).

The murine ATDC5 chondrocyte cell line has been shown to undergo the temporal sequence of events that occur during longitudinal bone growth *in vivo* and, thereby, provides an excellent model to study the molecular mechanisms underlying regulation of growth plate maturation and endochondral bone formation (Shukunami *et al.* 1997, Horiguchi *et al.* 2000, Farquharson 2003). The local effect of pro-inflammatory cytokines on the growth plate has also been studied in a fetal organ-culture model of bone growth, where it has been reported that they reduce proteoglycan synthesis and restrict longitudinal growth (van Bezooijen *et al.* 2002, Martensson *et al.* 2004). Given that there are clear differences between the prenatal and postnatal regulation of growth it would be beneficial to examine these effects in a postnatal model of bone growth. In addition, the potential for spontaneous recovery in chondrogenesis or longitudinal growth following cytokine exposure is, to date, unknown.

In this study, the ATDC5 cell line was used to characterise and compare the effects of IL-1 β , IL-6 and TNF α on cell proliferation, differentiation and apoptosis. Explant studies were also undertaken to examine their effect on the longitudinal growth of neonatal murine metatarsals. Subsequently, these models were used to examine the potential for spontaneous recovery of chondrocyte dynamics and longitudinal growth following temporary exposure to the pro-inflammatory cytokines.

Materials and Methods

Chondrocyte cell culture

The ATDC5 chondrocyte cell line was sourced from the RIKEN cell bank (Ibaraki, Japan) and maintained as described by Atsumi *et al.* (1990). Cells were maintained in T175 tissue-culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of 250 000 cells/flask in a maintenance medium of Dulbecco's modified Eagles' medium/Ham's F12 (Invitrogen) supplemented with 5% FCS (Invitrogen), 10 μ g/ml human transferrin, 3×10^{-8} M sodium selenite (Sigma), sodium pyruvate (1 mM; Invitrogen) and gentamycin (50 μ g/ml; Invitrogen). For individual experiments, semi-confluent cultures were passaged with trypsin-EDTA (Sigma) and cultured (day 0) at a density of 6000 cells/cm² in multi-well plates (Costar, High Wycombe, Bucks, UK) in a differentiation medium that consisted of maintenance medium supplemented with insulin (10 μ g/ml; Sigma). Incubation was at 37 °C in a humidified atmosphere of 95% air/5% CO₂ and the medium was changed every second/third day. In all experiments, unless otherwise stated, cytokines were added to chondrocyte cultures from days 13 to 17. During this period the cells express established markers of the chondrocyte differentiated phenotype (Mushtaq *et al.* 2002).

Chondrocyte number, proliferation, differentiation and matrix production Murine TNF α (Autogen Bioclear, Calne, Wilts, UK), IL-1 β (Autogen Bioclear) and IL-6 (Autogen Bioclear) (+IL-6 soluble receptor (IL-6R) at matching concentrations (R&D Systems Europe Ltd, Abingdon, Oxon, UK)) were added to the cells at a final concentration of 100, 10, 1.0 and 0.1 ng/ml and compared with control cultures, which received vehicle only. The cytokines were also added to cells in all available combinations at a final concentration of 10 ng/ml. The rate of chondrocyte proliferation was assessed in cells cultured in 48-well plates over a 24-h period starting on day 13. On day 14, the chondrocytes were incubated with 0.2 μ Ci/ml [³H]thymidine (37 MBq/ml; Amersham Pharmacia Biotech) for the last 2 h of the culture period. The amount of radioactivity incorporated into trichloroacetic acid-insoluble precipitates was measured (Farquharson *et al.* 1999). Cell protein content was assessed using a detergent compatible (D_C) protein assay reagent (Bio-Rad Laboratories Inc) based on the Lowry procedure (Lowry *et al.* 1951) and lyophilised bovine plasma γ -globulin was used as standard (Bio-Rad Laboratories Inc). Proteoglycan synthesis was evaluated by staining cells with Alcian Blue (Shukunami *et al.* 1997, Mushtaq *et al.* 2002). Cells were cultured in 12-well plates, and exposed to the cytokines on day 13 and day 15. On day 17, the cells were washed twice with PBS, fixed in 95% methanol for 20 min and stained with 1% Alcian Blue 8 GX (Sigma) in 0.1 M HCl overnight and rinsed with distilled water. Alcian Blue-stained cultures were extracted with 1 ml 6 M guanidine-HCl for 6 h at room temperature. The optical density (O.D.) was determined at 630 nm using a spectrophotometer (Multiskan Ascent; Thermo Electron Corporation, Vantaa, Finland).

Analysis of chondrogenic gene expression On days 13 and 15, IL-6 (+IL-6R at matching concentrations), IL-1 β and TNF α were added at a final concentration of 10 ng/ml to cells cultured in six-well plates. The experiment was stopped on day 17 and total RNA was extracted from chondrocytes by repeated aspiration through a 25 gauge syringe needle in 0.5 ml Ultraspec (Biotechx, Houston, TX, USA). Following extraction with chloroform, RNA in the aqueous phase was precipitated with isopropanol and bound to RNA Tack resin (Biotechx) following the manufacturer's protocol. After washing with 75% ethanol, the RNA was eluted in 100 μ l ribonuclease-free water (Houston *et al.* 1999). For each sample, total RNA content was assessed by absorbance at 260 nm and purity by A260/A280 ratios, which were 1.9–2.0 in all cases. All preparations were diluted to a concentration of 50 ng/ μ l and stored at –70 °C. Gene expression was analysed by semi-quantitative RT-PCR (Jefferies *et al.* 1998, 2000, Farquharson *et al.* 1999, Houston *et al.* 1999). Aliquots of 500 ng RNA (or an equivalent volume of water as a control)

were reverse transcribed in 20 µl reactions containing cDNA equivalent to 10 ng RNA and 200 nM gene-specific primers in 11.1 × PCR buffer (Jefferies *et al.* 1998). Primers for 18S (20 cycles, 488 bp) (Ambion, Huntingdon, Cambs, UK); collagen type II (30 cycles, 172 bp, forward 5'-CAC ACT GGT AAG TGG GGC AAG ACC-3', reverse 5'-GGA TTG TGT TGT TTC AGG GTT CGGG-3'); collagen type X (35 cycles, 583 bp, forward 5'-AGG CAA GCC AGG CTA TGG AA-3', reverse 5'-GCT GTC CTG GAA AGC CGT TT-3'; aggrecan (35 cycles, 218 bp, forward 5'-CGA GAA TGA CAC CTG CTA GG-3', reverse 5'-AAG AAG ACA GGA CCA GGA AGG-3') were used. The cycling profile was 1 min at 92 °C (first cycle, 2 min), 1 min at 55 °C and 1 min at 70 °C. The number of cycles performed was carefully titrated to ensure that the reactions were in the exponential phase. Reaction products were analysed on 1.5% agarose gels in the presence of ethidium bromide (250 µg/l) and a digital image of each gel was captured using a gel documentation system (Bio-Rad Laboratories Inc).

Apoptosis Apoptosis of the cells was measured using a caspase-3 colorimetric assay (R&D Systems) with spectrophotometric quantification at 405 nm. TNFα, IL-1β and IL-6 (+IL-6R) were added to the cells at a final concentration of 10 ng/ml and compared with control cultures. Apoptosis was assessed following the manufacturer's protocol in cells cultured in six-well plates over a 24-h period starting on day 16.

Recovery experiments in ATDC5 ATDC5 cells were exposed to IL-1β, TNFα and IL-1β+TNFα in combination (10 ng/ml) at day 6. The cytokines were subsequently replaced with differentiation medium either on day 8 after an exposure period of 2 days or on day 13 after an exposure period of 7 days. Proteoglycan synthesis was assessed at day 20 and compared with that in controls and with that in cells continuously exposed to cytokines from day 6 to day 20.

Organ culture

The middle three metatarsals were aseptically dissected from 2-day-old neonatal Swiss mice. Bones were cultured at 37 °C in a humidified atmosphere of 95% air/5% CO₂ in 24-well plates for a total of 8 days. Each culture well contained 300 µl of α-minimum essential medium (Invitrogen) supplemented with 1 mmol/l β-glycerophosphate (Sigma), 0.05 mg/ml L-ascorbic acid phosphate (Wako Pure Chemicals Ltd, Neuss, Germany), 0.05 mg/ml gentamycin, 1.25 µg/ml fungizone (Invitrogen) and 10% FBS. TNFα, IL-1β and IL-6 (+IL-6R) were added individually and in combination at a final concentration of 10 ng/ml for the full 8 days. To study spontaneous recovery from cytokine treatment, metatarsals were exposed for the first 2 or 4 days of the 8-day culture period to TNFα and

IL-1β treatment (individually and in combination); for the remainder of the 8-day culture period the metatarsals were exposed to culture medium only. In all experiments, the medium was changed every second/third day. The influence of resorption on length was studied by adding etidronate at a final concentration of either 10 µM or 1 µM, in combination with IL-1β (10 ng/ml) for an 8-day culture period. The experimental protocol was approved by Roslin Institute's Animal Users Committee and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals.

Morphometric analysis Digital images of the metatarsals were captured every second day of culture and viewed on a Nikon Eclipse TE3000 microscope (Nikon, Kingston upon Thames, Surrey, UK), using a digital camera (DS Camera Head DS-5 M; Nikon). The total length of the bone through the centre of the mineralising zone was determined using image analysis software (DS Camera Control Unit DS-L1; Nikon). All results are expressed as a percentage change from harvesting length, which was regarded as baseline.

For the determination of the size (in the direction of longitudinal growth) within the growth region of the distinct chondrocyte maturational zones, the metatarsals were fixed in 70% ethanol, dehydrated and embedded in paraffin wax (Mushtaq *et al.* 2004). Sections (10 µm in thickness) were stained with haematoxylin and eosin using standard protocols. The sizes of the proliferating, hypertrophic and mineralising zones of the metatarsals were determined using image analysis software (Nikon).

Cell proliferation and dry weight determination On day 6 of culture [³H]thymidine was added (final concentration 10 µCi/ml) to each metatarsal for the last 6 h of culture. After washing in PBS, the metatarsals were extracted in trichloroacetic acid (twice for 30 min), acetone (twice for 30 min) and ether (three times for 30 min) and air dried overnight at room temperature. After the determination of dry weight (Sartorius Micro, Gottingen, Germany) the tissue was solubilised (NCS-II tissue solubiliser; Amersham Biosciences) and the DNA incorporating [³H]thymidine was determined using a scintillation counter (Mushtaq *et al.* 2004). The cell proliferation data are expressed as [³H]thymidine (d.p.m.) per metatarsal.

Statistical analysis

All experiments were performed at least twice. General linear model analysis and the Student's *t*-test were used to assess the data. All data are expressed as the mean ± S.E.M. of six observations within each experiment. Statistical analysis was performed using Minitab 14 (Minitab Inc, State College, Philadelphia, PA, USA). *P* < 0.05 was considered to be significant.

Results

Chondrocyte cell culture

Cell proliferation Compared with control cultures, IL-1β reduced cell proliferation, as assessed by [³H]thymidine uptake, at every concentration studied (Table 1; *P*<0.001). However, TNFα only reduced cell proliferation at the higher concentrations of 100 ng/ml and 10 ng/ml (Table 1; *P*<0.001). IL-6 did not affect cell proliferation at any of the concentrations studied. No additive or synergistic effects on proliferation were observed when these cytokines were added in combination.

Proteoglycan synthesis Compared with control cultures, IL-1β significantly reduced proteoglycan synthesis, as indicated by Alcian Blue staining at every concentration studied (Table 1; *P*<0.001). Again, TNFα only reduced proteoglycan synthesis at the higher concentrations of 100 ng/ml and 10 ng/ml (Table 1; *P*<0.001). IL-6 did not significantly alter proteoglycan synthesis at any of the concentrations studied. No additive or synergistic effects on proteoglycan synthesis were observed when these cytokines were added in combination.

Apoptosis Apoptosis, as determined by caspase-3 activity, was significantly increased by exposure to both TNFα (Table 1; *P*<0.001) and IL-1β (Table 1; *P*<0.01) (both 10 ng/ml) compared with control cells. Furthermore, caspase-3 activity was significantly greater in cells treated with TNFα compared with IL-1β (*P*<0.001). Caspase-3 activity was not significantly increased in cells exposed to IL-6.

Cell number In comparison with control cultures, a significant reduction in cell number, as assessed by cell protein quantification, only occurred following treatment with TNFα (10 ng/ml) (*P*<0.01). Exposure to IL-1β or IL-6 (both 10 ng/ml) did not result in a significant reduction in cell number (Table 1). No additive or synergistic effects on cell number were observed when these cytokines were added in combination.

Chondrogenic gene expression TNFα and IL-1β (10 ng/ml) markedly reduced aggrecan, collagen II and collagen X gene expression (Fig. 1). The expression of aggrecan appeared to be particularly decreased by both TNFα and IL-1β treatment. Cells treated with IL-6 did not show a clear reduction in levels of aggrecan, collagen II and collagen X gene expression.

Recovery Morphological assessment of the ATDC5 cells revealed that exposure to TNFα (10 ng/ml) induced cell death and detachment from the culture plastic. Hence, none of the recovery periods were associated with a detectable rise in proteoglycan synthesis. Proteoglycan synthesis was not affected following 2 days of exposure to

Table 1 Effect of TNFα, IL-1β and IL-6 on thymidine uptake (O.D. at 630 nm), caspase-3 activity (O.D. at 405 nm/protein) and cellular protein (mg) in ATDC5 cell culture

	TNFα (ng/ml)				IL-1β (ng/ml)				IL-6 (ng/ml)			
	100	10	1	Control	100	10	1	Control	100	10	1	Control
Thymidine uptake (d.p.m.)	193.4*** (18.6)	269.6*** (38.6)	597.5.4 (518.9)	6210.5 (338.4)	620.0*** (34.1)	789.6*** (49.9)	795.7*** (35.1)	981.8*** (20.8)	7314.7 (196.5)	7236.1 (216.0)	6802.2 (285.4)	6279.7 (141.6)
Alcian Blue uptake (O.D.)	0.10** (0.01)	0.06*** (0.01)	0.55 (0.02)	0.47 (0.04)	0.11*** (0.01)	0.12*** (0.01)	0.12*** (0.01)	0.13*** (0.01)	0.45 (0.04)	0.39 (0.03)	0.57 (0.04)	0.43 (0.03)
Caspase-3 activity (O.D./mg protein)	ND	1.31*** (0.12)	ND	0.19 (0.01)	ND	0.36** (0.02)	ND	ND	0.19 (0.01)	0.16 (0.01)	ND	ND
Cellular protein (mg)	ND	0.34** (0.02)	ND	0.59 (0.03)	ND	0.57 (0.9)	ND	ND	0.59 (0.03)	0.58 (0.04)	ND	ND

S.E.M. in parentheses, *n*=6, ND, not determined. ***P*<0.01 and ****P*<0.001 compared with control.

Figure 1 Semiquantitative RT-PCR analysis of aggrecan, collagen II and collagen X expression in ATDC5 cells following exposure to TNF α , IL-1 β and IL-6 at 100 ng/ml and 10 ng/ml, compared with control (C) samples. B, blank.

IL-1 β (Fig. 2). However, by the time the cells were exposed to IL-1 β (10 ng/ml) for 7 days, a 41% reduction in Alcian Blue staining was evident in comparison with control cells ($P<0.001$). In the cells where 2 days of exposure to IL-1 β (10 ng/ml) was followed by a 12-day cytokine-free recovery period, proteoglycan synthesis at the end of the 14 days was 12% less in the treated cells

compared with the control ($P<0.05$). This difference in proteoglycan synthesis at the end of 14 days was greater at 38% when the treatment period was prolonged to 7 days, with a shorter recovery period ($P<0.001$). However, at 14 days, proteoglycan synthesis in these cells which had been treated for 7 days and subsequently had a 7-day recovery period was greater by 16% when compared with the cells which were continuously treated with IL-1 β for 14 days ($P<0.05$).

Neonatal metatarsal organ culture

Longitudinal growth A reduction in metatarsal growth was first noted after 4 days of exposure to TNF α (38%; $P<0.01$) and IL-1 β (50%; $P<0.001$) compared with control metatarsals (Fig. 3A). In combination, TNF α and IL-1 β reduced metatarsal growth by 66% of control values after 4 days of exposure ($P<0.001$). At the end of the 8-day culture period, TNF α reduced growth by 45% ($P<0.01$) and IL-1 β reduced growth by 71% ($P<0.001$) compared with control metatarsals (Fig. 3A). Treatment with IL-1 β with and without TNF α induced shrinking and folding of the metatarsals. In combination, TNF α and IL-1 β had an additive effect and reduced metatarsal growth by 110% of control values ($P<0.001$). By 6 days the combination of TNF α and IL-1 β had resulted in an actual reduction of absolute length. Addition of etidronate at 1 μ M or 10 μ M did not alter the IL-1 β -induced reduction in metatarsal growth (Fig. 3B). IL-6 did not have any significant effect on metatarsal growth.

Chondrocyte maturational zones TNF α and IL-1 β in combination resulted in a significant decrease in the length

Figure 2 Effect of duration of IL-1 β on Alcian Blue uptake (O.D. at 630 nm, means $\pm$ S.E.M., $n=6$) in ATDC5 cells. Cells were continuously exposed to IL-1 β and assayed at 2, 7 and 14 days (IL-1 β ■ and control □). In addition, cells were exposed to IL-1 β for 2 days or 7 days and allowed to recover for 12 or 7 days respectively and were assayed at 14 days. Percentile differences from respective controls are indicated where a significant reduction in Alcian Blue uptake was observed. * $P<0.05$; *** $P<0.001$.

Figure 3 Percentage change in length of postnatal murine metatarsals treated with (A) control medium, IL-6, TNF α , TNF α +IL-6, IL-1 β , IL-1 β +IL-6, TNF α +IL-1 β +IL-6 and TNF α +IL-1 β over an 8-day period (all 10 ng/ml) ($n=6$), (B) control medium, IL-1 β (10 ng/ml) and IL-1 β +etidronate (1 μ M and 10 μ M) over a 7-day period ($n=4$), (C) control medium, TNF α , IL-1 β , TNF α +IL-1 β (all 10 ng/ml) continuously for 8 days, or for 2 days only, followed by 6 days of cytokine-free recovery ($n=6$) and (D) control medium, TNF α , IL-1 β , TNF α +IL-1 β continuously for 8 days, or for 4 days only, followed by 4 days of cytokine-free recovery. Values are means $\pm$ S.E.M. ** $P<0.01$; *** $P<0.001$ versus control at day 8.

of the proliferating zone compared with controls ($P<0.001$) (Table 2). The length of the mineralising zone was not altered by any of the cytokine treatments. The hypertrophic zone was significantly reduced compared with controls by TNF α , IL-1 β and TNF α +IL-1 β treatments (Table 2; $P<0.001$). Furthermore, IL-1 β and TNF α +IL-1 β

reduced the length of the hypertrophic zone more markedly than TNF α alone (Table 2 and Fig. 4; $P<0.001$).

Cell proliferation and dry weight Compared with control metatarsals, there was a significant reduction in [3 H]thymidine uptake in metatarsals treated with TNF α

Table 2 Effect of TNF α , IL-1 β and TNF α +IL-1 β on metatarsal [3 H]thymidine uptake (d.p.m.), dry weight (μ g) and lengths of the proliferating, mineralising and hypertrophic zones (μ M)

Treatment	Proliferating zone (μ M)	Mineralising zone (μ M)	Hypertrophic zone (μ M)	[3 H]thymidine uptake (d.p.m.)	Dry weights (μ g)
Control	154 (9)	60 (8)	60 (8)	7977 (1149)	258 (45)
TNF α	148 (15)	61 (13)	43 (7)***	2688 (312)***	136 (20)*
IL-1 β	143 (16)	69 (8)	8 (3)***	4273 (1171)*	140 (21)*
TNF α +IL-1 β	93 (20)***	60 (8)	12 (2)***	2161 (300)***	100 (12)**

S.E.M. in parentheses, $n=6$. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with control.

Figure 4 (A) Hypertrophic zone of a control metatarsal and (B) markedly reduced hypertrophic zone following exposure to TNF α and IL-1 β in combination. P, proliferative zone; H, hypertrophic zone; M, mineralisation zone. Bar=100 μ m.

($P<0.001$), IL-1 β ($P<0.05$) and TNF α +IL-1 β ($P<0.05$) (Table 2). Metatarsal weight was also significantly reduced compared with controls by TNF α , IL-1 β (both $P<0.05$) and TNF α +IL-1 β ($P<0.01$) (Table 2).

Recovery Spontaneous recovery over the 8 days of metatarsal growth was assessed by exposing the bones for either 2 days or 4 days to cytokines, followed by a 6-day or a 4-day period of cytokine-free recovery period respectively (Fig. 3C and D). At day 8, metatarsals that had been exposed to IL-1 β or TNF α for the first 2 days had grown a similar amount as the control bones. However, histological analysis revealed that, at the end of the recovery period, the size of the hypertrophic zones of the metatarsals exposed to IL-1 β for 2 days remained significantly smaller compared with controls ($P<0.001$) (Table 3). The size of the hypertrophic zones exposed to TNF α for 2 days was not significantly different from

Table 3 Effect of exposure to TNF α and IL-1 β (2 days) followed by a recovery period (6 days) on the lengths of the proliferating, mineralising and hypertrophic zones (μ m)

Treatment	Proliferating zone (μ m)	Mineralising zone (μ m)	Hypertrophic zone (μ m)
Control	128 (8)	62 (3)	73 (6)
TNF α recovery	124 (6)	68 (2)	67 (2)
IL-1 β recovery	125 (12)	69 (2)	25 (4)***

S.E.M. in parentheses, $n=6$. *** $P<0.001$ compared with control.

controls. The percentage change in the length of the metatarsals that had been exposed to a combination of IL-1 β and TNF α for 2 days was 59% less compared with controls ($P<0.01$) by the end of the recovery period, at 8 days (Fig. 3C).

Metatarsals that had been exposed to TNF α only for the first 4 days had grown a similar amount as the control bones after the 4-day recovery period. However, the change in length of metatarsals that were exposed to IL-1 β for 4 days followed by 4 days of recovery in the absence of the cytokine was 52% of controls by the end of the recovery period ($P<0.001$) (Fig. 3D). This lack of recovery was more marked after 4 days of a combination of IL-1 β and TNF α where the percentage change in length was 87% lower than control metatarsals ($P<0.001$).

Discussion

The current study clearly showed that IL-1 β and TNF α , but not IL-6, not only have a dramatic, direct effect on chondrocyte dynamics but they also alter the potential for spontaneous recovery. Whilst the mouse metatarsal is a highly physiological model of longitudinal growth (Mushtaq *et al.* 2004), concerns about the applicability of the fetal model for studying postnatal growth led us to develop a novel, postnatal mouse metatarsal model for studying the local regulation of catch-up growth in conditions associated with postnatal growth retardation. By using this model of longitudinal growth, and combining it with a study of the effect of cytokines on the ATDC5 cell line, we have considerably extended our understanding of the effect of pro-inflammatory cytokines at the level of the growth plate.

In the cell culture studies, we found multiple strands of evidence suggesting that individual pro-inflammatory cytokines have varying effects on growth plate chondrocytes. First, a greater amount of apoptosis occurred following TNF α exposure compared with IL-1 β exposure. Although both cytokines reduced proliferation, IL-1 β was much more potent, exerting effects at all concentrations examined, whereas TNF α only exerted

effects at the higher concentrations. Our findings were reinforced by gene expression studies, which showed that TNF α and IL-1 β markedly reduced the mRNA expression of aggrecan, collagen II and collagen X. The expression of aggrecan at both the protein and gene level appeared to be particularly sensitive to both TNF α and IL-1 β which are reported to stimulate the synthesis of protein-degrading enzymes such as collagenases and stromelysins in cartilage (Reboul *et al.* 1996, Ohta *et al.* 1998). Consistent with these observations, we observed a marked reduction in overall proteoglycan synthesis following TNF α and IL-1 β exposure. Our previous studies have shown that proteoglycan synthesis is very sensitive to glucocorticoid exposure (Mushtaq *et al.* 2002). Together with collagen type II, proteoglycans and, in particular, aggrecan, provide a scaffold and stimuli for chondrocyte attachment, migration and differentiation and, thereby, maintain the structural organisation of the growth plate (Bateman *et al.* 1996). The contribution of aggrecan to bone growth has been demonstrated in a number of murine transgenic models. Cartilage matrix deficiency (*cmd*) is a result of a 7 bp deletion in exon 5 of the aggrecan gene and results in mice with short limbs (Watanabe *et al.* 1994). Similarly delayed bone formation and progressive dwarfism have been recorded in mice null for aggrecan link protein (Watanabe & Yamada 1999). The growth plate cartilage of these mice was characterised by decreased numbers of hypertrophic chondrocytes, which may contribute to the short limbs observed.

Recovery from TNF α exposure in ATDC5 cells could not be studied because of its toxicity to the cells when added during the chondrogenesis period of maturation (Mushtaq *et al.* 2002). In contrast, exposure to IL-1 β was not toxic and allowed detailed studies of the recovery of proteoglycan synthesis. By limiting the duration of exposure to a variable number of days, these studies showed that even a short 2-day period of exposure led to a long-term reduction in proteoglycan synthesis. Although there was some recovery in proteoglycan synthesis after this short period of exposure, this partial recovery was not evident in the cells exposed for longer periods. Interestingly, although there was a long-term reduction in proteoglycan synthesis after the 2-day period of treatment, no differences were observed in proteoglycan synthesis between the treated and control cells at 2 days. This suggests that cytokine-induced effects may manifest themselves at a later stage following cessation of the exposure. A similar phenomenon has also been noted following the removal of glucocorticoid exposure to growth plate chondrocytes (Mushtaq *et al.* 2002).

The reduction in longitudinal bone growth observed following TNF α and IL-1 β treatment of postnatal metatarsals was more marked with the latter cytokine. The initial marked reduction in longitudinal bone growth followed by a reduction in absolute length after a few days of continuous exposure to TNF α and IL-1 β in combina-

tion was particularly remarkable. The failure to ameliorate these effects with etidronate suggest that it is unlikely that these observed effects were due to cytokine-stimulated osteoclast activity (Manolagas 1995). Our studies showed clear evidence that the growth-inhibitory effects are due to a combination of effects on matrix synthesis, chondrocyte proliferation and a reduction in the hypertrophic zone. Whilst treatment with either TNF α or IL-1 β reduced proliferation by comparable amounts, IL-1 β seemed to have the predominant effect on the hypertrophic chondrocytes which are the principal determinant of longitudinal bone growth (Noonan *et al.* 1998).

The metatarsal recovery studies showed that a 2-day exposure to either TNF α or IL-1 β alone did not impair subsequent bone growth. However, a 2-day exposure to a combination of these two cytokines reduced subsequent bone growth, demonstrating a synergistic effect of TNF α and IL-1 β as reported by Martensson *et al.* (2004). Our experiments clearly showed that catch-up growth did not occur following combined exposure to TNF α and IL-1 β or following a longer, 4-day, period of exposure to IL-1 β . Four days of exposure to TNF α did not impair subsequent bone growth, indicating that only relatively long-term exposure to TNF α is detrimental to bone growth. In both the ATDC5 and metatarsal recovery studies, however, it is possible that recovery could have occurred given a longer time period of observation.

The results of this study concur with and extend previous reports highlighting the effects of inflammatory cytokines on growth plate chondrocytes. In cultures of rabbit growth plate chondrocytes, IL-1 β decreased alkaline phosphatase activity during the hypertrophic stage and suppressed an increase in cell size and type X collagen expression, suggesting an inhibition of chondrocyte terminal differentiation and hypertrophy (Kato *et al.* 1993). TNF α induced apoptosis in chick growth plate chondrocyte cultures (Aizawa *et al.* 2001) and suppressed cartilaginous nodule formation and the accumulation of cartilage-specific proteoglycan reduction in the ATDC5 cell line (Horiguchi *et al.* 2000). Interestingly, Horiguchi *et al.* (2000) noted that TNF α (10 ng/ml) increased [3 H]thymidine uptake in the ATDC5 cell line which is in contrast to our data. This reason for this discrepancy is unclear; however, the reduction in chondrocyte proliferation noted in the ATDC5 cell line was confirmed in the metatarsal model. TNF α has also been shown to synergise with IL-17 to reduce proteoglycan synthesis in fetal mouse metatarsals (van Bezooijen *et al.* 2002).

In both models reported in our studies IL-6 did not have any significant inhibitory effects. Furthermore, the cell culture studies suggest a possible pro-proliferative effect of IL-6, although this was not a consistent finding. This lack of inhibitory response is also consistent with the findings in another study (Martensson *et al.* 2004). It is, therefore, possible that IL-6 may mediate growth abnormalities through systemic effects, whereas TNF α and

IL-1 β may act locally at the level of the growth plate. In this postnatal, murine metatarsal model of longitudinal growth, IL-1 β and TNF α both had an inhibitory effect on growth at 10 ng/ml. However, in the fetal rat metatarsal model, IL-1 β had a positive effect on growth at that concentration and TNF α did not have any effect (Martensson *et al.* 2004). Our observations not only suggest that the growth-inhibitory effects of TNF α and IL-1 β may be mediated through different cellular mechanisms and may be age dependent but they also emphasise the importance of studying more than one model of bone growth.

The partial recovery observed following cytokine exposure is comparable with that reported following cessation of glucocorticoid administration to growth plate chondrocytes (Baron *et al.* 1992, Mushtaq *et al.* 2002). Our previous studies have suggested that longitudinal growth and especially chondrocyte hypertrophy in the metatarsal model of growth is highly dependent on insulin-like growth factor-I (IGF-I) action (Mushtaq *et al.* 2004). It is possible that the growth-inhibitory effects of inflammatory cytokines and glucocorticoids are mediated via similar signalling mechanisms such as the phosphatidylinositol 3-kinase pathway (Kenchappa *et al.* 2004, Chrysis *et al.* 2005). Alternatively, cytokines may induce a state of IGF-I resistance by reducing IGF-I signal transduction in chondrocytes by inhibiting insulin receptor substrate-1 phosphorylation (Broussard *et al.* 2004) by the up-regulation of the suppressor of cytokine-signalling protein-3 expression (Shi *et al.* 2004). If this is the case, our experiments indicate that a persistent state of IGF-I resistance may exist following the removal of the insult and this possibility requires further investigation. Our data may also explain the lack of catch-up growth observed in rats administered IGF-I following experimental colitis (Ballinger *et al.* 2000). In children with juvenile idiopathic arthritis, high-dose treatment with GH leads to a rise in systemic levels of IGF-I and has been reported to reduce the worsening growth retardation but does not seem to result in normalisation of height (Simon *et al.* 2003). Furthermore, several studies of final height of adults with childhood onset inflammatory bowel disease show that these adults may be relatively short (Castile *et al.* 1980, Griffiths *et al.* 1993, Markowitz *et al.* 1993, Alemzadeh *et al.* 2002, Sawczenko *et al.* 2003). Our data would suggest that even a relatively short period of exposure to pro-inflammatory cytokines, especially when in combination, may have an irreversible effect on growth plate chondrogenesis.

Whilst many previous studies have examined the effects of pro-inflammatory cytokines on articular chondrocytes relatively few have investigated growth plate chondrocytes and none has investigated the potential for spontaneous recovery following exposure. Our results extend the data from previous chondrocyte culture studies and the findings will allow a more focused approach towards

studying the mechanisms underlying cytokine-induced growth alterations, as well as investigating the potential benefit of growth-promoting therapy.

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