Expression and Role of Interleukin-6 in Distraction Osteogenesis

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Abstract. Distraction osteogenesis is a special form of bone healing in which well-controlled distraction stresses and consequent tensile strains within callus tissue induce very efficient new bone formation. Proinflammatory cytokines are involved during the early phase of fracture healing and callus remodeling. Temporal expression patterns of proinflammatory cytokines were assessed in Sprague-Dawley rat tibial models of distraction osteogenesis and acute lengthening, and only interleukin-6 (IL-6) was found to be specifically induced during the distraction phase. IL-6 immunoreactivity was detected not only in hemopoietic cells and osteoblasts but also in the spindle-shaped cells of the fibrous interzone, where most of the tensile strains are concentrated. *In vitro* study revealed that IL-6 did not affect the proliferation of C3H10T1/2 cells, mouse bone marrow stromal cells (MSCs), or MC3T3-E1 cells; but its blocking antibody reduced the proliferation of C3H10T1/2 cells and MSCs. The mRNA expression of COL1A1 and osteopontin were not changed by IL-6 or its blocking antibody, but the alkaline phosphatase activities of MC3T3-E1 cells were increased by IL-6 and decreased by its blocking antibody. These findings indicate that IL-6 is a proinflammatory cytokine that responds to tensile strain during distraction osteogenesis. IL-6 negatively affects the proliferation of primitive mesenchymal cells, whereas the differentiation of more mature osteoblastic lineage cells is enhanced by IL-6 *in vitro*. IL-6 appears to be one of the cytokines involved in the complex network of signal cascades evoked during distraction osteogenesis and may differentially affect immature and mature osteoblastic lineage cells.

Key words: Interleukin-6 — Distraction osteogenesis — Proinflammatory cytokine

Fracture healing involves a cascade of events that begins with an inflammatory reaction [1], in which macrophages and other immune cells are recruited to the fracture site and release several factors including interleukin-1 (IL-1), IL-6, and tumor necrosis factor α (TNF-α) [2, 3]. These proinflammatory cytokines are associated with the innate tissue response to injury or microbial challenge but are also known to enhance extracellular matrix synthesis, stimulate angiogenesis, and recruit endogenous mesenchymal cells to the injury site [4]. At the end of the fracture repair sequence, the remodeling of fracture callus is crucial and required for the restoration of mechanical integrity. IL-1, IL-6, and TNF-α have been shown to play important regulatory roles in bone remodeling and homeostasis [5−9] and, through a variety of mechanisms, to regulate osteoclast activity either by stimulating hemopoietic progenitor cells to differentiate into mature osteoclasts or by activating existing osteoclasts.

Distraction osteogenesis is a special form of bone healing in which well-controlled distraction stresses and consequent tensile strains within callus tissues produce new bone formation at an unprecedented rate. After its introduction by Ilizarov in the 1950s [10], distraction osteogenesis has widely been used clinically for limb lengthening, correction of deformity, and reconstruction of large bone defects. Moreover, the technique has sparked both clinical enthusiasm and basic research because it regenerates new bone in a unique way. Contrary to the previously held notion that bone forms in response to compression, the so-called method of Ilizarov demonstrates that a carefully performed osteotomy followed by well-controlled distraction at an optimal rhythm and rate can induce new bone formation more efficiently than any other method. It is also well known that intense neoangiogenesis takes place along the entire limb segment when it is subjected to distraction osteogenesis [11−13]. Many angiogenic factors have been found to be induced during the distraction process and to contribute to angiogenesis and subsequent new bone formation [14, 15].

Distraction osteogenesis may be divided into three stages: (1) the latency period, when inflammatory reactions caused by corticotomy subside and the repair process begins; (2) the distraction phase, when the strains induced by gradual distraction activate a series of signaling cascades that induce intense angiogenesis and new bone formation; and (3) the consolidation phase, when newly regenerated bone bridges the distraction gap

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and undergoes remodeling to achieve the strength of host bone. Various growth and angiogenic factors have been studied in the context of distraction osteogenesis [14−19], and these authors believed that proinflammatory cytokine signaling cascades are involved in the process. Cillo et al. [20] studied the effect of mechanical strain on the mRNA expression of growth factors and cytokines in an osteosarcoma cell line (SaOS-2) and observed that IL-6 mRNA expression was induced after 24 hours of stretching. This finding suggests that proinflammatory cytokines such as IL-6 are induced by cell strain and may play a role in new bone formation or remodeling. To study this process, we designed experiments to answer the following questions: (1) Which proinflammatory cytokines are induced during distraction osteogenesis? (2) What are the spatial expression patterns of cytokines induced by this process? (3) What are the effects of these cytokines on the cells involved in new bone formation?

Materials and Methods

Thirty-five male 16-week-old Sprague-Dawley rats weighing 350−400 g were used in this study. The animal experiments were in compliance with the *Guiding Principles in the Care and Use of Animals* and approved by the Animal Care Committee at Seoul National University Hospital. Rats were divided into two groups based on surgical treatment: a distraction osteogenesis (DO) group and an acute lengthening (AL) group. The distraction osteogenesis model in the rat tibia has been previously described [21]. Briefly, the left tibia was fixed with a pair of mini-monofixators that enabled gradual lengthening after mid-diaphyseal osteotomy. Distraction began on postoperative day (POD) 7. Fragments were distracted at a rate of 0.5 mm/day in two steps per day from POD 7 to 13. Animals were killed on PODs 1, 3, 5, 7, 9, 11, 14, and 21. In the AL group, the same surgical procedure was performed as in the DO group but the osteotomy underwent an immediate 4 mm distraction. Animals were killed on PODs 1, 3, 5, 7, 14, and 21.

Quantitation of mRNA Expression Using RNase Protection Analysis

Mid-diaphyseal sections of the tibiae, including regenerated tissue in the distraction gap, and 5 mm of bone segments proximal and distal to it were excised with intact periosteum and stored in liquid nitrogen until required for analysis. For assays, bone tissue was mixed with solution D (guanidium thiocyanate 4 M, sodium acetate 25 mM, sarcosyl 0.5%) and ground in liquid nitrogen using a 6700 Freezer/Mill (Spex, Edison, NJ). Total RNA was then extracted using the acid phenol method and precipitated with alcohol. mRNA expression during fracture healing was quantitatively assessed by ribonuclease protection analysis, as described previously [22]. Two multiprobe template sets for rat cytokines (rCK-1 and rCK-2; Pharmingen, San Diego, CA) were used to generate single-stranded ^{32}P-labeled cRNA probes. The target genes of these probes included TNF-α, TNF-β, interferon-γ (IFNγ), IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-18, macrophage inhibiting factor (MIF), and the so-called housekeeping genes *L32* and glyceraldehyde-3-

phosphate dehydrogenase (*GAPDH*). RNase protection products were fractionated on a denaturing 6% acrylamide gel, and autoradiographic bands of the RNase protection products were quantified and normalized vs. *L32* and *GAPDH* mRNA.

Immunohistochemistry

Tibial segments from POD 7 (no distraction) and POD 11 (distraction for 4 days) specimens of the DO group were harvested after being perfused with 4% paraformaldehyde solution. Following overnight fixation, tissues were decalcified with 10% ethylenediaminetetraacetic acid (EDTA) buffer. IL-6 immunohistochemical studies were performed using the ABC method (LSAB-2 rat system; Dako, Carpentaria, CA) using monoclonal antibody for IL-6 (Santa Cruz Biotechnology, Santa Cruz, CA).

In Vitro Effect of IL-6 and of Its Blocking Antibody on Murine Cells

In vitro studies were performed using bone marrow cells and cell lines from mouse rather than rat equivalents due to the availability of species-specific ligand IL-6 and its blocking antibody (Sigma-Aldrich, St. Louis, MO). Bone marrow-derived cells were harvested by curettage of the marrow cavities of long bones from Balb/c mice. C3H10T1/2, a cell line with characteristics of mesenchymal stem cells, and MC3T3-E1, an osteoblast cell line derived from mouse calvaria, were obtained from the American Type Culture Collection (Rockville, MD). All cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% of each of penicillin and streptomycin (Life Technologies, Gaithersburg, MD) at 37°C in a humidified 5% CO_2 atmosphere. Cell proliferation assays, mRNA assays for osteoblast markers, and alkaline phosphatase assays were performed on the second-passage cells.

Cell Proliferation Assay

Cells were plated at 5×10^3/mL into 96-well plates and cultured for 48 hours. They were then treated with various concentrations of IL-6 (Sigma, St. Louis, MO; 0, 0.1, 1, 10, and 100 ng/mL) or anti-mouse IL-6 antibody (Sigma) [23] (0, 0.1, 1, 10, and 100 μg/mL) in serum-free medium for 24 hours. Cell proliferation assays were performed using CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay (Promega, Madison, WI). The cells were then incubated with 333 mg/L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulfophenyl)-2H-tetrazolium (MTS) and 25 μmol/L phenazine methosulfate solution for 3 hours at 37°C in a humidified 5% CO_2 atmosphere. The absorbance of the soluble formazan produced by cellular reductions of MTS was measured at 490 nm.

Osteoblastic Marker mRNA Assay

Cells were plated at 1×10^6/mL into 100 cm^2 plates containing osteogenic medium (DMEM with 10% FBS, 100 nM dexamethasone, 10 mM sodium-glycerol phosphate, and 0.05 mM ascorbic acid-2-phosphate). They were treated with IL-6 at 0.1 or 10 ng/mL or anti-mouse IL-6 antibody at 0.1 or 10 μg/mL for 7 days; media were changed every 48 hours. Total RNA was extracted, and single-stranded ^{32}P-labeled cRNA probes were generated from linearized plasmids containing mouse osteopontin, type II collagen (COL2A1), and type I collagen (COL1A1) (all from Pharmingen) as a custom-made template set. Probes for the *L32* and *GAPDH* genes were used for the internal control genes.
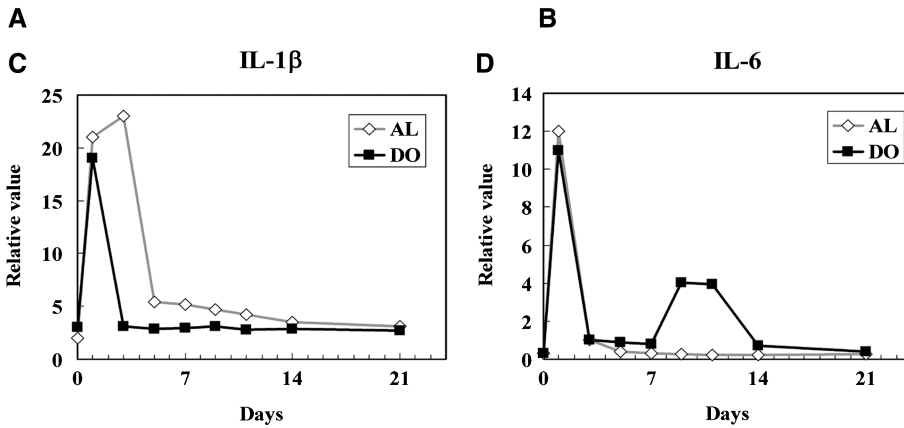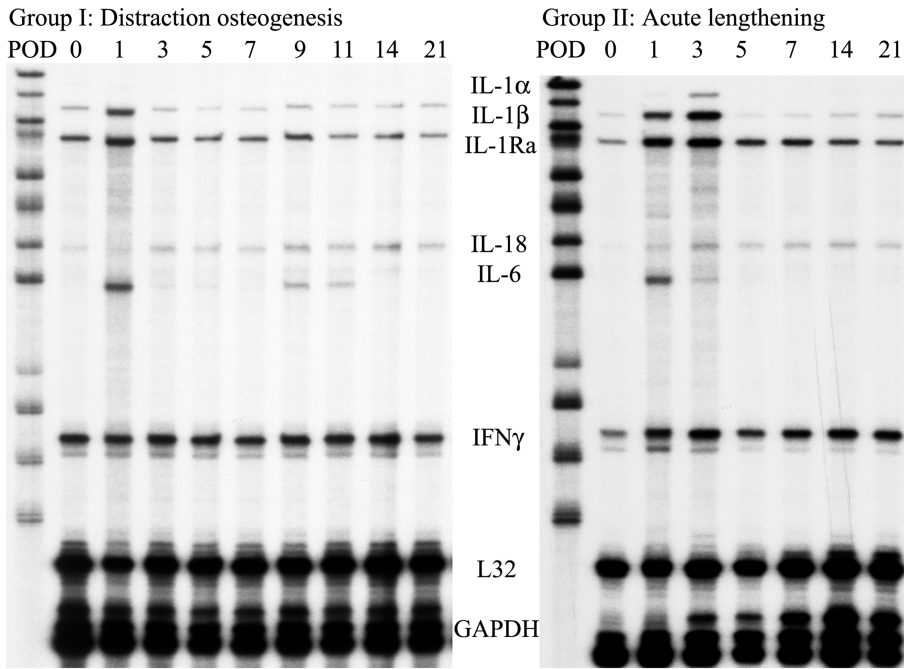
Fig. 1. RNase protection assay for mRNA expression of proinflammatory cytokines during distraction osteogenesis (**A**) and acute lengthening (**B**). (**C**, **D**) Graphic analysis of the relative mRNA levels of IL-1β and IL-6.

Alkaline Phosphatase Activity Assays

Cells were plated at 3×10^4/well into 24-well plates for analysis of alkaline phosphatase activity. After 48 hours in control medium, cells were treated with various concentrations of IL-6 (0, 0.01, 0.1, 1, or 10 ng/mL; Sigma) or anti-mouse IL-6 antibody (0, 0.01, 0.1, 1, or 10 μg/mL; Sigma) for 14 days. Cellular alkaline phosphatase activities were determined as previously described [24] and normalized vs. protein concentrations, which were measured using the Lowry method [25]. Data are expressed as ratios of micromoles of inorganic phosphate cleaved by alkaline phosphatase in 30 minutes/μg of protein.

Statistical Analysis

Results of the cell proliferation assay and alkaline phosphatase activity assays were analyzed using Student's *t*-test. Those data at each concentration of the ligand or its blocking antibody were compared with their baseline values. Calculation was performed using SPSS 12.0 for Windows (SPSS, Chicago, IL). $P < 0.05$ was considered significant.

Results

Expression of Proinflammatory Cytokine mRNA

Of the cytokines tested, the mRNAs for IL-1α, IL-1β, IL-6, IL-18, and IFNγ produced detectable RNase protection assay signals (Fig. 1A, B), whereas TNF-α and TNF-β did not (data not shown). IL-1α was detectable only during acute lengthening, and the temporal patterns of expression of IL-18 and IFNγ were not so distinctive.

In the DO group, the mRNA expression of IL-1β showed acute upregulation at POD 1 and then returned to its preoperative level from POD 3 throughout the experimental period. In the AL group, upregulation of IL-1β persisted until POD 3 and then subsided to its preoperative level (Fig. 1C). In the DO group, IL-6 showed a unique temporal expression pattern. It was
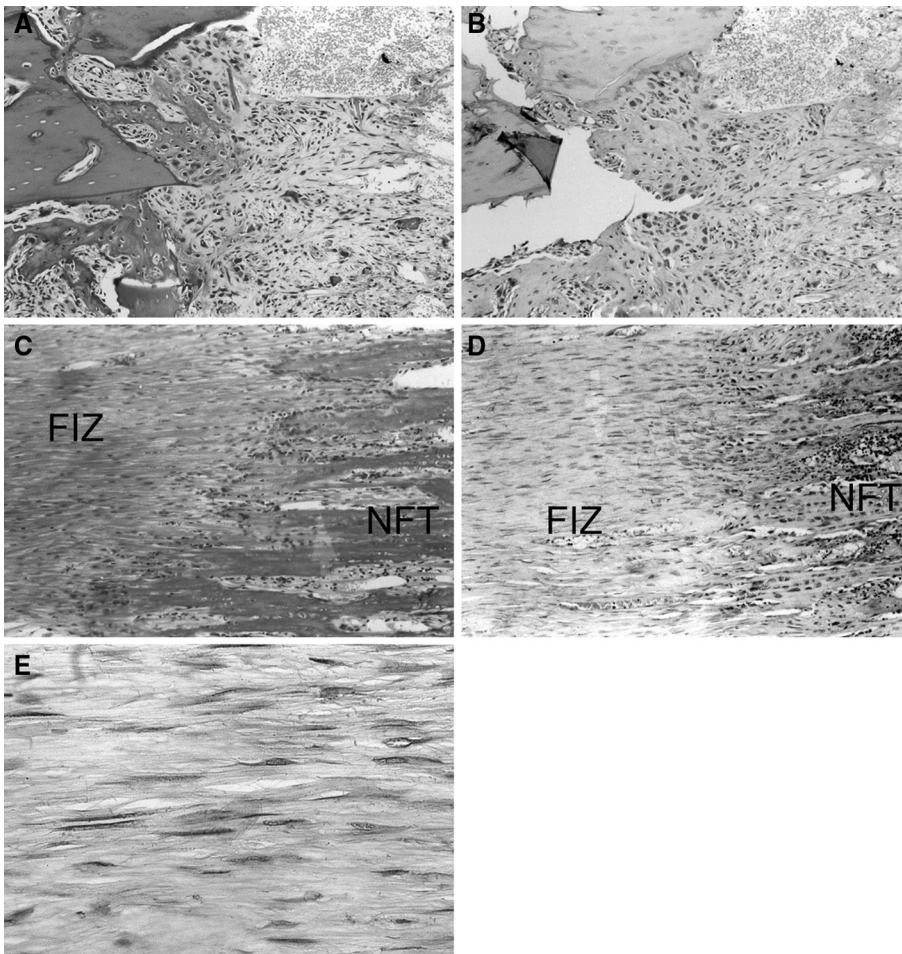
Fig. 2. (**A**, **B**) POD 7 without distraction showed IL-6 immunoreactivity in chondroid cells and osteoblastic cells lining the bony callus. (**C**, **D**) The DO group at POD 11 showed IL-6 immunoreactivity in osteoblastic cells at the newly formed trabeculae (NFT) and in spindle-shaped fibroblast-like cells in the fibrous interzone (FIZ). (**E**) High-power view of the fibrous interzone. (**A**, **C**) Hematoxylin and eosin staining, (**B-E**) immunostaining for IL-6. (**A**, **B**) ×100, (**C**, **D**) ×40, (**E**) ×200.

upregulated on POD 1, subsided to its preoperative level, and then its expression was reactivated on PODs 9 and 11, which coincided with the distraction phase. It then resubsided during the consolidation period. In the AL group, IL-6 mRNA expression persisted at its preoperative level during the second and third postoperative weeks (Fig. 1D).

Immunohistochemistry

Immunohistochemistry was performed in order to characterize the spatial expression pattern of IL-6 before distraction (POD 7) and during the distraction phase (POD 11). In POD 7 specimens, IL-6 immunoreactivity was detected in marrow cells, in osteoblastic cells lining newly formed trabeculae, and in chondroid cells around the chondroosseous callus. In contrast, undifferentiated mesenchymal cells showed no IL-6 immunoreactivity. In POD 11 specimens, the characteristic histological pattern of distraction osteogenesis was observed involving a fibrous interzone, a primary matrix front, microcolumn formation, and subsequent remodeling into mature diaphyseal bone. In POD 11 specimens, IL-6 immunoreactivity was detected not only in osteoblasts lining the trabeculae of the primary mineralization front and mi-

crocolumns but also in the spindle-shaped mesenchymal cells at the fibrous interzone (Fig. 2).

In Vitro Effects of IL-6

The *in vitro* effects of IL-6 on murine cells and on the C3H10T1/2 and MC3T3-E1 cell lines were tested in order to understand the role of IL-6 upregulation during the distraction phase of distraction osteogenesis. Mouse bone marrow-derived stromal cells (MSCs) and C3H10T1/2 and MC3T3-E1 cells were treated with IL-6 or its blocking antibody. None of the cells tested showed significant changes in proliferation after treatment with concentrations of IL-6 ranging 0−100 ng/mL. On the other hand, anti-IL-6 blocking antibody increased the proliferation of all three cell types. The mesenchymal stem cell-like cell line C3H10T1/2 responded at the lowest concentration of 1.0 µg/mL, while the mature osteoblast-like cell line MC3T3-E1 responded only at the relatively high concentration of 100 µg/mL. In terms of response, mouse MSCs were intermediate between these two cell lines (Fig. 3).

Osteoblastic phenotypic expression, as determined by expression of mRNA for osteopontin and COL1A1, was not affected by either IL-6 or anti-IL-6 antibody treat-
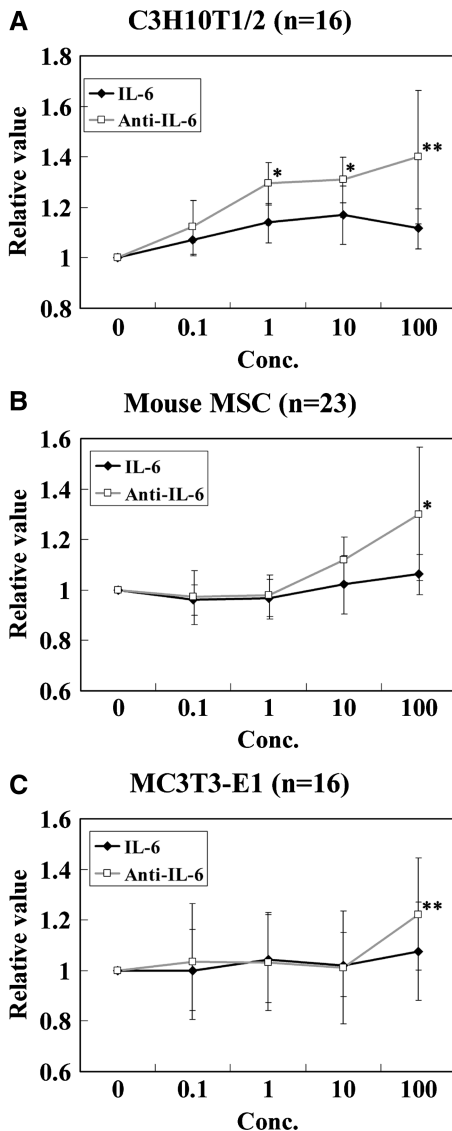
A **C3H10T1/2 (n=16)**

B **Mouse MSC (n=23)**

C **MC3T3-E1 (n=16)**

Fig. 3. Effect of IL-6 (ng/mL) and its blocking antibody (µg/mL) on the proliferation of (**A**) C3H10T1/2 cells, (**B**) MSCs from Balb/c mice, and (**C**) MC3T3-E1 cells. *$P < 0.05$ and **$P < 0.001$.

ment for 7 days. Although it was difficult to compare the expression levels of these genes in different cells, in C3H10T1/2 cells osteopontin mRNA appeared to be expressed at lower levels than in MC3T3-E1 cells or MSCs (Fig. 4).

Changes in alkaline phosphatase activity due to 2 weeks of IL-6 treatment or treatment with its blocking antibody varied in the different cells. It was interesting to note that in C3H10T1/2 cells and mouse MSCs alkaline phosphatase activity was suppressed by IL-6 or its blocking antibody, especially at high concentrations (10 ng/mL of IL-6 and 10 µg/mL of blocking antibody). In C3H10T1/2 cells, significant suppression of alkaline phosphatase activity was observed after treatment with IL-6 concentrations of 1.0 ng/mL or higher. On the

other hand, mouse MSCs started to significantly suppress alkaline phosphatase activity at an IL-6 concentration of 0.01 ng/ml or at a blocking antibody concentration of 0.1 µg/mL. In contrast to these two cells types, MC3T3-E1 cells responded differently to IL-6 and its blocking antibody. Alkaline phosphatase activity was increased by IL-6 dose-dependently from 0.01 to 10 ng/mL. On the other hand, its activity increased after treatment with anti-IL-6 antibody at concentrations up to 0.1 µg/mL and then significantly decreased as treatment concentrations increased (0.1−10 µg/mL) (Fig. 5).

Discussion

This study demonstrates that IL-6 is upregulated in response to the mechanical strain of distraction during distraction osteogenesis. During the inflammatory phase of fracture healing, proinflammatory cytokines such as IL-1, IL-6, and TNF-α are upregulated in osteoblastic lineage cells as well as macrophages and other immune cells [2]. Our first question was whether the force of distraction can reactivate the expression of proinflammatory cytokine during the distraction phase of the procedure. Cillo et al. [20] studied the effects of mechanical strain on growth factor and cytokine mRNA expression. They applied a tensile stretch to the mineralizing human osteoblast-like cell line SaOS-2. The investigators found that IL-6 mRNA was induced within 24 hours of stretching but that IL-1 was not induced by stretching stimuli. These findings suggest that IL-6 is sensitive to a distraction strain, which concurs with the in vivo findings of the current study. In the distraction osteogenesis model used here, both IL-1β and IL-6 mRNA expression were upregulated immediately after corticotomy but then returned to baseline levels rapidly during the postoperative period. However, IL-6 alone was reexpressed during the distraction phase. The temporal expression pattern of IL-6 during distraction osteogenesis supports the notion that it is induced by surgical or blunt trauma to bone and by the strain of distraction osteogenesis.

TNF-α mRNA expression remained silent during the study period in the distraction osteogenesis model but was reported in a previous study to be immediately upregulated after administering a fracture using a guillotine-type apparatus [2]. As corticotomy in the DO group was performed in a much less traumatic manner than with the guillotine-type fracture model, TNF-α mRNA expression appears to be induced only by a more substantial trauma. It is noteworthy that IL-1β and IL-6, in contrast, were upregulated even by corticotomy.

The second question posed concerned the identities of the cells that respond to distraction strain and express IL-6. It is well known that IL-6 is expressed in a variety
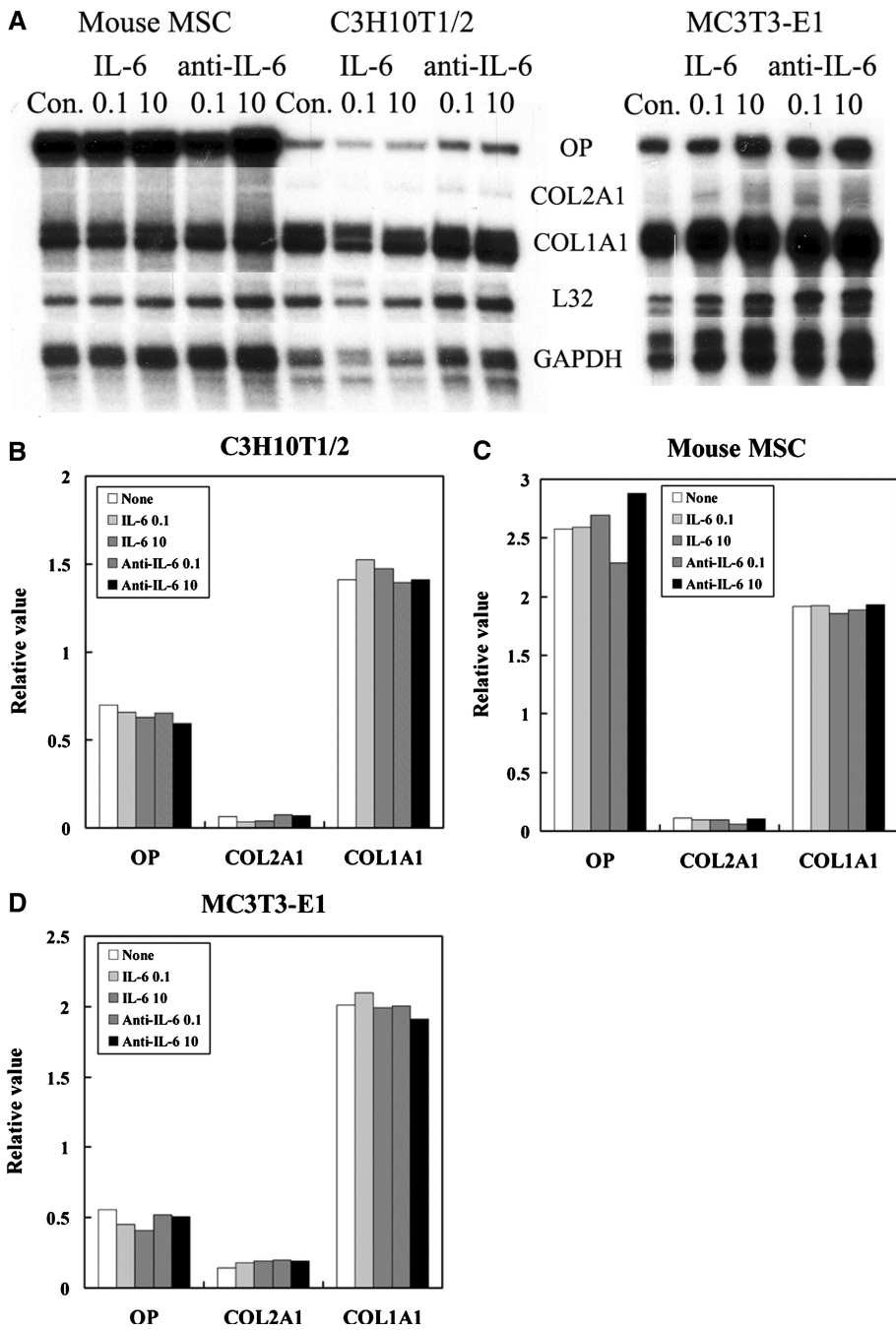
Fig. 4. RNase protection assay for mRNA expression of osteoblastic marker genes at treatment with IL-6 (ng/mL) and anti-IL-6 blocking antibody (μg/mL). (**A**) Autoradiographic image on 6% polyacrylamide gel electrophoresis sequence. (**B-D**) Graphic depiction of relative mRNA levels. Con, nontreated control; OP, osteopontin; L32, a housekeeping gene.

of cells, including stimulated monocytes, macrophages, T and B lymphocytes, fibroblasts, chondrocytes, and osteoblasts [26]. The distraction gap and its surrounding tissue in distraction osteogenesis are composed of a variety of cell types and tissues, which include a fibrous interzone, a primary matrix front, microcolumns, and host bone formed centripetally from the center of the distraction gap [27]. Three-dimensional finite element analyses have revealed that tensile strains are highest at the fibrous interzone [28], and our immunohistochemical data show that spindle-shaped cells at the fibrous interzone express IL-6 during the distraction phase. These findings support the notion that the fibrous

interzone responds to distraction strain by expressing IL-6. Moreover, this *in vivo* result concurs with previously reported *in vitro* findings in which tension strain upregulated IL-6 expression in SaOS-2 cells [20].

These results led us to investigate the effect of IL-6 on osteoblastic lineage cells. IL-6 is a multifunctional cytokine that has profound effects on hematopoietic lineage cells, such as osteoclasts and T and B lymphocytes, and mesenchymal lineage cells, such as chondrocytes and osteoblasts. Investigations into the role of IL-6 in bone metabolism have focused on its stimulation of osteoclastic activity [29, 30]. Regarding the effect on cells of mesenchymal origin, IL-6 seems to be associated with
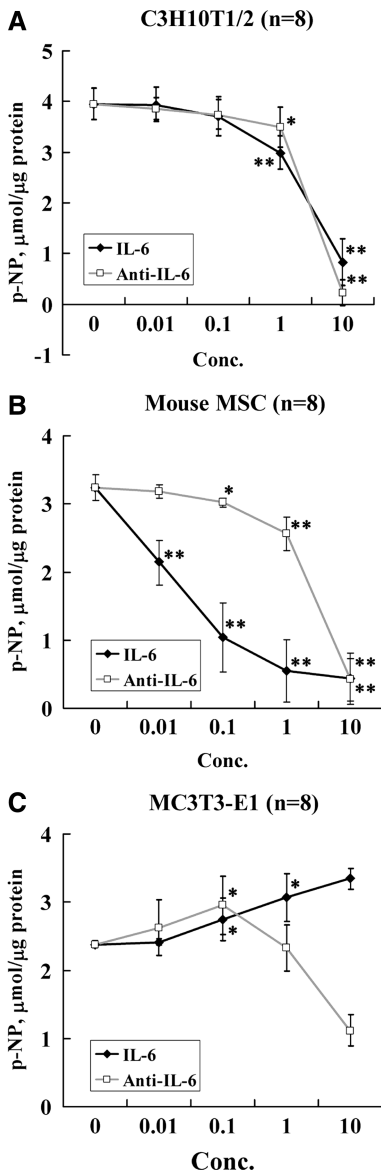
A C3H10T1/2 (n=8)

B Mouse MSC (n=8)

C MC3T3-E1 (n=8)

Fig. 5. Effect of IL-6 (ng/mL) and its blocking antibody (μg/mL) on alkaline phosphatase activity in each cell type. $*P < 0.05$ and $**P < 0.001$.

endochondral ossification because IL-6-deficient mice displayed mild cartilage destruction in response to antigen-induced arthritis [31]. However, new bone formation in distraction osteogenesis occurs mainly via intramembranous ossification [32]; thus, the direct effects of IL-6 on mesenchymal cells and osteoblastic lineage cells should be more important.

Although it is well known that IL-6 is produced by osteoblasts, which also express its receptor [33], the direct effect of IL-6 on osteoblasts is controversial. Fang and Hahn [34] showed that IL-6 treatment stimulated a dose-dependent increase in [³H]TdR incorporation, increased cell numbers, and increased the secretion of prostaglandin E_2 in UMR-106-01 cells (a rat osteoblastic osteosarcoma cell line) but decreased protein synthesis. On the other hand, Littlewood et al. [35] showed that recombinant human IL-6 and sheep anti-human IL-6 antibody had no proliferative or differentiating effects on ROS 17/2.8 cells (osteoblast-like cells). Kim et al. [36] showed that IL-6 treatment did not affect proliferation or mRNA expression of osteoblastic markers in human MSCs.

In the present study, we tested IL-6 and its blocking antibody in three different cell types and found that it did not have a proliferative effect on the three cell types tested, whereas anti-IL-6 blocking antibody increased cell numbers in all three types: C3H10T1/2 cells, mouse MSCs, and MC3T3-E1 cells. Interestingly, this effect was more obvious at lower concentrations in the primitive cell line (C3H10T1/2) than in the mature osteoblast cell line (MC3T3-E1). These findings suggest that endogenous IL-6 may negatively control the proliferation of primitive mesenchymal cells in response to distraction strain. Moreover, the effect of endogenous IL-6 appears to have reached a plateau as additional exogenous IL-6 treatment did not have any additive effect. We investigated the effect of IL-6 on osteoblastic differentiation by observing both the mRNA expression of marker genes and alkaline phosphatase activity. mRNA expression of osteoblastic marker genes was unaffected by either the addition of exogenous IL-6 or blocking endogenous IL-6. However, it was interesting to note that alkaline phosphatase activity changed in response to these stimulations. Specifically, it was reduced by anti-IL-6 blocking antibody in all three cell types tested, especially at higher concentrations. In more primitive cells, such as C3H10T1/2 and mouse MSCs, IL-6 reduced alkaline phosphatase activity in a dose-dependent manner. It is difficult to interpret these similar responses to ligand and its blocking antibody in these primitive cells, but it probably reflects the complexity of the IL-6 response. The mature osteoblast-like cell line MC3T3-E1 increased alkaline phosphatase activity on increasing IL-6 concentration. These findings suggest that IL-6 works differently on primitive mesenchymal cells and mature osteoblasts, and it is believed likely that IL-6 plays a positive role in the differentiation of mature osteoblastic lineage cells.

Limitations of this experiment are that production of endogenous IL-6 was not assayed and the mechanism of anti-IL-6 blocking antibody was not clear. It was reported that MC3T3-E1 cells and human bone marrow stromal cells produced IL-6 protein in the basal level condition [37, 38], and reverse-transcription polymerase chain reaction showed that all three cell types or cell lines used in this study expressed IL-6 mRNA (data not shown). However, there still exists a possibility that anti-IL-6 blocking antibody binds to molecules other than IL-6 and resulted in the changes observed.

In summary, this study shows that the proinflammatory cytokine IL-6 is upregulated in response to the

strain associated with distraction and appears to be one of the cytokines or growth factors involved in the complex network and signaling cascade evoked during distraction osteogenesis. Moreover, it may act differentially on immature and mature osteoblastic lineage cells during the process of new bone formation. The proliferation of primitive mesenchymal cells may be negatively controlled by IL-6, whereas the differentiation of mature osteoblastic lineage cells seems to be enhanced by this cytokine.

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