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Expression of interleukin-17B in mouse embryonic limb buds and regulation by BMP-7 and $bFGF^{abla}$

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Abstract

Interleukin-17B (IL-17B) is a member of interleukin-17 family that displays a variety of proinflammatory and immune modulatory activities. In this study, we found that IL-17B mRNA was maximally expressed in the limb buds of 14.5 days post coitus (dpc) mouse embryo and declined to low level at 19.5 dpc. By immunohistochemical staining, the strongest IL-17B signals were observed in the cells of the bone collar in the primary ossification center. The chondrocytes in the resting and proliferative zones were stained moderately, while little staining was seen in the hypertrophic zone. Furthermore, in both C3H10T1/2 and MC3T3-E1 cells, the IL-17B mRNA was up-regulated by recombinant human bone morphogenetic protein-7, but down-regulated by basic fibroblast growth factor via the extracellular signal-regulated kinase pathway. This study provides the first evidence that IL-17B is expressed in the mouse embryonic limb buds and may play a role in chondrogenesis and osteogenesis. © 2004 Elsevier Inc. All rights reserved.

Keywords: Interleukin-17; Bone morphogenetic protein; Fibroblast growth factor; Limb buds; Bone development

Interleukin-17 (IL-17) is a family of cytokines including six homologous molecules, named IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F [1–6]. The prototype member IL-17A (or IL-17) was originally named CTLA-8 after being cloned from activated T cells, which shares 57% homology to the protein of the open reading frame 13 gene of the T lymphotropic herpesvirus saimiri [7]. The other five members of this family share 20–50% homology to IL-17A [1]. The receptor for IL-17A is IL-17R, which is extensively expressed in various tissues or cells tested, in contrast to the exclusive expression of IL-17A in activated T cells [8,9]. There are four additional receptor-like molecules that share homology to IL-17R, i.e., IL-17Rh1 (also named IL-17BR), IL-17RL (also named IL-17RC), IL-17RD (also named similar expression to fgf genes, Sef), and IL-17RE [1]. IL-17Rh1 was shown to bind to IL-17B but with higher affinity to IL-17E [10-12]. IL-17RD (or Sef) has been found to bind fibroblast growth factor (FGF) receptors and inhibit the FGF receptor-mediated extracellular signal-regulated kinase (ERK) pathway [13–20]. Neither the receptors for IL-17C, IL-17D, and IL-17F cytokines have been identified, nor the cytokine ligands for the receptor-like molecules IL-17RL, IL-17RD, and IL-17RE. Nevertheless, the IL-17 cytokines have been reported to play proinflammatory and immune modulatory functions [1-6].

 $^{^{\}star}$ *Abbreviations:* IL-17, interleukin-17; BMP-7, bone morphogenetic protein-7; bFGF, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase; Sef, similar expression to fgf genes; PTHrP, parathyroid hormone-related protein.

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IL-17B was reported to share about 21% homology to IL-17A at the amino acid level, with a predicted molecular mass for the mature monomer of about 20 kDa [10,11]. In adult human tissues, variable levels of IL-17B mRNA were detected by Northern blot in pancreas, small intestine, stomach, testis, spinal cord, prostate, colon mucosal lining, ovary, trachea, uterus, adrenal gland, and substantia nigra, while no signal was detected in T cells even by reverse transcriptionpolymerase chain reaction (RT-PCR) amplification [10,11]. Murine IL-17B mRNA, about 88% homologous to human IL-17B, was detected in mouse brain, heart, testis, and less in lung, liver, and skeletal muscle [11]. Recently, murine IL-17B mRNA was detected in the whole mouse embryo: first a weak signal at stage day 11, then a peak at stage day 15, followed by a decreased level at stage day 17 [21]. By in situ hybridization or immunohistochemistry, IL-17B mRNA or protein was detected in the neurons of mouse or human brains and spinal cords [21]. So far, the only in vitro biological activity reported on recombinant human IL-17B was the induction of TNF α , IL-1 β , IL-6, IFN- γ , and granulocyte colony-stimulating factor in the human leukemia monocytic THP-1 cells [10], but not in human foreskin fibroblasts [10], HeLa, CHO, or 293T cells [11]. The only in vivo biological activity reported was the stimulation of polymorphonuclear lymphocyte infiltration into the peritoneal cavity after intraperitoneal injection of recombinant human IL-17B, probably through an indirect unknown mechanism [11]. The link of IL-17B to Charcot-Marie-Tooth disease was not established [21].

Previously, we reported that we identified IL-17B from the articular cartilage of 2- to 3-month-old calf joints [1]. However, we could not detect IL-17B in adult cow cartilage (unpublished observation). This led us to speculate that IL-17B is predominantly expressed during the early stages of cartilage and bone development. Therefore, we conducted the present study on mouse embryos and neonatal mice.

Materials and methods

Mouse embryos and neonatal mice. This study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis, according to National Institutes of Health guidelines. Timed pregnant CD-1 mice were obtained from the Charles River Laboratories (Wilmington, MA) and kept at the animal facilities of University of California, Davis. The pregnant mice from 9.5 to 17.5 dpc and neonatal mice born at 19.5 dpc were euthanized by CO_2 asphyxiation. Embryos and neonatal mice were dissected, and their forelimbs and hindlimbs were collected into RLT lysis buffer (Qiagen, Valencia, CA) for RNA isolation or RIPA lysis buffer for protein isolation. Frozen sections of 15.5 and 17.5 dpc mouse embryos were also obtained from the University of Michigan Center for Organo-genesis (Ann Arbor, MI) for immunohistochemistry.

RNA isolation and real-time RT-PCR. Total RNA was extracted from Polytron homogenized limbs using RNeasy Mini Kit (Qiagen) with on-membrane DNase I digestion to avoid genomic DNA contamination. cDNA was made from 5 µg of total RNA using Superscript First-Strand Synthesis System with oligo(dT) primers (Invitrogen, Carlsbad, CA). Mouse IL-17B and glyceraldehyde-3phosphate dehydrogenase (GAPDH) primers were from Applied Biosystems. Real-time quantitative PCR was done in triplicate on the cDNA with an ABI 7700 Sequence Detector and Sybr-Green reagents (Applied Biosystems) following the recommended protocols. Results were normalized to GAPDH levels using the formula ΔC_t (cycle threshold) = C_t of IL-17B – C_t of GAPDH. Since 10.5 dpc embryonic limbs expressed the lowest level of IL-17B, $\Delta\Delta C_t$ was calculated using the formula $\Delta\Delta C_t = \Delta C_t$ of x dpc – ΔC_t of 10.5 dpc. The data were presented as fold change of IL-17B mRNA compared to 10.5 dpc embryonic limbs, where fold = $2^{\Delta\Delta C_t}$. IL-17B mRNA expression of the cell lines was detected similarly.

Protein isolation, immunoprecipitation, and Western blot. Protein was extracted from Polytron homogenized limbs in RIPA lysis buffer [50 mM sodium fluoride, 0.5% Igepal CA-630 (NP-40), 10 mM sodium phosphate, 150 mM sodium chloride, 25 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1.2 mM sodium vanadate] supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) [22]. The protein concentrations were measured using the Protein Assay kit (Bio-Rad). One microgram of goat anti-IL-17B antibodies (H-16, Santa Cruz Biotechnology, Santa Cruz, CA) was added to 150 mg protein in 1 ml RIPA buffer and incubated for 1 h at 4 °C, followed by adding 10 µl of protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences) and incubation for overnight at 4 °C. RIPA lysis buffer only and recombinant human IL-17B (rhIL-17B, R&D Systems) were included as negative and positive controls, respectively. After two washes with RIPA lysis buffer and two washes with PBS, the beads-bound antibodies and IL-17B were eluted with protein loading buffer by boiling 5 min, subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane by electroblotting (Bio-Rad). The membranes were blocked with 5% nonfat dried milk in TBST (25 mM Tris-HCl, 125 mM NaCl, and 0.1% Tween 20) for 2 h and probed with goat anti-IL-17B antibodies overnight and then horseradish peroxidaseconjugated secondary antibodies for 1 h. The results were visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

Immunohistochemistry. Frozen sections of mouse embryos were fixed in acetone at -20 °C for 10 min, blocked for endogenous peroxidase with 3% H₂O₂ for 30 min and then normal blocking serum for 30 min. The sections were incubated with goat anti-IL-17B antibodies (1:100) overnight at 4 °C. For negative control, primary antibodies were replaced with normal serum. The VECTSTAIN elite ABC Reagent (PK-6105) and DAB Substrate Kit (Vector Laboratories, Burlingame, CA) were used to stain the sections according to the manufacturer's protocol. One set of consecutive sections was pretreated with 25 mM EDTA before incubation with antibodies, in order to rule out the nonspecific binding caused by bone minerals. Another set of consecutive sections was stained with 1% toluidine blue for histological examination.

Cell culture. C3H10T1/2 (a mouse embryo mesenchymal cell line), MC3T3-E1 (a mouse preosteoblast cell line, clones 14, 24, and 30), and C2C12 (a mouse myoblast cell line) were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, in a 37 °C, 5% CO₂ humidified incubator. About 0.3 million cells in 3 ml of complete culture medium in 60 mm dishes were treated with or without 1–300 ng/ml recombinant human bone morphogenetic protein-7 (BMP-7, a gift from Dr. T.K. Sampath, Creative BioMolecules, Hopkinton, MA) or basic FGF (bFGF, R&D Systems), or the combination of BMP-7 and bFGF. Where indicated, up to 50 µM of a selective MEK inhibitor PD98059 (Sigma–Aldrich, St. Louis, MO) was added to the cells 30 min prior to the treatment. The cells were harvested for RNA in RLT lysis buffer 3 h to 3 days after treatment.

Results and discussion

IL-17B mRNA was expressed in mouse embryonic limb buds

IL-17 cytokines form a unique novel family, of which the prototype member IL-17A has been studied extensively [1–6]. However, reports on IL-17B were very limited [10–12,21]. Our finding that IL-17B was expressed in calf articular cartilage but not in adult cow cartilage led us to hypothesize that IL-17B is perhaps predominantly expressed in early stages of bone development. To test our hypothesis, we isolated total RNA from mouse embryonic limb buds and limbs of neonatal mice. Real-time quantitative RT-PCR revealed that mouse IL-17B mRNA was barely detectable at 10.5 dpc limb buds (actual C_t of IL-17B was 27.5, while GAPDH was 17.5) and increased slightly at 11.5 dpc (4-fold, compared to 10.5 dpc) (Fig. 1A). At these stages, mesenchymal cells



Fig. 1. IL-17B mRNA and protein were expressed in mouse embryonic limb buds. (A) Real-time quantitative RT-PCR of IL-17B mRNA was done in triplicate with RNA from the limb buds of CD-1 mouse embryos at different stages. Data were presented as fold changes compared to 10.5 dpc. (B) Immunoprecipitation was done with protein lysates from limb buds using anti-IL-17B antibodies and probed for IL-17B. rhIL-17B and lysis buffer alone were used as positive and negative controls.

in the limb buds are proliferating rapidly [23]. IL-17B mRNA increased significantly at 12.5 dpc (64-fold) upon mesenchymal condensation, and dramatically at 13.5 dpc (256-fold) when the condensed mesenchymal cells differentiated into chondrocytes. Interestingly, IL-17B mRNA reached the peak level (588-fold) at 14.5 dpc when the primary ossification centers first appeared in the long bones of mouse embryonic limbs, and attenuated to lower levels (158- to 104-fold) with the further growth of limbs from 15.5 to 17.5 dpc. Furthermore, IL-17B mRNA returned to a very low level (7-fold) upon birth at 19.5 dpc. Our data on IL-17B mRNA expression in the mouse embryonic limb buds seemed consistent with the dynamics found in the whole embryo study [21]. In addition, a low level of IL-17B mRNA was detected in the body of 9.5 dpc embryos, while variable levels of IL-17B mRNA were detected in the skulls, brains, bodies, and skins of embryos at different stages (data not shown).

IL-17B protein was expressed in mouse embryonic limb buds

To determine if IL-17B protein was expressed in the mouse embryonic limb buds, we initially tried to detect IL-17B from the protein lysates by Western blot. However, we failed to see any signal even when 150 µg of total protein lysates was loaded, while our positive control rhIL-17B was readily detectable (data not shown). Nevertheless, the Western blot confirmed that the goat anti-IL-17B antibodies were very specific, as no nonspecific signal showed up. We then pooled the limb buds of each stage and used 150 mg protein lysates for immunoprecipitation assay. As shown in Fig. 1B, mouse IL-17B protein was detected as approximately 22 kDa bands from 14.5 to 17.5 dpc limbs, while a faint band was visible at 13.5 dpc. The protein size of mouse IL-17B was larger than that predicted from the amino acid sequence, which might be due to glycosylation or other modifications, while the rhIL-17B was produced from bacteria, therefore its size was as predicted. IL-17B protein expression also had a peak at 14.5 dpc, which corresponded to the peak of IL-17B mRNA at this stage. However, we failed to detect any signal of IL-17B protein from 10.5, 11.5, 12.5, and 19.5 dpc limb buds or limbs (data not shown), possibly due to their lower levels of protein expression as shown by their lower levels of mRNA expression.

Localization of IL-17B protein in mouse embryonic limb buds

As mouse embryonic limb buds contain various tissues especially at older stages, it was important to know which cells expressed IL-17B. Representatives of immunohistochemistry are shown in Fig. 2 (15.5 dpc: a–d;



Fig. 2. Localization of IL-17B in mouse embryo. Consecutive sections of 15.5 dpc (A–D) and 17.5 dpc (E–H) were stained with toluidine blue for histological examination (A,E,G) and with anti-IL-17B antibodies for IL-17B (B,C,D,F,H). Note the strong staining of the bone collar cells (solid arrow in B,C,D,F) and moderate staining of chondrocytes (empty arrow in C,F,H). Magnification: $25 \times$ for (A,B,E,G); $100 \times$ for (C,F,H); $200 \times$ for (D).

17.5 dpc: e–h). The chondrocytes were stained blue or dark blue by toluidine blue, while the hypertrophic chondrocytes were not stained (Figs. 2A, E, and G). Under low power field (Fig. 2B), strong IL-17B signals were seen in the cells of the bone collar in the primary ossification center, typically in the mid-shaft region of ulna. The identity of these cells was not characterized, but they were probably osteoblasts [24]. The chondrocytes in the resting and proliferative zones were stained moderately, while little staining was seen in the hypertrophic

zone. Under higher magnification fields (Figs. 2C and D), the staining pattern was more obvious. Moderate IL-17B signals were also observed on the muscle cells and keratinocytes, but not fibroblasts in the subcutaneous or interstitial regions (Figs. 2B–D). The chondrocytes of ribs (Fig. 2F) and tarsals (Fig. 2H) were also stained moderately, as well as the lung (Fig. 2F). Similar IL-17B signals were detected when the sections were pre-treated with 25 mM EDTA before incubation with antibodies, which ruled out the possibility that the

signals were nonspecific bindings caused by bone minerals (data not shown). In addition, we also observed IL-17B signals in the membranous primordia of skull bones, brain, and primordia of follicles of vibrissae (data not shown).

IL-17B was expressed in C3H10T1/2 and MC3T3-E1 cells

As we demonstrated that IL-17B mRNA and protein were expressed in the mouse embryonic limb buds, especially in the cells of the bone collar in the primary ossification center, we were very interested to know if IL-17B was expressed in the osteogenic cell lines. C3H10T1/2 (a mouse embryo mesenchymal cell line), MC3T3-E1 (a mouse preosteoblast cell line), and C2C12 (a mouse myoblast cell line) have the potential to differentiate into chondrocytes or osteoblasts under different culture conditions [25–28]. So we first examined if these cells expressed IL-17B or not. By real-time quantitative RT-PCR, we found both C3H10T1/2 and MC3T3-E1 cells (clones 14, 24, and 30) expressed a low level of IL-17B mRNA, but C2C12 cells did not express any detectable IL-17B mRNA (Fig. 3A). It was possibly because C2C12 cells were more committed to myoblastic differentiation, although they retained some potential for osteoblastic differentiation [28]. This was in line with our suspicion that IL-17B was more involved in bone development.

IL-17B expression was up-regulated by BMP-7 but down-regulated by bFGF

It is well known that the early bone development is controlled by many morphogens and growth factors, such as BMPs, FGFs, Wnts, hedgehog proteins, and parathyroid hormone-related protein (PTHrP) [24]. So we tested if IL-17B expression was regulated by these morphogens and growth factors. By real-time quantitative RT-PCR, we found that IL-17B mRNA expression in C3H10T1/2 cells was up-regulated by BMP-7 in a time- and dose-dependent manner (Figs. 3B and C). There was a 40- to 64-fold increase of IL-17B mRNA upon 20–300 ng/ml BMP-7 treatment for 48 h, while no significant increase was detected when the cells were treated for 3–10 h or with BMP-7 at a dosage of 10 ng/ml or less.

In contrast, 20 ng/ml of bFGF down-regulated IL-17B mRNA expression in C3H10T1/2 cells in a time-dependent manner, starting as early as 3 h and completely blocking IL-17B mRNA expression by 72 h (Fig. 4A). Moreover, bFGF inhibited IL-17B mRNA expression in a dose-dependent manner (Fig. 4B). It seemed that bFGF was a very effective inhibitor, as 1 ng/ml bFGF inhibited about 95% of IL-17B mRNA expression by 48 h, and higher dosage essentially blocked the expres-



Fig. 3. Expression of IL-17B in osteogenic cell lines and its upregulation by BMP-7. Real-time quantitative RT-PCR of IL-17B mRNA was done in triplicate with RNA from the cells untreated (A), C3H10T1/2 cells treated with 20 ng/ml BMP-7 for up to 48 h (B), or C3H10T1/2 cells treated with 1–300 ng/ml BMP-7 for 48 h (C). Data were presented as $\Delta\Delta C_t$ compared to C2C12 cells (A) or fold changes compared to control (B,C).

sion of IL-17B. Interestingly, when C3H10T1/2 cells were treated simultaneously with 100 ng/ml BMP-7 and increasing dosage of bFGF (1–10 ng/ml), BMP-7 antagonized bFGF's inhibition of IL-17B mRNA expression, causing a net increase of 28% at 1 ng/ml bFGF. However, 5–10 ng/ml of bFGF overcame the stimulatory effect of 100 ng/ml of BMP-7, resulting in a net decrease of 55–90% of IL-17B mRNA expression (Fig. 4C). Although our finding that IL-17B mRNA



Fig. 4. Down-regulation of IL-17B mRNA expression in C3H10T1/2 cells by bFGF. Real-time quantitative RT-PCR of IL-17B mRNA was done in triplicate with RNA from the cells treated with 20 ng/ml of bFGF for up to 72 h (A), treated with 1–100 ng/ml bFGF for 48 h (B), or treated by 1–5 ng/ml bFGF with or without 100 ng/ml BMP-7 (C). Data were presented as fold changes compared to control.

expression was regulated by BMP-7 and bFGF in an antagonistic manner was unexpected, it was not surprising as BMPs and FGFs antagonize each other in the regulation of many genes, such as ectodin and Dach1 [26,29].

MC3T3-E1 is a mouse preosteoblast cell line, several clones of which have been characterized with variable potentials to differentiate into osteoblasts [30]. Like in C3H10T1/2 cells, we found that BMP-7 up-regulated



Fig. 5. Regulation of IL-17B mRNA expression in MC3T3-E1 cells by BMP-7 and bFGF, and in C3H10T1/2 cells by PD98059. Real-time quantitative RT-PCR of IL-17B mRNA was done in triplicate with RNA from the MC3T3-E1 cells (clones 14, 24, and 30) treated with 100 ng/ml BMP-7 or 5 ng/ml bFGF for 48 h (A), and the C3H10T1/2 cells treated with 25–50 μ M PD98059 with or without 1 ng/ml bFGF for 48 h (B). Data were presented as fold changes compared to control.

and bFGF down-regulated IL-17B mRNA expression in several clones of MC3T3-E1 cells, namely, clone 14, 24, and 30 (Fig. 5A), although the magnitude of regulation was less dramatic compared to C3H10T1/2 cells. It was noteworthy that sonic hedgehog or parathyroid hormone did not affect the IL-17B mRNA expression (data not shown).

Down-regulation of IL-17B by bFGF was mediated via the ERK pathway

It is well known that bFGF binds to FGF receptor and activates extracellular signal-regulated kinase (ERK) pathway, which can be inhibited by a selective MEK inhibitor PD98059 [31,32]. Since we found that bFGF was an inhibitor of IL-17B mRNA expression, we speculated that if we inhibited the ERK pathway by PD98059, we would be able to restore IL-17B mRNA expression. Indeed, we found pre-treatment of C3H10T1/2 cells with 25–50 μ M of PD98059 restored the IL-17B mRNA expression that was reduced by 1 ng/ml bFGF, resulting in a net increase of 50–200% compared to the control cells (Fig. 5B). Furthermore, 25–50 μ M of PD98059 alone induced 3- to 7-fold increase of IL-17B mRNA expression by inhibition of the basal level activity of the ERK pathway (Fig. 5B). Our data indicated that inhibition of IL-17B expression by bFGF was mediated through the ERK pathway.

In conclusion, the present study provides the first evidence that IL-17B is expressed in the mouse embryonic limb buds. The dynamic expression pattern of IL-17B is correlated to the process of chondrogenesis and osteogenesis, which implies the role of IL-17B in the early bone development. The strongest IL-17B expression in the bone collar cells and in the two osteogenic cell lines (C3H10T1/2 and MC3T3-E1) suggests that IL-17B might play a role in the differentiation of mesenchymal cells or preosteoblasts into osteoblasts, which is further underscored by our findings that the IL-17B mRNA is up-regulated by BMP-7, but down-regulated by bFGF via the ERK pathway in the two osteogenic cell lines. It is not clear whether IL-17B is a mediator of BMP-7 or bFGF signaling and whether IL-17B has any direct effect on the cells studied. The actions of IL-17B in vitro and in transgenic/knockout mice model in vivo are being studied. In addition, our findings that IL-17B is also expressed in other non-skeletal embryonic tissues suggest that IL-17B might have certain function in the general process of organogenesis. To the best of our knowledge, this is the first time that a cytokine from a generally proinflammatory IL-17 family has been implicated in organogenesis [1-6].

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