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Expression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 (CTGF/Hcs24/CCN2) during distraction osteogenesis

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Abstract To investigate the localization and expression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24/CCN family member 2 (CTGF/Hcs24/CCN2) during distraction osteogenesis in the rat femur, we studied a total of 54 male rats (11 weeks old). We performed osteotomy in the midshaft of the right femur. After 7 days (lag phase), distraction was started, at the rate of 0.25 mm/12 h for 21 days (distraction phase) by using a small external fixator, and this was followed by a 7-day consolidation phase. Localization and expression of CTGF/Hcs24 during distraction osteogenesis in the femur were examined by immunostaining, in situ hybridization, and reverse transcriptase polymerase chain reaction (RT-PCR). Immunostaining showed the localization of CTGF/Hcs24 in various cells located in the bone-forming area around the osteotomy site. During the distraction phase, in situ hybridization showed that CTGF/Hcs24 mRNA was expressed not only in hypertrophic chondrocytes and osteoblasts but also in fibroblast-like cells and mesenchymal cells at sites of endochondral ossification, and not only in osteoblasts but also in pre-osteoblasts and fibroblast-like cells at sites of intramembranous ossification. RT-PCR showed higher level expression of CTGF/Hcs24 mRNA in the distracted group than in the nondistracted group. These results revealed an elevated pattern of CTGF/Hcs24 mRNA expression during distraction osteogenesis, and suggest that CTGF/Hcs24 may play some roles in the endochondral and intramembranous ossification processes that occur during distraction osteogenesis.

Key words distraction osteogenesis · CTGF/Hcs24/CCN2 · immunostaining · in situ hybridization · RT-PCR

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Introduction

Connective tissue growth factor (CTGF) is a cysteine-rich secretory protein of 36–38 kDa and belongs to the CCN family, which consists of six distinct members (CTGF/Fisp12, Cyr61/Cef10, Nov, rCOP-1/WISP-2, ELM-1/WISP-2, and WISP-3) [1–3]. Previously, we cloned cDNA of the hypertrophic chondrocyte specific gene no. 24 (*hcs24*) from a human chondrosarcoma-derived chondrocytic cell line, HCS-2/8 [4–7] and found it to be identical to that of CTGF. Although the nomenclature "CCN1–6" for CCN genes and proteins was proposed recently [8], it is not widely used yet [3,9]; so we have used "CTGF/Hcs24/CCN2" only in the title of this article, and "CTGF/Hcs24" in the text. The expression of CTGF/Hcs24 mRNA was highest in hypertrophic chondrocytes [10], but it has also been detected in osteoblasts [11], fibroblasts [12], and vascular endothelial cells [13,14]. We also reported that recombinant CTGF/Hcs24 (rCTGF/Hcs24) promoted the proliferation and differentiation of chondrocytes [15,16] and osteoblasts in culture [17] and angiogenesis both in vivo and in vitro [18] and that CTGF/Hcs24-specific receptors were present on chondrocytic cells [19] and osteoblasts [17]. Recently, we also reported the function of CTGF in bone and cartilage tissues in vivo [20]. CTGF/Hcs24 was shown to be upregulated during fracture healing in a mouse model [21], suggesting that CTGF/Hcs24 may play some roles in endochondral and intramembranous ossification in vivo.

Distraction osteogenesis is the process of generation of viable bone by the gradual separating of osteotomized bone edges, and it has been believed that gradual distraction does not prevent, but actually promotes osteogenesis and the growth of surrounding soft tissues [22,23]. Over the past few years, it has been shown by several in vitro studies that both the proliferation and the differentiation of chondrocytes and osteo-

blasts are increased in response to mechanical stress [24,25], and it has also been shown by several in vivo studies that the expression of some kinds of growth factors is increased during the process of distraction osteogenesis [26–29]. These results suggest that applied mechanical stress during this process may stimulate the proliferation and differentiation of cells participating in newly formed callus and thus promote ossification. However, the exact mechanism by which the mechanical stress stimulates ossification during distraction osteogenesis is unknown.

In the distraction osteogenesis model, cells at various stages of differentiation are localized in order along the tension vector. Histologically, at least two modes of ossification can be observed during distraction osteogenesis: intramembranous and endochondral ossification [27–32]. Thus, we can examine the cellular and molecular events occurring in several cell types at various stages of differentiation during these modes of ossification. Although the fracture-healing model is useful for observation of the process of ossification, as we reported before [21], it is somewhat difficult to evaluate the cellular and molecular events exactly in this model, because the cells at various stages in their differentiation are located at random during this process. Therefore, we consider the distraction osteogenesis model to have an advantage over the fracture-healing model.

Although it is known that CTGF is expressed in bone [33] and cartilage tissues [10,20,21,34], little is known about the possible regulation of CTGF in the process of distraction osteogenesis. In this study, we investigated the localization and the expression of CTGF/Hcs24 and CTGF/Hcs24 mRNA by immunostaining, in situ hybridization, and reverse transcriptase-polymerase chain reaction (RT-PCR), and we discuss their roles in the process of distraction osteogenesis in this rat model.

Materials and methods

Experimental model for distraction osteogenesis

A total of 54 male Wistar rats (11 weeks old) were studied in this series of experiments. A monolateral external fixator (Hoffman Mini Lengthening System; Stryker, Plan-les-Ouates, Switzerland) was fixed surgically on the lateral aspect of right femur with four 1.5-mm half-pins (Fig. 1) [28,29]. The rats were anesthetized by intramuscular administration of ketamine. A longitudinal skin incision was made on the lateral aspect of the right femur, and the fascia was cut longitudinally, and after the muscles had been separated, the right femur was exposed. Four half-pins were inserted into the axis of the femoral shaft. The four half-pins were clamped to the external fixator and then a transverse

Fig. 1. A rat with a monolateral external fixator on the right femur

osteotomy was made gently between the second and third half-pins. After this procedure, the muscles were returned to their normal position and the skin incision was sutured. After the operation, the rats were maintained in cages with free access to food and water. For the distracted group, there was a lag phase of 7 days after osteotomy; a distraction phase of 21 days, during which gradual distraction was performed at a rate of 0.25 mm every 12 h; and a consolidation phase, during which the external fixator remained in place with no distraction. For the nondistracted group, distraction was not performed after osteotomy. The animals were killed at various stages for histologic examination and extraction of RNA. The experimental procedures were undertaken in compliance with the guiding principles for the care and use of laboratory animals described in *The American Journal of Physiology*.

Tissue preparation

To prepare samples for safranin O fast-green staining, immunostaining, and in situ hybridization, we killed a total of 20 rats at various stages of lengthening (Table 1). The arteries were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and the distracted femurs were dissected with the surrounding soft tissues and fixed overnight in the same solution with the external fixator still in place. The samples were then dehydrated with a series of ethanol, decalcified with 20% ethylene-diamine tetraacetate (EDTA), and then embedded in paraffin according to the standard procedure. Longitudinal sections of 6.0-μm thickness were cut on a microtome and mounted on slide glasses. They were stored at 4°C until used for staining with fast-green, immunostaining, and in situ hybridization.

Immunostaining

The paraffin sections were soaked in xylene to remove the paraffin and rehydrated in a graded series of alcohol (100% to 50%). After deparaffinization, the sections

Table 1. Time course and number of rats used in this study

	Days after osteotomy				
	0	7	18	28	35
	Lag phase		Distraction phase		Consolidation phase
	Osteotomy	Beginning of distraction		End of distraction	
			Distracted group	(*n*)	(*n*)	(*n*)
			Histologic examination	5	5	5
			RNA extraction	5	5	5
Histologic examination		$n = 5$				
RNA extraction	$n = 6$	$n = 5$	Nondistracted group	(*n*)	(*n*)	
			RNA extraction	4	4	

were rinsed in tap water for 2 min. After trypsin treatment, endogenous peroxidase activity was quenched by immersion in 10 mmol/l phosphate-buffered saline (PBS; pH 7.6) with 0.3% hydrogen peroxide for 30 min. After a rinse in 10 mmol/l PBS (pH 7.6) for 5 min, the sections were incubated for 30 min with diluted normal blocking serum, which was prepared from the species in which the secondary antibody was made.

Anti-CTGF/Hcs24 antibodies were raised in a rabbit by immunization with a synthetic peptide of CTGF/Hcs24, as described previously [21], and used as primary antibodies. The sections were incubated for 1 h with a 1: 150 dilution of primary antibodies at room temperature. After having been rinsed in PBS, the sections were sequentially incubated with diluted biotinylated secondary antibody (goat anti-rabbit IgG) solution for 30 min. The slides were next washed for 5 min in PBS and then incubated with ABC reagent (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) for 30 min. The reaction was visualized by immersing the sections in diaminobenzidine hydrochloride and the substrate, 0.6% hydrogen peroxide, for 1–2 min. The sections were finally counterstained with Mayer's hematoxylin to visualize the nuclei, then rinsed in tap water for 10 min, dehydrated in a graded series of alcohol (70% to absolute methanol) and through xylene, and mounted.

Negative control

The negative control sections were prepared from the same tissue block as that used for immunostaining. Instead of the anti-CTGF/Hcs24 antibodies, nonimmune rabbit IgG was used as the primary antibody.

Probe preparation

For the generation of sense and antisense mouse CTGF (mCTGF) probes, a full-length cDNA of mCTGF (1.1 kb), which was obtained by RT-PCR [10], was subcloned into pGEM-T plasmids (Promega, Madison, WI, USA). Digoxigenin (DIG)-labeled sense and antisense CTGF riboprobes were synthesized with T7 or SP6 polymerase and DIG-11-UTP by using a DIG RNA labeling kit (Roche Molecular Systems, Mannheim, Germany).

In situ hybridization

In situ hybridization was performed at 52°C as described previously [35]. The endogenous alkaline phosphatase activity was blocked by exposure to 70°C for 15 min in the prehybridization mixture containing 1 mM tetramisole-hydrochrolide. After hybridization, the color reaction was performed with antidigoxigenin-alkaline phosphatase-conjugated antibody, nitroblue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolyphosphate (BCP).

RNA Extraction

For the preparation of samples for RT-PCR, a total of 34 rats were killed (Table 1). The lengthened segments, with small pieces of both bone fragments, but without surrounding soft tissues, were dissected from each sample, snap-frozen in liquid nitrogen, and smashed by hammering vigorously. Total RNA was isolated from the smashed samples by using ISOGEN Reagent (Nippon Gene, Tokyo, Japan), following the manufacturer's instructions.

RT-PCR

Total RNA (1 mg) was reverse-transcribed to cDNA by using oligo d (T) with reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan). Then, the cDNAs of CTGF were amplified with LA-Taq DNA polymerase (Takara, Tokyo, Japan), and the cDNAs of G3PDH were also amplified as a control with the same enzyme. The sets of the synthesized primers described below

were used for amplification. The numbers in parentheses are the expected sizes of the PCR products: CTGF/Hcs24, sense 5′-AAATTGCCAAGCCTGTCAAG-3′ and antisense 5′-GTCCCTTACTCCCTGGCTTT-3′ (286 bp); G3PDH, sense 5′-ACCACAGTCCATGCCATCAC-3′ and antisense 5′-TCCACCACCCTGTTGCTGTA-3′ (450 bp). The amplification conditions were as follows: for CTGF cDNA, 94°C (5 min) for 1 cycle, followed by 94°C (1 min), 60°C (1 min), and 72°C (1 min) for 34 cycles, and a final incubation at 72°C for 5 min. For G3PDH, the conditions were the same, except that the cDNA was amplified for 30 cycles. The PCR products were electrophoresed onto a 2% agarose gel containing ethidium bromide and visualized under ultraviolet light. Photographs of the stained gels were taken and analyzed quantitatively with NIH Image (National Institutes of Health (NIH), MD, USA). Under these conditions, the ratio of the RT-PCR product of CTGF to that of G3PDH increased constantly and linearly. Therefore, the results were normalized by the amount of the RT-PCR products of G3PDH.

Statistical analysis

The values for results of RT-PCR were expressed as the means ± SD of four determinations for each PCR product. Statistical comparisons between the distracted and nondistracted groups were performed by using the nonpaired *t*-test with StatView software for Macintosh, version 5.0. (SAS Institute, Cary, NC, USA).

Results

Radiological findings

A monolateral external fixator was fixed surgically on the lateral aspect of the right femur with four 1.5-mm half pins (Fig. 1). Figure 2 shows the radiographs of rat femur lengthened during the process of the distraction osteogenesis. As seen on the radiograph obtained at 7 days after osteotomy, the proximal and distal bone fragments were fixed tightly by the external apparatus, and callus was barely visible around the osteotomized bone edges. At 11 and 21 days after the beginning of distraction, an apparent callus around the osteotomized bone edges became visible on the radiograph. But continuity of proximal and distal callus could not be seen, and there was a central radiolucent zone between them. By 7 days after the end of distraction, both sites of callus had become larger, calcified, and partially fused; and the central radiolucent zone had become smaller.

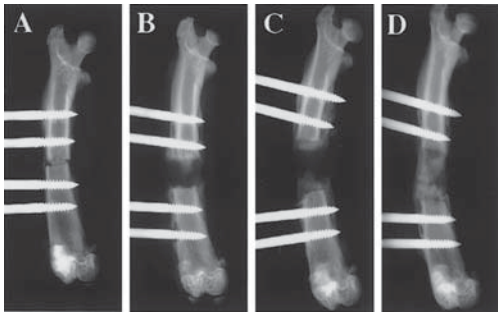

Fig. 2. Radiographs of the lengthened femur at 7 days after osteotomy, just before distraction was started (**A**), after 11 days of distraction (**B**), after 21 days of distraction (**C**), and 7 days after the end of distraction (**D**)

Histologic features of distraction osteogenesis

Areas of cartilage formation within the various calluses were confirmed by safranin O fast-green staining.

Lag phase

At 7 days after osteotomy, the cortex at the osteotomized bone edge was surrounded by an external callus consisting of regenerating cartilage and newly formed trabecular bone (Fig. 3A). Chondrocytes were observed in the regions of regenerating cartilage that were stained light red with safranin O (Fig. 3B,C), and endochondral ossification was observed. Thus, the histologic events observed during this phase basically resembled those in the early stage of fracture healing.

Distraction phase

By 11 days after the beginning of distraction (18 days after osteotomy), the callus had clearly become larger and was observed around the proximal and distal osteotomized bone edges (Fig. 4A). There was a fibrous interzone including newly formed blood vessels at the center of the lengthened area. Bone formation was observed at both sides of the central fibrous interzone. Next to the fibrous region, there was a cartilage area (Fig. 4C). The cartilage had been invaded by capillaries; osteoblastic cells, which had come through the capillaries, were seen around the newly formed bone (Fig. 4B). Thus, endochondral ossification was seen predominantly between the osteotomy sites at the early stage of distraction.

By 21 days after the beginning of distraction (28 days after osteotomy), the newly formed trabecular bone had become larger, and the cartilage area, which had been expansive in the lag and early distraction phases, had clearly become smaller (Fig. 5A). The fibroblast-like cells in the fibrous interzone had been converted into bone-forming cells directly producing new bone. Osteoblasts, pre-osteoblasts, and fibroblast-like cells were located longitudinally in the order of their stage of

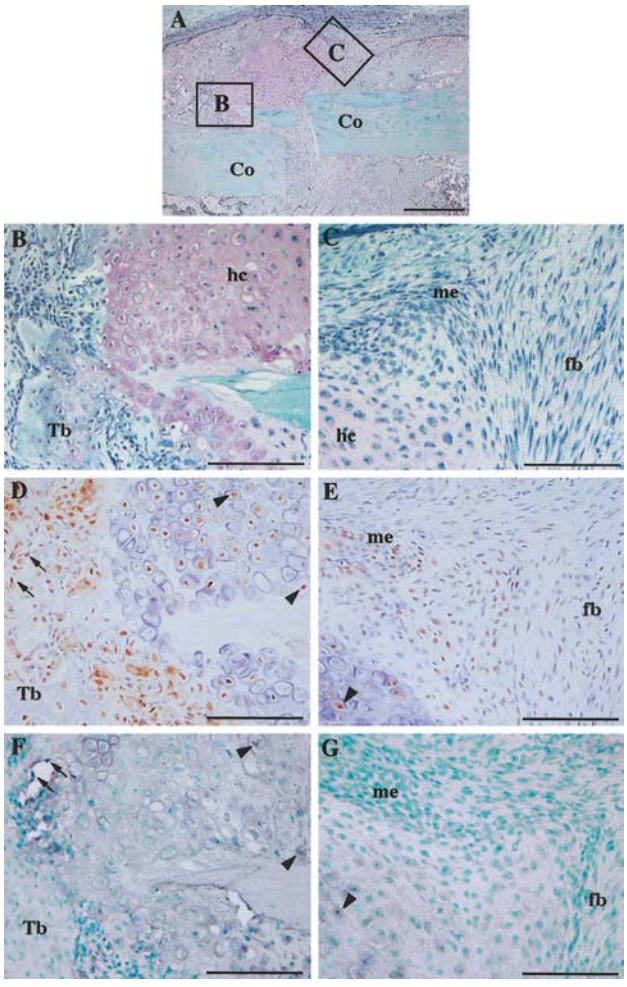

Fig. 3. Histological features and localization of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 (CTGF/Hcs24) protein and mRNA on day 7 after osteotomy. **A** Safranin O fast-green staining. **B**, **C** Safranin O fast-green staining (high-power views of the rectangular areas marked in **A**). **B**, **D**, **F** and **C**, **E**, **G** are sequential sections. **D**, **E** Strong signal of CTGF/Hcs24 protein was detected in hypertrophic chondrocytes and osteoblasts. **F**, **G** Moderate signal of CTGF/Hcs24 mRNA was detected in hypertrophic chondrocytes and osteoblasts. Specific regions are indicated as follows: *Co*, cortical bone; *hc*, zone of hypertrophic chondrocytes; *Tb*, trabecular bone; *me*, condensation of mesenchyme; *fb*, fibroblast-like cells. *Arrowheads* indicate CTGF/Hcs24 protein or mRNA presence in hypertrophic chondrocytes. *Arrows* indicate CTGF/Hcs24 protein or mRNA presence in osteoblasts. *Bars*, 1000 μm in **A** and 250 μm in **B–G**

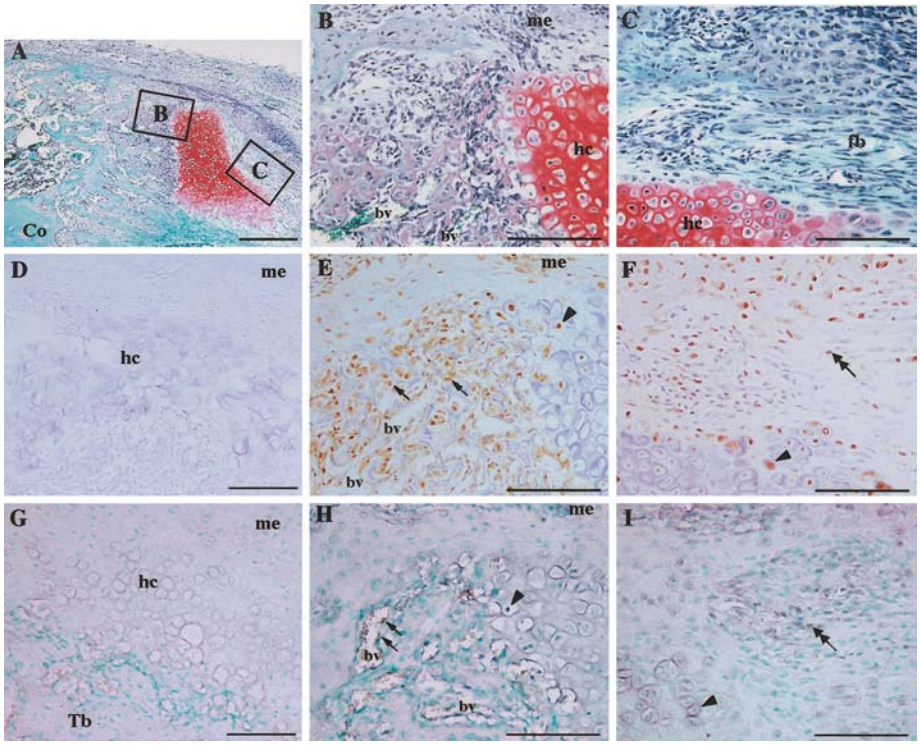

Fig. 4. Histological features and localization of CTGF/Hcs24 protein and mRNA on day 11 after the beginning of distraction (18 days after osteotomy). **A** Safranin O fast-green staining. **B**, **C** Safranin O fast-green staining (high-power views of the *rectangular areas* marked in **A**). **B**, **E**, **H** and **C**, **F**, **I** are sequential sections. **E**, **F** CTGF/Hcs24 protein was detected in hypertrophic chondrocytes, osteoblasts, mesenchymal cells, and fibroblast-like cells. **H**, **I** CTGF/Hcs24 mRNA was detected not only in hypertrophic chondrocytes and osteoblasts but also in mesenchymal cells and fibroblast-like cells. **D** Staining with nonimmune rabbit IgG was negative in all cells. **G** Sense probes detected no signals in any cells. Specific regions are indicated as follows: *Co*, cortical bone; *Tb*, trabecular bone; *hc*, zone of hypertrophic chondrocytes; *me*, condensation of mesenchyme; *bv*, blood vessel; *fb*, fibroblast-like cells; *Arrowheads* indicate CTGF/Hcs24 protein or mRNA presence in hypertrophic chondrocytes. *Double-headed arrows* indicate CTGF/Hcs24 protein or mRNA presence in fibroblast-like cells. *Arrows* indicate CTGF/Hcs24 protein or mRNA presence in osteoblasts. *Bars*, 1000 μm in **A** and 250 μm in **B–I**

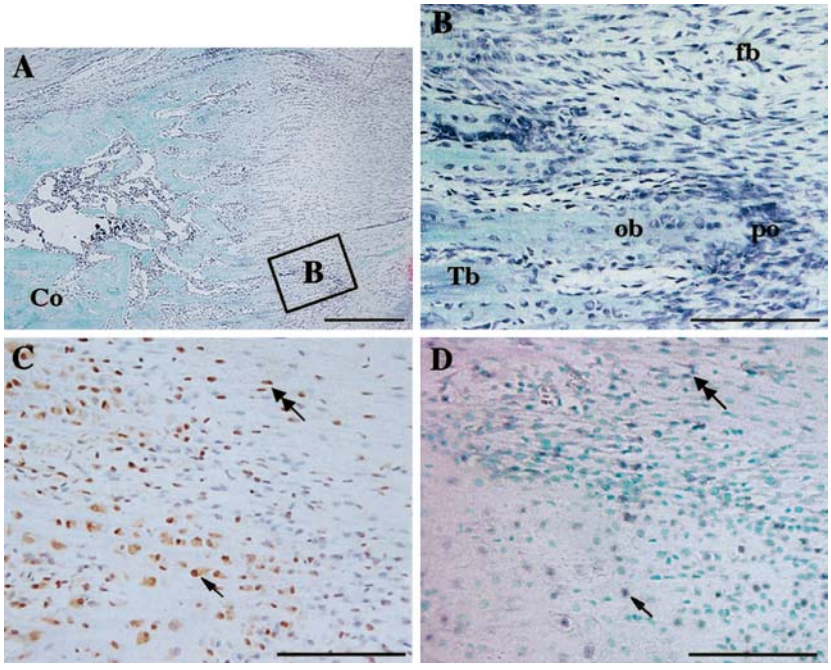

Fig. 5. Histological features and localization of CTGF/Hcs24 protein and mRNA on day 21 after the beginning of distraction (28 days after osteotomy). **A** Safranin O fast-green staining. **B** safranin O fast-green staining high-power view of the *rectangular area* marked in **A**. **B–D** are sequential sections. **C** CTGF/Hcs24 protein was detected in osteoblasts, pre-osteoblasts, and fibroblast-like cells. **D** CTGF/Hcs24 mRNA was detected not only in osteoblasts but also in pre-osteoblasts and fibroblast-like cells. Specific regions are indicated as follows: *Co*, cortical bone; *Tb*, trabecular bone; *fb*, fibroblast-like cells; *po*, pre-osteoblasts; *ob*, osteoblasts. *Double-headed arrows* indicate CTGF/Hcs24 protein or mRNA presence in fibroblast-like cells. *Arrows* indicate CTGF/Hcs24 protein or mRNA presence in osteoblasts. *Bars*, 1000 μm in **A** and 250 μm in **B–D**

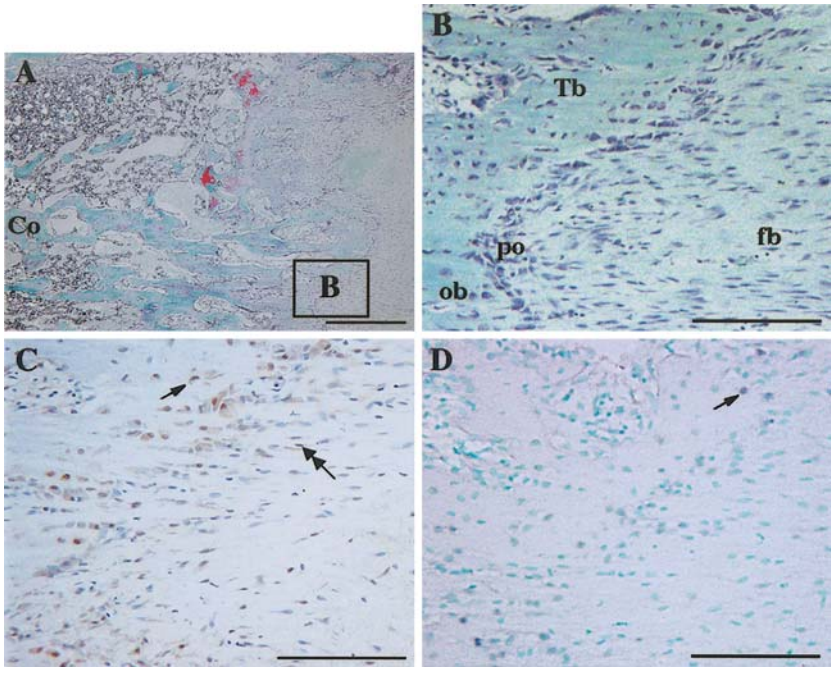

Fig. 6. Histological features and localization of CTGF/Hcs24 protein and mRNA on day 7 after the end of distraction (35 days after osteotomy). **A** Safranin O fast-green staining. **B** safranin O fast-green staining high-power view of the *rectangular area* marked in **A**. **B–D** are sequential sections. **C** Moderate signal of CTGF/Hcs24 protein was detected in osteoblasts. **D** Moderate signal of CTGF/Hcs24 mRNA was detected in osteoblasts, but the signal was weak in fibroblast-like cells and pre-osteoblasts. Specific regions are indicated as follows: *Co*, cortical bone; *Tb*, trabecular bone; *po*, pre-osteoblasts; *ob*, osteoblasts; *fb*, fibroblast-like cells. *Double-headed arrow* indicates fibroblast-like cells. *Arrows* indicate osteoblasts. *Bar*, 1000 μm in **A** and 250 μm in **B–D**

differentiation (Fig. 5B). Thus, the cartilage area became smaller and intramembranous ossification was seen predominantly between the osteotomy sites at the advanced stage of distraction.

Consolidation phase

At 7 days after the end of distraction (35 days after osteotomy), the histologic features in this period resembled those of the remodeling phase of fracture healing. Although the proximal and distal new bone had fused partially in the middle of the fibrous interzone, the fusion was not yet complete, and intramembranous ossification was mainly seen there (Fig. 6A,B).

Immunostaining and in situ hybridization

A quantitative evaluation of the localization of CTGF/Hcs24 protein and mRNA in the lengthened femur is

Table 2. Localization of CTGF/Hcs24 protein and mRNA

	Days after osteotomy			
	7	18	28	35
	← Distraction phase →			
Endochondral ossification				
Mesenchymal cells and fibroblast-like cells	+/±[a]	++/+		
Hypertrophic chondrocytes	++/+	++/+		
Osteoblasts	++/+	++/+		
Intramembranous ossification				
Fibroblast-like cells and pre-osteoblasts			+/+	±/±
Osteoblasts			++/+	+/±

−, no signal (0%); ±, weak signal (0–40% of the cells positive); +, moderate signal (40%–70%); ++, strong signal (70%–100%)
CTGF/Hcs24, connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24
[a]Frequency of positive cells (protein/mRNA)

given in Table 2. The frequency of CTGF/Hcs24-positive cells was classified into four groups, i.e., −, no signal (0%); ±, weak signal (0–40%); +, moderate signal (40%–70%); and ++, strong signal (70%–100%), based on the observation of 20 rats.

Lag phase

At 7 days after osteotomy, positive staining for CTGF/Hcs24 was observed in the cells participating in bone-forming areas. At the sites of endochondral ossification, chondrocytes and osteoblasts showed strong positive immunostaining (Fig. 3D,E), and moderate signals by in situ hybridization (Fig. 3F,G).

Distraction phase

At 11 days after the beginning of distraction, at sites of endochondral ossification, mesenchymal cells, fibroblast-like cells, hypertrophic chondrocytes, and osteoblasts around capillaries invading cartilage area showed strong positive immunostaining (Fig. 4E,F). On the other hand, moderate signals were detected not only in hypertrophic chondrocytes and osteoblasts but also in mesenchymal cells and fibroblast-like cells by in situ hybridization in the same area (Fig. 4H,I). Interestingly, the expression of CTGF/Hcs24 mRNA in fibroblast-like cells and mesenchymal cells in endochondral ossification was clearly enhanced after the beginning of distraction. As for the negative control in which nonimmune rabbit IgG was used as the primary antibody, no signal was detected in the cells in the bone-formation area (Fig. 4D). Sense probes detected no signal in any of the cells in the area of bone formation (Fig. 4G).

At 21 days after the beginning of distraction, in the areas of intramembranous ossification, fibroblast-like cells and pre-osteoblasts showed moderate positive staining, and osteoblasts being embedded in newly formed trabecular bone showed strong positive immunostaining (Fig. 5C). On the other hand, moderate signals were detected not only in osteoblasts but also in fibroblast-like cells and pre-osteoblasts, by in situ hybridization, in the same area (Fig. 5D).

Consolidation phase

At 7 days after the end of distraction, although CTGF/Hcs24 continued to be localized in areas of bone formation in spite of the end of distraction, the ratio of CTGF/Hcs24-positive cells decreased compared with that for the distraction phase. At sites of intramembranous ossification, osteoblasts showed moderate positive immunostaining, and pre-osteoblasts and fibroblast-like cells showed weak positive immunostaining (Fig. 6C), and a weak signal was detected in osteoblasts by in situ hybridization in the same region (Fig. 6D). Interestingly, the expression of CTGF/Hcs24 mRNA in pre-osteoblasts and fibroblast-like cells in intramembranous ossification was clearly reduced after the end of distraction.

Similar results were obtained for all five rats in each distracted group. The frequency of CTGF/Hcs24-positive cells during distraction osteogenesis is summarized in Table 2.

Detection of CTGF mRNA by RT-PCR

The expression level of CTGF mRNA in the lengthened femur was disclosed by RT-PCR with synthetic primers specific for CTGF. The sizes of the PCR products were consistent with those expected from their primer design (Fig. 7A). Expression of CTGF mRNA was increased during the lag phase. In the nondistracted group, after the expression of CTGF mRNA showed a peak level at 7 days after osteotomy, it declined gradually to a lower level. However, in the distracted group, the expression further increased slightly after the beginning of distraction and was maintained at this level during the distraction phase. Then, it decreased clearly after the end of distraction (Fig. 7B). The maintenance of this elevated expression in the distracted group presented a striking contrast to the decrease in that in the nondistracted group.

Discussion

We made a rat model of femur lengthening to investigate the localization and expression of CTGF/Hcs24 during distraction osteogenesis. We demonstrated that the lengthening of the rat femur displayed histologic and radiographic findings consistent with those of other

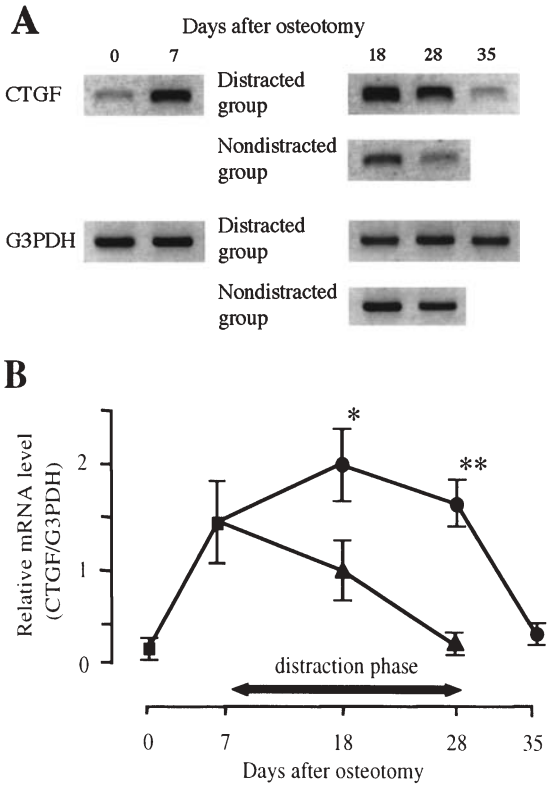

Fig. 7. A Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the expression of CTGF mRNA in distracted femurs and nondistracted femurs. **B** Changes in mRNA expression of CTGF/Hcs24 in rat femurs after osteotomy. The changes in mRNA expression in the distracted group (*closed circles*) and nondistracted group (*closed triangles*) are shown. Days 0 and 7 after osteotomy are indicated by *rectangles*. *Points and bars* are means and SD for quadruplicate determinations of each PCR product on agarose gels. $*P < 0.05$, $**P < 0.01$, significantly different from the nondistracted group

previous reports [27–32]. Thus, we believe that our model is valid to investigate the cellular and molecular events occurring during distraction osteogenesis.

Using immunostaining, we demonstrated that CTGF/Hcs24 was localized in various cells participating in the ossification during distraction osteogenesis. CTGF/Hcs24 was detected in nuclei as well as in cytoplasm. In this regard, CTGF/Hcs24 has been reported to be accumulated around the mitotic machinery [36]. In addition, Wahab et al. [37] reported that CTGF was internalized from the cell surface and translocated into the nucleus. At sites of endochondral and intramembranous ossification, CTGF/Hcs24 was detected in chondrocytes and osteoblasts, as previously reported in fracture healing [20]. Because CTGF/Hcs24 stimulates the proliferation and differentiation of chondrocytes [15] and osteoblasts [17] in vitro, we speculate that CTGF/Hcs24 could promote chondrogenesis and osteogenesis in vivo through its direct action on these cells. CTGF/Hcs24 was also detected in fibroblast-like cells in the fibrous interzone. The origin of these cells located in this fibrous interzone is unknown, but they appeared to retain the potential to proliferate and to differentiate into chondrocytes and osteoblasts during distraction osteogenesis. This finding supports earlier findings that CTGF/Hcs24 was localized in fibroblasts [38] and stimulated matrix production [12], and suggests that CTGF/Hcs24 may play some roles in the production of new matrix to fill the distraction gap and to migrate either to the primary matrix front or to other locations.

The localization of CTGF/Hcs24 mRNA in the lengthened femur was detected by in situ hybridization. From a comparison of the pattern of CTGF/Hcs24 mRNA with the patterns of protein distribution, we found that the localization of CTGF/Hcs24 mRNA did not accord with that of the protein. Because CTGF/Hcs24 is a secreted type of protein and CTGF/Hcs24 synthesized in these cells acts in an autocrine fashion, and in a paracrine manner toward other cells [9,15,17], this protein may be located not only in cells producing CTGF/Hcs24 mRNA and protein but also in cells not producing them. Therefore, we suspected that the distribution of CTGF/Hcs24 protein occurred widely and differed from the localization of the mRNA. Moreover, mRNA expression and protein production do not occur at the same time; protein is synthesized after the expression of the mRNA. Therefore, we suspected that, although CTGF/Hcs24 protein was strongly detected in osteoblasts (Fig. 5C), the localization of CTGF/Hcs24 mRNA was predominant in cells that are possibly osteoblast precursor cells (Fig. 5D).

An interesting feature of CTGF/Hcs24 mRNA expression during distraction osteogenesis was the expansiveness of the expression pattern compared with the pattern seen during embryogenesis or fracture healing. The expression of CTGF/Hcs24 mRNA in vivo was earlier investigated by in situ hybridization; in the articular cartilage and embryogenesis of the mouse, CTGF/Hcs24 mRNA was detected in proliferating and hypertrophic chondrocytes [10,34]. During fracture healing of the mouse rib, it was detected in proliferating and hypertrophic chondrocytes, vascular endothelial cells, active osteoblasts, and cells in fibrous tissue [21]. However, during the distraction phase in this study, CTGF/Hcs24 mRNA was detected not only in hypertrophic chondrocytes and osteoblasts but also in preosteoblasts, fibroblast-like cells, and mesenchymal cells that are possibly their precursor cells. Moreover, RT-PCR demonstrated the elevated expression of CTGF/Hcs24 mRNA in the lengthened segments of the rat femur. It is believed that the mechanical stress induced by the distraction procedure is responsible for the progression of osteogenesis [22,23], and many researchers have investigated the effect of this mechanical stress on

bone and soft tissue in vitro and vivo. It was shown that mechanical stress induced overexpression of CTGF mRNA in fibroblasts in vitro [39,40]. We also reported that CTGF might play some role, especially in osteocytes, in the response of bone to mechanical stress in vivo [33]. Although the mechanism by which the mechanical stress caused by distraction acts on these cells is not clear, the up-regulated expression of CTGF/Hcs24 mRNA during distraction osteogenesis appears to be a part of it.

In conclusion, CTGF/Hcs24 mRNA was more highly expressed in the rat femur during distraction osteogenesis than during fracture healing, and CTGF/Hcs24 was detected in various cells around the osteotomized site, particularly in proliferating and differentiating cells during this process. Our results support the view that CTGF/Hcs24 is an important regulator in chondrogenesis and osteogenesis in vivo, and suggest that the expression of CTGF/Hcs24 mRNA is upregulated by the mechanical stress induced by the distraction procedure, and that the produced CTGF/Hcs24 promotes endochondral and intramembranous ossification in the processes of distraction osteogenesis.

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References

1. Bork P (1993) The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett 327:125–130
2. Brigstock DR (1999) The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. Endocr Rev 20:189–206
3. Takigawa M, Nakanishi T, Kubota S, Nishida T (2003) Role of CTGF/HCS24/ecogenin in skeletal growth control. J Cell Physiol 194:256–266
4. Ohba Y, Goto Y, Kimura Y, Suzuki F, Hisa T, Takahashi K, Takigawa M (1995) Purification of an angiogenesis inhibitor from culture medium conditioned by a human chondrosarcoma-derived chondrocytic cell line, HCS-2/8. Biochim Biophys Acta 1245:1–8
5. Takigawa M, Tajima K, Pan HO, Enomoto M, Kinoshita A, Suzuki F, Takano Y, Mori Y (1989) Establishment of a clonal human chondrosarcoma cell line with cartilage phenotypes. Cancer Res 49:3996–4002
6. Takigawa M, Pan HO, Kinoshita A, Tajima K, Takano Y (1991) Establishment from a human chondrosarcoma of a new immortal cell line with high tumorigenicity in vivo, which is able to form proteoglycan-rich cartilage-like nodules and to respond to insulin in vitro. Int J Cancer 48:717–725
7. Zhu J, Pan HO, Suzuki F, Takigawa M (1994) Proto-oncogene expression in a human chondrosarcoma cell line: HCS-2/8. Jpn J Cancer Res 85:364–371
8. Brigstock DR, Goldschmeding R, Katsube KI, Lam SC, Lau LF, Lyons K, Naus C, Perbal B, Riser B, Takigawa M, Yeger H (2003) Proposal for a unified CCN nomenclature. Mol Pathol 56:127–128
9. Takigawa M (2003) CTGF/Hcs24 as a multifunctional growth factor for fibroblasts, chondrocytes, and vascular endothelial cells. Drug News Perspect 16:11–21
10. Nakanishi T, Kimura Y, Tamura T, Ichikawa H, Yamaai Y, Sugimoto T, Takigawa M (1997) Cloning of a mRNA preferentially expressed in chondrocytes by differential display-PCR from a human chondrocytic cell line that is identical with connective tissue growth factor (CTGF) mRNA. Biochem Biophys Res Commun 234:206–210
11. Xu J, Smock SL, Safadi FF, Rosenzweig AB, Odgren PR, Marks SCJ, Owen TA, Popoff SN (2000) Cloning the full-length cDNA for rat connective tissue growth factor: implications for skeletal development. J Cell Biochem 77:103–115
12. Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR (1996) Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. J Invest Dermatol 107:404–411
13. Lin J, Liliensiek B, Kanitz M, Schimanski U, Böhrer H, Waldherr R, Martin E, Kauffmann G, Ziegler R, Nawroth PP (1998) Molecular cloning of genes differentially regulated by TNF-α in bovine aortic endothelial cells, fibroblasts and smooth muscle cells. Cardiovasc Res 38:802–813
14. Shimo T, Nakanishi T, Kimura Y, Nishida T, Ishizeki K, Matsumura T, Takigawa M (1998) Inhibition of endogenous expression of connective tissue growth factor by its antisense oligonucleotide and antisesnse RNA suppresses proliferation and migration of vascular endothelial cells. J Biochem 124:130–140
15. Nakanishi T, Nishida T, Shimo T, Kobayashi K, Kubo T, Tamatani T, Tezuka K, Takigawa M (2000) Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. Endocrinology 141:264–273
16. Nishida T, Kubota S, Nakanishi T, Kuboki T, Yosimichi G, Kondo S, Takigawa M (2002) CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, stimulates proliferation and differentiation, but not hypertrophy of cultured articular chondrocytes. J Cell Physiol 192:55–63
17. Nishida T, Nakanishi T, Asano M, Shimo T, Takigawa M (2000) Effects of CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, on the proliferation and differentiation of osteoblastic cells in vitro. J Cell Physiol 184:197–206
18. Shimo T, Nakanishi T, Nishida T, Asano M, Kanayama M, Kuboki T, Tamatani T, Tezuka K, Takemura M, Matsumura T, Takigawa M (1999) Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo. J Biochem 126:137–145
19. Nishida T, Nakanishi T, Shimo T, Asano M, Hattori T, Tamatani T, Tezuka K, Takigawa M (1998) Demonstration of receptors specific for connective tissue growth factor on a human chondrocytic cell line (HCS-2/8). Biochem Biophys Res Commun 247:905–909

20. Nakanishi T, Yamaai T, Asano M, Nawachi K, Suzuki M, Sugimoto T, Takigawa M (2001) Overexpression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 decreases bone density in adult mice and induces dwarfism. Biochem Biophys Res Commun 281:678–681
21. Nakata E, Nakanishi T, Kawai A, Asaumi K, Yamaai M, Asano M, Nishida T, Mitani S, Inoue H, Takigawa M (2002) Expression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 (CTGF/Hcs24) during fracture healing. Bone 31:441–447
22. Ilizalov GA (1989) The tension-stress effect on the genesis and growth of tissues. Part 1. The influence of stability of fixation and soft-tissue preservation. Clin Orthop 238:249–281
23. Ilizalov GA (1989) The tension-stress effect on the genesis and growth of tissues. Part 2. The influence of the rate of and frequency of distraction. Clin Orthop 239:263–285
24. Brighton CT, Stanford B, Gross SB, Leatherwood DF, Williams JL, Pollack SR (1991) The proliferative and synthetic response of isolated calvarial bone cells of rat to cyclic biaxial mechanical strain. J Bone Joint Surg Am 73:320–331
25. Neidlinger WC, Winkle HJ, Claes L (1994) Cyclic stretching of human osteoblasts affects proliferation and metabolism: a new experimental method and its application. J Orthop Res 12:70–78
26. Lammens J, Liu Z, Aerssens J, Dequeker J, Fabby G (1998) Distraction bone healing versus osteotomy healing: a comparative biochemical analysis. J Bone Miner Res 13:279–286
27. Rauch F, Lauzier D, Croteau S, Travers R, Glorieux FH, Hamdy R (2000) Temporal and spatial expression of bone morphogenetic protein-2, -4, and -7 during distraction osteogenesis. Bone 26:611–617
28. Sato M, Yasui N, Nakase T, Kawahata H, Sugimoto M, Hirota S, Kitamura Y, Nomura S (1998) Expression of bone matrix proteins mRNA during distraction osteogenesis. J Bone Miner Res 13:1221–1231
29. Sato M, Takahiro O, Nakase T, Hirota S, Kitamura Y, Nomura S, Yasui N (1999) Mechanical tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. J Bone Miner Res 14:1084–1095
30. Kojimoto H, Yasui N, Goto T, Matuda S, Simomura Y (1988) Bone lengthening in rabbits by callus distraction. J Bone Joint Surg Br 70:543–549
31. Perrien DS, Brown EC, Aronson J, Skinner RA, Montague DC, Badger TM, Lumpkin CK Jr (2002) Immunohistochemical study of osteopontin expression during distraction osteogenesis in the rat. J Histochem Cytochem 50:567–574
32. Yasui N, Sato M, Takahiro O, Kimura T, Kawahata H, Kitamura Y, Nomura S (1997) Three modes of ossification during distraction osteogenesis in the rat. J Bone Joint Surg Br 79:824–830
33. Yamashiro T, Fukunaga T, Kobashi N, Kamioka H, Nakanishi T, Takigawa M, Yamamoto T (2001) Mechanical stimulation induces CTGF expression in rat osteocytes. J Dent Res 80:461–465
34. Nawachi K, Inoue M, Kubota S, Nishida T, Yosimichi G, Nakanishi T, Kanyama M, Kuboki T, Yatani H, Yamaai T, Takigawa M (2002) Tyrosine kinase-type receptor ErbB4 in chondrocytes: interaction with connective tissue growth factor and distribution in cartilage. FEBS Lett 528:109–113
35. Meyer D, Yamaai T, Garratt A, Riethmacher-Sonnenberg E, Kane D, Theill LE, Birchmeier C (1997) Isoform-specific expression and function of neuregulin. Development 124:3575–3586
36. Kubota S, Hattori T, Shimo T, Nakanishi T, Takigawa M, (2000) Novel intracellular effects of human connective tissue growth factor expressed in Cos-7 cell. FEBS Lett 474:58–62
37. Wahab N, Brinkman H, Mason R (2001) Uptake and intracellular transport of the connective tissue growth factor: a potential mode of action. J Biochem 359:89–97
38. Igarashi A, Okochi H, Bradham DM, Grotendorst GR (1993) Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. Mol Biol Cell 4:637–645
39. Kessler D, Dethlefsen S, Haase I, Plomann M, Hirche F, Krieg T, Eckes B (2001) Fibroblasts in mechanically stressed collagen lattices assume a "Synthetic" phenotype. J Biol Chem 276:36575–36585
40. Schild C, Trueb B (2002) Mechanical stress is required for high-level expression of connective tissue growth factor. Exp Cell Res 274:83–91