主論文

Impaired expression of non-collagenous bone matrix protein mRNAs during fracture healing in ascorbic acid-deficient rats

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Running title: Expression of bone matrix protein in ascorbic acid-deficient rats

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ABSTRACT

In scorbutic patients, fractures are slow to heal because of impaired collagen synthesis. To investigate the influence of impaired collagen synthesis on the differentiation and proliferation of osteogenic and chondrogenic cells, we examined the expression of genes encoding bone matrix proteins, including osteonectin (ON), osteopontin (OPN), osteocalcin (OC), and matrix Gla protein (MGP), as differentiation markers for osteogenic and chondrogenic cells during fracture healing in Osteogenic Disorder Shionogi (ODS) rats, which have a hereditary defect in the ability to synthesize ascorbic acid (Asc). In ODS rats without Asc supplementation, intramembranous ossification was completely inhibited. Although a few fibroblast-like cells expressing ON mRNA were observed, no OPN mRNA-expressing cells were detected. During endochondral ossification, a small amount of metachromaic staining cartilage appeared at the fracture site, but there was no provisional calcification zone in the cartilage. Chondrocytes expressed ON and MGP mRNAs, but not OPN mRNA. When Asc was given to these rats, callus formation was soon detected around the fracture site, while OPN mRNA was expressed by differentiated osteoblasts and hypertrophic chondrocytes. Our data indicate that impaired collagen synthesis due to Asc deficiency inhibited the increase of ON and MGP mRNA-expressing cells as well as the appearance of OPN mRNA-expressing cells. Since OPN is considered to play an important role in normal and pathological mineralization, lack of OPN mRNA expression accompanying impaired collagen synthesis may have a role in defective mineralization and delayed fracture healing in scurvy.

Key words: fracture healing, ascorbic acid, osteopontin, non-collagenous bone matrix protein, scurvy

INTRODUCTION

A deficiency of ascorbic acid (Asc: vitamin C) in humans causes subperiosteal hemorrhages, thinning of the diaphysis of long bones, and changes of the metaphysis. In addition, bone fractures in scorbutic patients are very slow to heal [1]. Since Asc is required for hydroxylation of the proline and lysine residues of pro-α-chains and stabilizes their triple helical structure before secretion of procollagen into the extracellular matrix [2,3], Asc deficiency impairs bone matrix synthesis and results in abnormal bone formation. When Asc is depleted from cultures of osteogenic MC3T3-E1 cells, the cells do not show an osteoblastic phenotype. Supplementation of Asc sequentially stimulates the synthesis of type I procollagen, the expression of alkaline phosphatase (ALP) mRNA, and the expression of osteocalcin (OC) mRNA by these cells [4,5]. Addition of 3,4-dehydroproline or *cis*-4-hydroxypro-line, which are inhibitors of collagen hydroxylation, to the culture system also inhibits the synthesis of type I collagen and blocks the enhanced expression of ALP and OC mRNAs [4,5]. These data suggest that collagen in bone matrix may play an important role in the regulation of osteoblastic differentiation through its interaction with osteoblasts via $\alpha_2\beta_1$ integrin [6]. Therefore, Asc deficiency is considered to result in abnormal differentiation of osteoblastic cells in vitro [4-6]. In cultured prehypertrophic sternal chondrocytes, however, Asc increases ALP activity and the type X collagen mRNA level, but inhibitors of collagen hydroxylation have no effect on the expression of these genes [7,8]. These results indicate that Asc controls the expression of bone-related genes in osteogenic cells through the formation of normal collagen matrix, while the expression of genes during chondrogenesis may be directly regulated by Asc rather than via collagen matrix formation. However, these results were obtained in vitro, so little is known about the in vivo differentiation and proliferation of osteogenic and chondrogenic cells in the presence of Asc deficiency.

The non-collagenous bone matrix proteins are considered to be useful markers to investigate the stage of differentiation of osteogenic and chondrogenic cells. Representative non-collagenous bone matrix proteins include osteonectin (ON),

osteopontin (OPN), OC, and matrix Gla protein (MGP). We have previously shown that the ON, OPN, and OC genes are sequentially expressed along with osteogenic cell differentiation in various types of developing bone [9]. During chondrogenesis, we have demonstrated that there is characteristic switching in the expression of ON and OPN mRNAs, i.e., ON gene expression is seen in immature and hypertrophic chondrocytes, while OPN gene expression occurs in hypertrophic chondrocytes from the zone of provisional calcification [9]. MGP mRNA is detectable in resting, proliferating, and late hypertrophic chondrocytes, but not in early hypertrophic chondrocytes and osteoblasts[10].

Since Asc deficiency during embryogenesis results in fetal death because of insufficient collagen synthesis in organs other than bone, whether Asc is important for the differentiation and proliferation of osteogenic and chondrogenic cells during embryonic osteogenesis and chondrogenesis has not been examined in vivo. We previously reported that the types of cells expressing ON, OPN, OC, and MGP mRNAs in fractured bones were similar to those found in embryonic bones [11]. Therefore, investigation of the effect of Asc deficiency on the expression of non-collagenous bone-related genes in an experimental fracture model could provide valuable information on the role of Asc in the differentiation and proliferation of osteogenic and chondrogenic cells during normal development of embryonic bones.

Normal rats can synthesize Asc, but Osteogenic Disorder Shionogi (ODS) rats have a hereditary defect of Asc synthesis due to the lack of L-gulonolactone oxidase, which catalyzes the conversion of L-gulono-γ-lactone into Asc [12,13]. When ODS rats are fed an ascorbic acid-free diet, the hydroxyproline level in polypeptides, which is known to be related to collagen synthesis [14], falls below that in normal rats after one week and is about one third of normal after two weeks [12]. Accordingly, collagen synthesis markedly decreased in these rats. When Asc is added to the drinking water, the scorbutic symptoms of these rats resolve in a few days. Thus, ODS rats are a valuable and convenient model for investigating the physiological significance of Asc.

In the present study, we examined the expression of ON, OPN, OC, and MGP mRNAs during the process of fracture healing in ODS rats to investigate the influence of impaired

collagen synthesis due to Asc deficiency on the differentiation and proliferation of osteogenic and chondrogenic cells.

MATERIALS AND METHODS

Experimental model of fracture healing

A total of 22 female, ODS rats (weighing 79.0 g-91.0 g, average 85.4g) of 5 week old were used in this study. Before the experiment, all rats were given a standard diet and water containing 1 mg/ml Asc of for 7 days and then the animals were divided into three groups. Group A was given the standard diet and water containing 1 mg/ml of Asc, while the other two groups (Group B and C) were given the standard diet and water without Asc. After 10 days, all rats gained weight (147 g in Group A, 144 g in Group B and C on the average), the bilateral eighth ribs of the rats in all groups were fractured, as described previously [15]. Briefly, each rat was anesthetized with ethyl ether, and the eighth ribs on both sides were exposed and cut vertical to the axis of the bone with scissors. During the operation, all animals were maintained in cages with free access to food and water. In Group C, rats received the standard diet and water containing 1 mg/ml of Asc from day 7 to day 15 after the operation. The experimental procedures were undertaken in compliance with the guidlines for the Care and Use of Animals described in the American Journal of Physiology.

Tissue preparation

Rats from Group A and Group B were sacrificed on days 2, 4, 7, 9, and 11 after the operation, while rats from Group C were sacrificed on day 15. A rectangular segment (4 mm horizontally × 2 mm vertically) was dissected from both eighth ribs together with the surrounding soft tissues and was fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4), decalcified with 20% EDTA in 0.1 M PB, and then embedded in paraffin. Longitudinal sections 4 μm thick were cut with a microtome and mounted on 3-aminopropyltriethoxysilane-coated slides for hematoxylin-eosin staining and in situ hybridization. The slides were stored at 4℃ until use.

Preparation of probes

The following complementary DNA (cDNA) clones were used as hybridization probes: a 1.0 kb fragment of mouse ON [16], a 1.2 kb fragment of mouse OPN [16], a 0.47 kb fragment of rat OC [11], and a 0.48 kb fragment of rat MGP [11].

In situ hybridization

The in situ hybridization technique used has been described previously [17,18]. Digoxigenin-labeled single-strand RNA probes were prepared for hybridization using a DIG RNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's instructions. Hybridization of ON, OPN, OC, and MGP mRNAs was performed at 50℃ for 16 hours, and the signals were detected using a nucleic acid detection kit (Boehringer Mannheim Biochemica, Mannheim, Germany).

The controls included (1) hybridization with the sense probes (2) RNase treatment before hybridization, and (3) use of neither the antisense RNA probe nor anti-digoxigenin antibody. All three types of control experiments showed no detectable signals.

RESULTS

<Fracture healing in Asc-deficient rats>

Groups B and C kept gaing weight at the rate of 3.1g per day on the average before surgery as well as Group A at the rate of 3.3 g per day. However, Group B and C began to lose weight at various times between 1 and 4 days after fracture. Group A gained weight throughout the exprimental period. Rats from Groups A and B were sacrificed on days 2, 4, 7, 9, and 11 after the operation. Paraffin sections containing the fracture sites were cut and subjected to routine histological examination and in situ hybridization.

On day 2 after surgery in Group A, the thickness of the periosteal layer was increased and fibroblast-like cells were observed in the surrounding muscle tissue near the fracture site. Hybridization with the ON cRNA probe revealed prominent signals in the proliferating periosteal cells and fibroblast-like cells near the fracture (Fig. 1, A and C). In Group B, on the other hand, the periosteal layer was not thickened and fibroblast-like cells were not observed around the fracture site. In addition, no ON mRNA-positive cells were detected at the fracture site (Fig. 1, B and D).

On day 4, the number of fibroblast-like cells around the fracture was increased in Group A. Primitive woven bone was seen in the medullary cavity and along the periosteum close to the fracture site (Fig. 2, A and C), and faintly metachromatic staining cartilage was also observed at the fracture site (not shown). MGP (Fig. 2 E) and ON (Fig. 2 G) mRNAs were detected in the fibroblast-like cells around the fracture site, while OPN and OC mRNAs were not detected in the area (Fig. 2 I).MGP (Fig. 2 E) and ON (Fig. 2 G) mRNAs were detected in the chondrocytes of immature cartilagenous tissue. A small number of fibroblast-like cells were present in the muscle tissue around the fracture site in Group B.

However, neither primitive woven bone nor cartilagenous tissue was observed (Fig. 2, B and D). In Group B, MGP mRNA was detected in the fibroblast-like cells (Fig. 2 F) as well as Group A (Fig. 2 E), and the very faint singal of ON mRNA was found (Fig. 2 H) in contrast to Group A (Fig. 2 G). In this period, no OPN and OC mRNAs were detected around fracture site in both Group A and B (Fig. 2, I and J).

From day 7 to day 11, the mass of newly formed cartilage enlarged and hypertrophic chondrocytes appeared within the metachromatic staining areas in Group A (Fig. 3, A and C). In addition, trabecular bone was formed through endochondral ossification near the fracture site (Fig. 3 A), while MGP (Fig. 3 E), ON (Fig. 3 G) , OPN (Fig. 3 I), and OC (not shown) mRNAs were expressed by osteoblastic cells on the surface of the trabecular bone. Within the cartilage, MGP mRNA was found in proliferating and hypertrophic chondrocytes (Fig. 3 E), ON mRNA was detected in proliferating chondrocytes (Fig. 3 G), and OPN mRNA was detected in chondrocytes at the zone of provisional calcification (Fig. 3 I). On the other hand, Group B showed no changes in periosteal thickness from days 7 to 9, and primitive woven bone was not found around the fracture site (Fig. 3 B). However, the number of fibroblast-like cells was increased slightly around the fracture (Fig. 3 B), and a small mass of metachromatic staining cartilage was observed in the periosteal region (Fig. 3 D).
From day 9 to day 11, neither the amount of cartilage nor the periosteum showed any increase in Group B (not shown), and woven bone was not detectable around the fracture site. Expression of ON mRNA was observed in fibroblast-like cells near the fracture (Fig. 3 H). In the cartilage, MGP (Fig. 3 F) and ON (Fig.3 H) mRNAs were expressed in the chondrocytes. However, OPN mRNA was not detected in the chondrocytes (Fig. 3 J) and no ON, OPN, or OC mRNA was detected in the periosteal cells (not shown).

<Reversal of impaired fracture healing process by Asc>

Group C rats lost weight at 20 % of their heighest weight attained at 7 days after surgery. From day 7 after surgery, they were given a standard diet and water containing 1 mg/ml of Asc. They began to gain weight from next day of Asc supplementation. After Asc supplementation for 8 days (on day 15 after surgery), the periosteum was thickened and new trabecular bone had formed in the periosteal region (Fig. 4 A). There were proliferating fibroblast-like cells and metachromatic staining cartilage was also observed around the fracture site, (Fig. 4, A and B). OPN mRNA was expressed by both the chondrocytes at the zone of provisional calcification and the osteoblastic cells throughout the newly formed bone (Fig. 4, C and D). ON mRNA was detected in the proliferating

chondrocytes, and MGP mRNA was found in all of the chondrocytes. The pattern of mRNA expression by chondrocytes was similar to that in Group A on day 9 (not shown). In addition, ON, OC, and MGP mRNAs were expressed by the osteoblastic cells throughout the newly formed bone, as was observed during fracture healing in Group A (not shown).

DISCUSSION

In the present study, we showed that impaired collagen synthesis due to Asc deficiency inhibits the appearance of OPN mRNA-expressing cells during both osteogenesis and chondrogenesis during fracture healing. A few polygonal cells which expressed ON mRNA appeared in Group B at a later stage, but these cells did not express OPN mRNA. These results indicate that both the in vivo differentiation and proliferation of OPN-expressing osteoblastic cells are also dependent on normal collagen synthesis in the presence of Asc. Since ON-and OC-expressing osteoblastic cells were also not observed in Group B, the differentiation of osteoblastic cells from preosteobalstic cells was virtually inhibited by Asc deficiency, as has been observed in previous in vitro studies [4,5]. In Group B, a small amount of metachromatic staining cartilage was observed at the fracture sites, but hypertrophic chondrocytes were not observed even 11 days after creation of the fracture. The chondrocytes expressed ON and MGP mRNAs, but not OPN mRNA. These results indicate that differentiation of ON or MGP mRNA-expressing chondrocytes into OPN mRNA-expressing hypertrophic chondrocytes was markedly inhibited.

Although the differentiation of ON mRNA-expressing fibroblast-like cells from undifferentiated mesenchymal cells and periosteal cells was observed after 9 days even in the absence of Asc, the number of ON mRNA-expressing fibroblast-like cells around the fracture site was much lower than that in the presence of Asc. When the proportion of ON mRNA- expressing fibroblast-like cells to all fibroblast-like cells was compared in Groups A and B, no significant difference was found (data not shown). These findings suggest that the proliferation of fibroblast-like cells was inhibited by depletion of Asc, but the differentiation of ON mRNA-expressing fibrolast-like cells from undifferentiated mesenchymal cells not affected.

We and other investigators have shown by in situ hybridization studies that OPN mRNA-expressing osteoblastic cells are located in the calcifying zone during embryogenic bone formation, callus formation during fracture healing, and ectopic bone fomation induced by bone morphogenic protein-4 [9, 16, 19, 20, 21]. OPN is also known to be involved in various types of pathological calcification, such as urinary calculus formation [22],

atherosclerosis [23], and calcification of breast cancer [24]. These studies suggest that OPN may play an important role in the mineralization process. In the present study, the lack of both endochondral and membranous ossification in the absence of Asc may have been due to inhibition of the expression of OPN mRNA. Although the molecular nature of the signal from the extracellular matrix has not been elucidated, it is possible to speculate that a matrix signal may eventually change the level of expression of a transcription factor and activate expression of the OPN gene. Several transcription factors are known to regulate OPN gene expression [25, 26, 27], so changes in the expression of these factors by osteoblastic cells in the presence of Asc should be further examined in the future. Previous investigations revealed that the mRNA levels for ALP and OC were decreased in calvaria bone of guinea pig in scurvy, while the OPN mRNA level was increased 2-3 times as much as normal [28]. The difference of our result to their study may cause the difference of normal bone and fractured bone.

In addition to inhibition of OPN gene expression, decreased insulin-like growth factor-I (IGF-I) activity may cause the impairement of fracture healing. IGF-I has been reported to stimulate collagen synthesis and accumulation of collagen mRNA in vitro [29], and IGF-I mRNA was also expressed during fracture healing [30, 31]. These studies indicated that IGF-I have an important role for bone formation and bone homeostasis. In guinea pig, lacking L-gulono-γ-lactone oxidase and unable to synthsize Asc as well as ODS rats [32], IGF-I action was decreased by induction of circulating insulin-like growth factor-binding protein-1 and 2 (IGFBP-1, 2) that are correlated with weight loss [33, 34]. In the present study, although the ODS rats without Asc supplementation gained weight before fracture, they began to lose weight in the early period after fracture. These data indicated that decreased IGF-I activity related to weight loss may be also one of major cause of the impairement of fracture healing in the ODS rats.

When Group C rats were given water containing 1mg/ml Asc from day 7 after the operation, the process of fracture healing resumed immediately. Endochondral ossification and membranous ossification were observed on day 15 after the operation. In addition, ON, OPN, and MGP mRNAs were expressed in the cartilage cells of the callus,

while ON, OPN, OC, and MGP mRNAs were detected in osteoblastic cells throughout the newly formed bone, as was observed in Group A on day 7. When scurvy patients are given an Asc-rich diet, spontaneous bleeding usually ceases within 24 h. Muscle and bone pain subsides quickly, and the gums begin to heal within 2 - 3 days [35]. Our results showed that similar rapid resumption of experimental fracture healing occurred after administration of Asc to ODS rats.

In summary, this study demonstrated the abnormal expression of bone matrix protein genes during the process of fracture healing in scorbutic ODS rats. Our findings may contribute to elucidation of the molecular mechanisms of abnormal bone formation and impaired fracture healing in scurvy patients.

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Figure legends

Figure 1.

Localization of ON mRNA by in situ hybridization on day 2 after fracture in rats from Group A (A, C) and Group B (B, D). (C, D) Higher magnification of the periosteal region (asterisks) in A and B respectively. (A and B: bar, 300μm; C and D: bar, 150μm.)

Figure 2.

Fracture healing at 4 days in Group A (A, C, E, G, I) and Group B (B, D, F, H, J). Sections were stained with hematoxylin-eosin (A-D), arrows in A and B indicated the fracture site. (C, D) Higher magnification of the periosteal region (asterisk) of A and B respectively. (E-J) Localization of mRNA encoding bone matrix proteins by in situ hybridization in Group A (E, G, I) and Group B (F, H, J).The probes used for in situ hybridization were for MGP (E, F), ON (G, H) and OPN (I, J). Primitive woven bone was seen in the medullary cavity and along the periosteum close to the fracture site (arrowheads in C), and immature cartilaginous tissue was also seen (arrow in C) in Group A. (A and B: bar, 250μm; C-J: bar, 100μm.)

Figure 3.

Fracture healing at 9 days in Group A (A, C, E, G, I) and Group B (B, D, F, H, J), and localization of mRNA encoding bone matrix proteins by in situ hybridization in Group A (E, G, I) and Group B (F, H, J). Sections were stained with hematoxylin-eosin (A, B) and toluidine blue (C, D). (C-J) Higher magnification of newly formed cartilage (asterisk) in A and B respectively. OPN mRNA was detected in the chondrocytes within the zone of provisional calcification (arrowheads in I) and in the osteoblastic cells on the surface of the trabecular bone in Group A, but no signals were detected in Group B (J). (A: bar, 400μm; B: bar, 240μm; C, E, G, I: bar, 100μm; D, F, H, J: 50μm.)

Figure 4.

Histological appearance of fracture healing after 8 days of Asc supplementation (day 15 after fracture creation) in Group C. Sections were stained with hematoxylin-eosin (A) and toluidine blue (B), or hybridized for OPN (C, D). OPN mRNA is expressed both by the chondrocytes within the zone of provisional calcification (arrowheads in C) and the osteoblastic cells throughout the newly formed bone (arrowheads in D). co: cortical bone of the fractured rib. nb: newly formed bone. (A and B: bar, 300μm; C: bar, 75μm; D: bar, 100μm.)

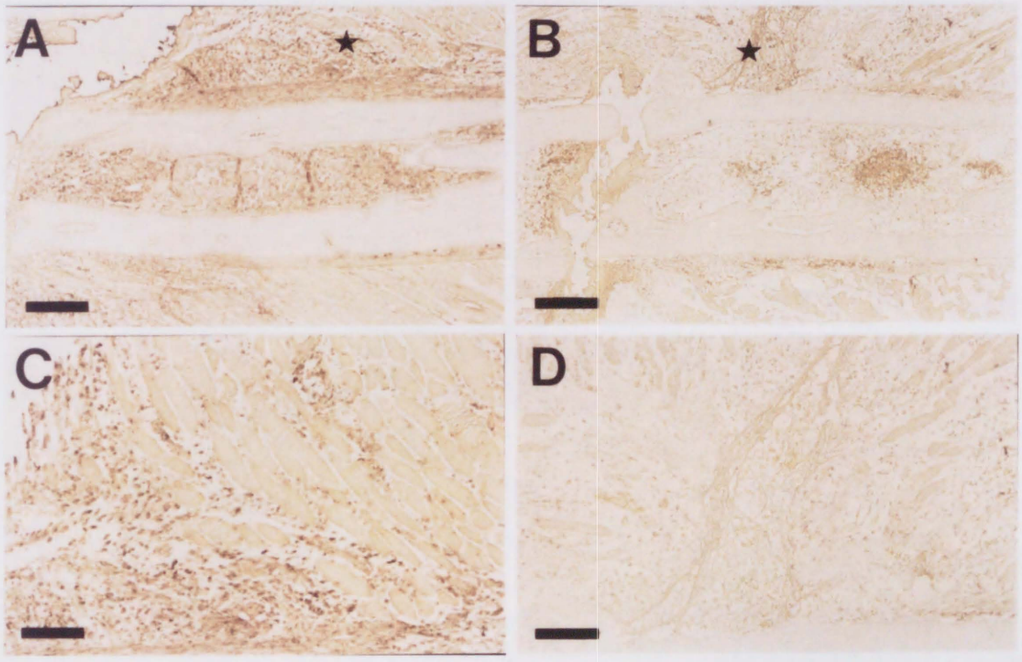
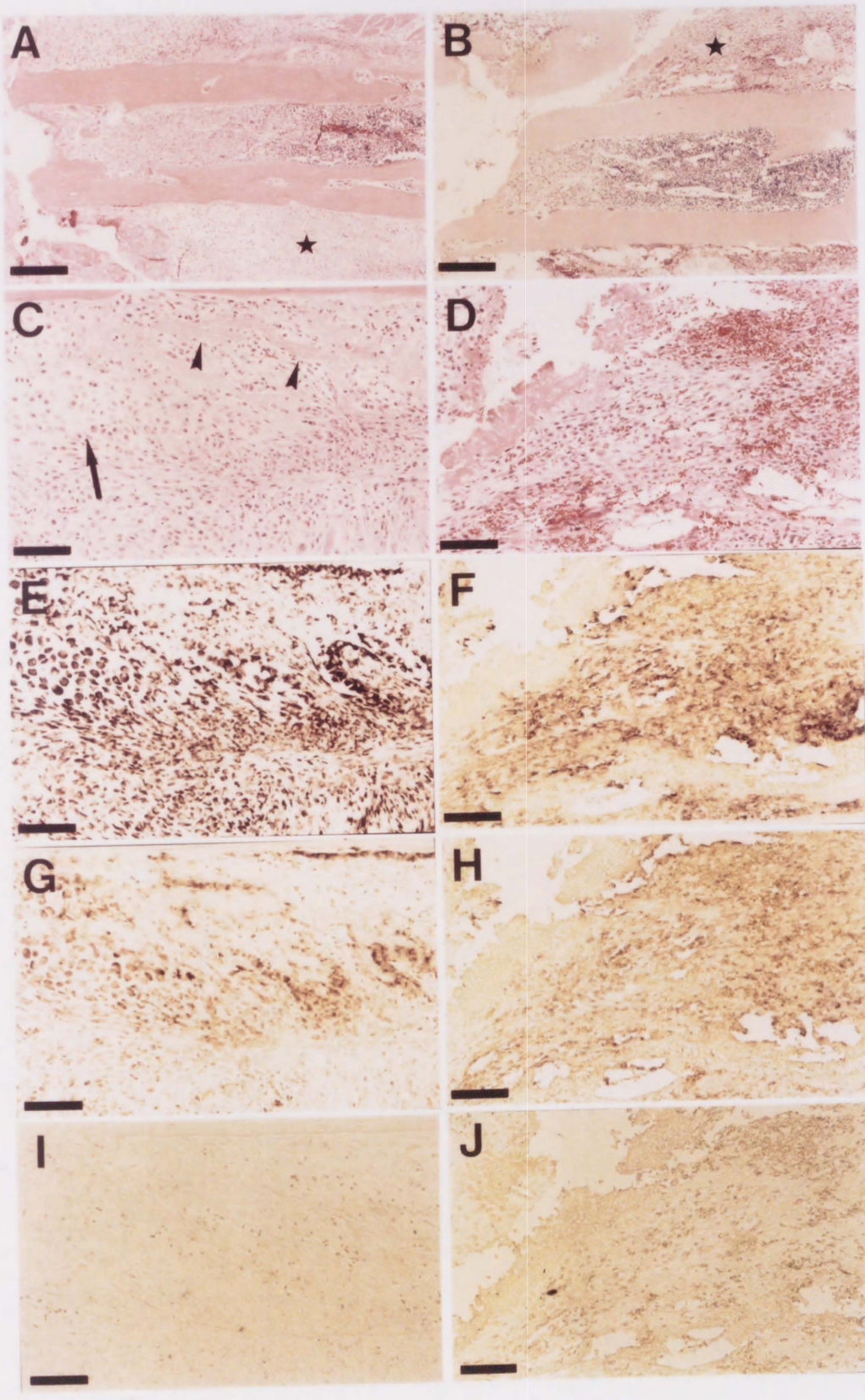


Fig. 1



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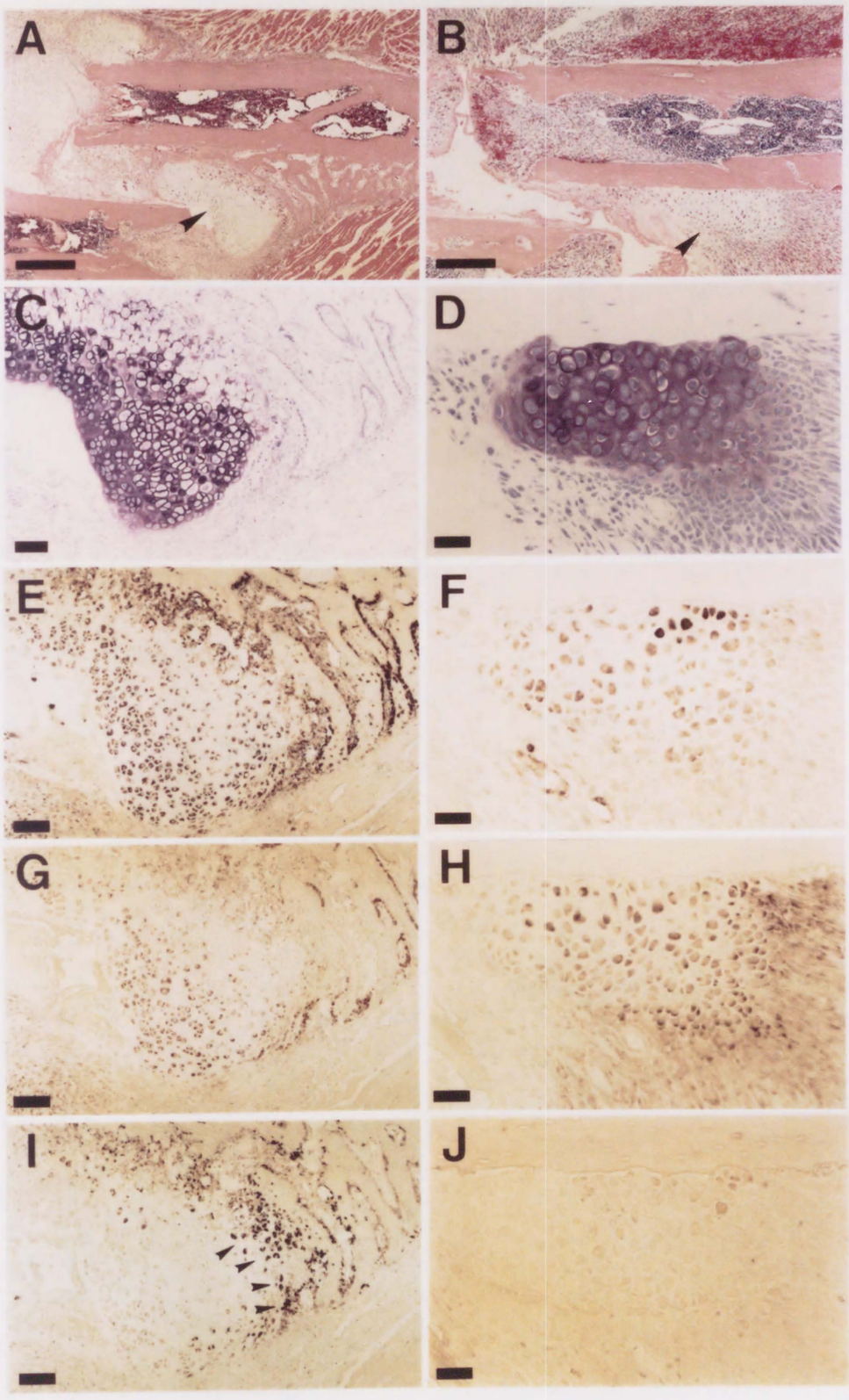

Fig. 3

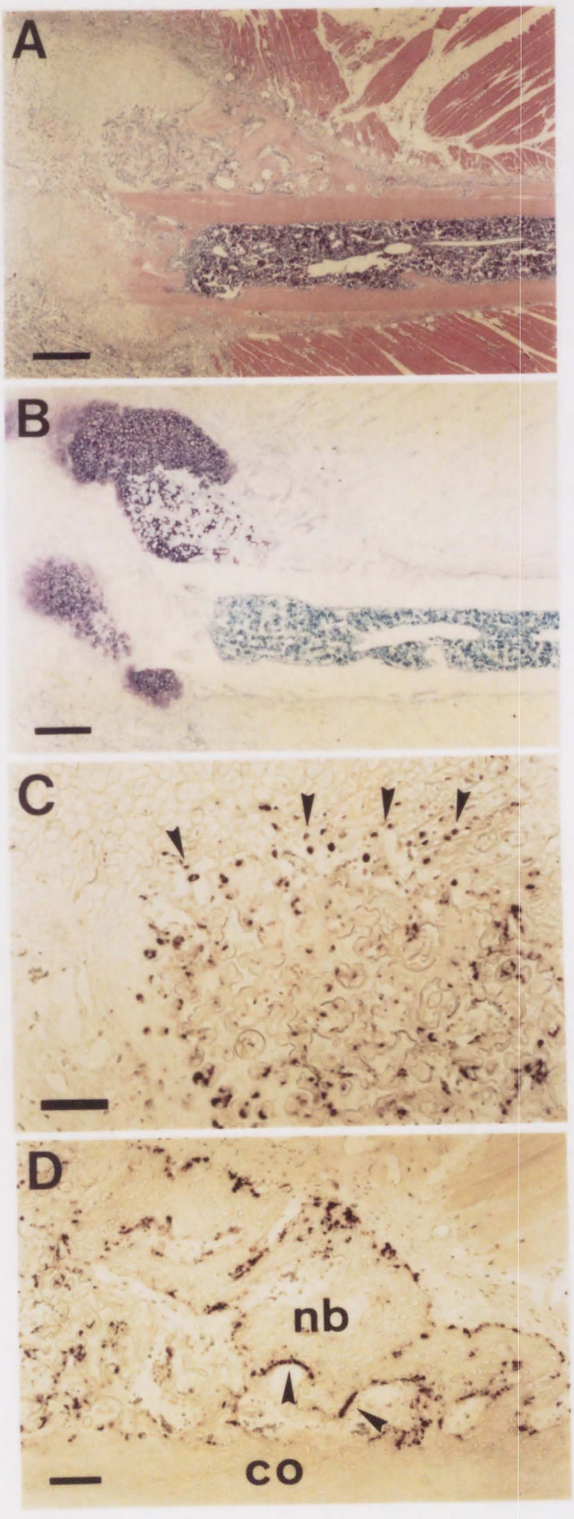

Fig. 4