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Fracture healing induces expression of the proto-oncogene *c-fos* in vivo

Possible involvement of the Fos protein in osteoblastic differentiation

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Here we report marked in vivo expression of the *c-fos* gene in the external soft callus (ESC) and periosteal hard callus (PHC) at the fracture site of adult rat tibia. Northern-blot analysis showed that the ESC expressed a high level of *c-fos* mRNA from post-fracture day 10 to day 28, the time when endochondral ossification progressed, and that the ossifying PHC also expressed *c-fos* mRNA. This *c-fos* expression was followed by sequential expression of the genes for alkaline phosphatase, osteopontin and osteocalcin, which are osteoblastic markers. Immunohistochemical analysis showed that the c-Fos protein was predominantly located in osteoblasts in the ossifying calluses.

Fracture healing; *c-fos*; Osteoblastic differentiation; Alkaline phosphatase

1. INTRODUCTION

The proto-oncogene *c-fos* encodes a nuclear protein that participates in transcriptional regulation of a variety of genes [1–3]. Studies on cultured cells have shown that expression of the gene is required in the early stage of cellular proliferation induced by growth signals [4–6]. Accumulating in vivo evidence also indicates that expression of the gene is associated with growth and differentiation of skeletal cells during embryonic development [7–12]. Deregulated expression of *c-fos* in transgenic mice interfered with normal bone development and induced bone tumors, suggesting a specific role of the gene in skeletal development [13,14].

The *c-fos* gene is reported to be transiently expressed in rat liver after partial hepatectomy [15–18], but lack of a reproducible experimental system has hampered studies on its expression in adult animals in vivo. In this work we developed a reproducible model of fracture healing using adult rats. After fracture of their tibiae, two types of callus were formed, a periosteal hard callus (PHC) and an external soft callus (ESC). Bone tissue was formed in the PHC by intramembraneous ossification and in the ESC by endochondral ossification, respectively. We examined expression of the *c-fos* gene in the PHC and ESC by Northern-blot analysis and immunohistochemistry, and found that: (1) expression of the gene was markedly stimulated and persisted for at least 4 days in the PHC and for two weeks in the ESC; (2) the c-Fos protein was located predominantly in osteoblasts in the ossifying calluses; and (3) expression of the gene preceded expression of osteoblastic marker genes, suggesting that *c-fos* expression is associated with osteoblastic differentiation in the calluses.

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2. MATERIALS AND METHODS

2.1. *Fracture model*

A hole was made at the anteromedial margin of the tibial plateau of anesthetized female Wistar rats of 7–8 months old. A stainless metal rod (0.5-mm diameter, Sankin, Osaka) was inserted through the hole into the medullary cavity down to the distal end of the bone. The tibial mid-shaft was then placed on a device with a blunt blade and broken by gentle manual compression. A sham-operated tibia served as a non-fractured control. The rats were allowed to recover after the operation and fed ad libitum on a standard diet. Animal protocols were conducted in accordance with the guidelines of Kyoto University.

2.2. *Total RNA extraction and Northern blot analysis*

Fractured and non-fractured tibiae were obtained by disarticulation at the knee and ankle joints and freed from surrounding soft tissues. The fracture calluses were then carefully dissected and separated into the ESC and PHC. A mid-shaft region of non-fractured tibiae was cut out, and bone marrow was obtained by flushing of the marrow cavity with normal saline. After homogenization, total RNA from each tissue was extracted by the acid guanidinium-phenol-chloroform method [19]. Denatured total RNA (20 μg) was separated by 1% agarose gel electrophoresis and transferred to a nylon membrane. Hybridization was performed at high stringency. The membrane was exposed to Kodak X-Omat film at −80°C for 24 h with a Cronex intensifying screen (du Pont, Wilmington, DE). The following hybridization probes were used: v-*fos*, a 1 kb *Pst*I fragment of pv-fos-1 [20]; purified synthetic oligonucleotides of 50-mer corresponding to the region of Ile^{146}-Ala^{162} of rat alkaline phosphatase cDNA

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[21], of 45-mer corresponding to the Ser^{112}–Thr^{126} region of rat osteopontin cDNA [22] and of 42-mer corresponding to the Cys^{23}–Ile^{36} region of rat osteocalcin cDNA [23].

2.3. *Immunohistochemical analysis*

The fractured bone specimens were fixed, decalcified by the method of Plank and Rychlo [24], embedded in paraffin, and cut into consecutive 6-μm thick longitudinal sections. The sections were treated with xylene to remove paraffin, rehydrated and incubated for 30 min at 25°C with a sheep polyclonal anti-c-Fos antibody (Cambridge Res. Biochem., Cambridge, MA) in phosphate-buffered saline containing 0.1% bovine serum albumin (crystalline grade, Armour, Kankakee, IL) [25]. The sections were subsequently incubated with biotinylated anti-sheep immunoglobulin G, rinsed, and then treated for 30 min with avidin-biotin-horseradish peroxidase complex (Vector, Burlingame, CA), followed by diaminobenzidine (DAB) treatment in DAB enhancement solution (HistoMark, Kirkegaad and Perry Lab., Gaithersburg, MD). Consecutive sections were also treated with non-immune sheep immunoglobulin G and immunostained otherwise identically to serve as a control.

3. RESULTS AND DISCUSSION

Fracture healing of the rat long bone involves formation of 3 different types of callus, i.e. PHC, ESC and endosteal callus, which are distinct in their locations and mode of ossification [26]. Use of intramedullary rods in our model precluded analysis of the endosteal callus. Fracture calluses were dissected out from the fracture site of adult rat tibiae and carefully separated into the PHC and ESC. We extracted total RNA from each callus and examined by Northern blot analysis the temporal profile of the *c-fos* gene expression over 28 days during the fracture healing process (Fig. 1). Histochemical analysis of the fracture calluses was also performed in parallel. Formation of non-lamellar woven bone in the PHC was initiated by post-fracture day 3 through rapid proliferation of the determined osteoprogenitor cells derived from the periosteum. The woven bone-filled PHC increased in size over the first 7–10 post-fracture days. Marked expression of *c-fos* mRNA was observed during this period in the PHC (Fig. 1a) in which osteoblasts were the predominant cellular component. Expression of the alkaline phosphatase (ALP) gene paralleled *c-fos* expression and was followed by expression of other osteoblastic marker genes, the osteopontin (OP) and osteocalcin (OC) genes from post-fracture day 7 to day 10 (Fig. 2).

In contrast to the PHC, formation of the ESC was in-

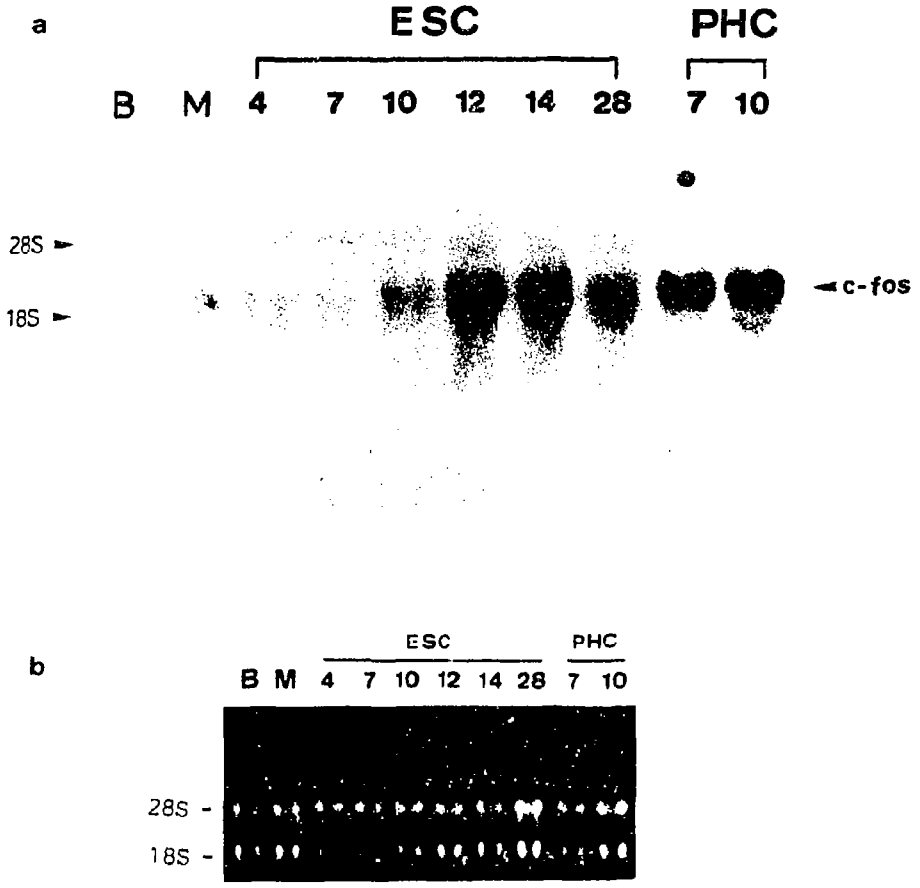

Fig. 1. Northern-blot analysis of RNA from rat fracture calluses and control bone. Samples of total RNA (20 μg/lane) from the following tissues were used: non-fractured control bone devoid of bone marrow, B; bone marrow from non-fractured control bone, M; ESCs on the indicated days after fracture, ESC; PHCs on the indicated days, PHC. In (a), the membrane was probed with a ^{32}P-labeled v-*fos* cDNA fragment. The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown in (b). The positions of 28S and 18S ribosomal RNA are indicated.

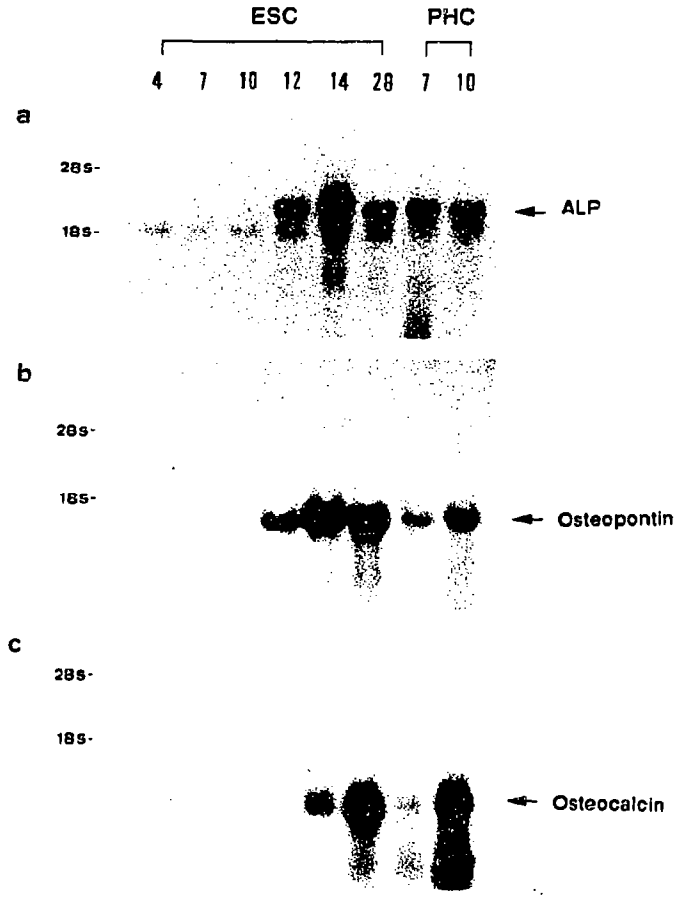

Fig. 2. Expression of ALP, osteopontin and osteocalcin mRNAs during fracture callus development. The membrane shown in Fig. 1 was rehybridized with the ALP (a), osteopontin (b) and osteocalcin (c) probes. The filter was completely dehybridized after each hybridization. The positions of 28S and 18S ribosomal RNA are indicated.

itiated by migration and proliferation of undifferentiated mesenchymal cells which first differentiated into chondrocytes. No appreciable expression of *c-fos* mRNA was detected before post-fracture day 10, by which time the ESC became completely occupied by cartilaginous tissue. Woven bone first appeared in the ESC near the ESC/HPC border, progressed toward the center of the ESC, and replaced the entire ESC by post-fracture day 28–35 (Fig. 3a). Dramatic expression of *c-fos* in the ESC occurred on post-fracture day 12 (Fig. 1a), persisted for at least two weeks and thereafter gradually decreased to a low level. Thus, expression of *c-fos* mRNA in the ESC temporarily coincided with massive progression of woven bone formation (Fig. 1a). Additionally, the *c-fos* gene expression preceded differentiation and maturation of osteoblasts as indicated by sequential induction of the expression of the ALP, OP and OC genes following *c-fos* gene expression (Fig. 2). An immunohistochemical study showed that osteoblasts in the ossifying ESC were the major site of expression of c-Fos protein (Fig. 3b and d). That most c-Fos-positive osteoblasts were tall and lined over the surface of the woven bone conforms further the notion that the *c-fos* expression is associated with osteoblastic differentiation in fracture calluses. Recent report by Closs et al. showed that cultured mouse mandibular condyles expressed *c-fos* mRNA in the zone of hypertrophic chondrocytes in vitro [27]. Mandibular condyle is unique in that in this particular tissue chondrocytes differentiate directly into osteoblasts. In our in vivo rat fracture model no appreciable staining of chondrocytes within the ESC was detected. Ossification of the fracture calluses was complete with marrow development by post-fracture day 40, followed by reconstruction of the deformed bone architecture. No expression of *c-fos* mRNA was detected in non-fractured control bone and bone marrow (Fig. 1a).

These results indicate that the *c-fos* gene is expressed in fracture calluses in the process of fracture healing of adult animals and plays a specific role in osteoblastic differentiation in vivo. Release of a variety of cultured cells from growth-arrest induces rapid and transient expression of *c-fos*, which is generally believed to play a role in cellular proliferation [4–6]. Owen et al. reported that expression of *c-fos* was associated with proliferation of cultured calvarial osteoblasts in vitro and that it preceded the ALP gene expression by two weeks [28]. The coordinate occupancy of AP-1 sites in the vitamin D-responsive elements of the ALP and OC genes argues for a role of c-Fos protein in suppression of the osteoblastic phenotype during proliferation of the cells [29]. In our in vivo rat fracture model, however, the ALP gene expression took place within a day or two in the ESC after initiation of the *c-fos* expression. The expression of the two genes occurred almost in parallel in the PHC (Figs. 1 and 2). Moreover, *c-fos* mRNA was not detected in the ESC during the first 7 days after fracture, when rapid proliferation of undifferentiated mesenchymal cells and chondrogenic differentiation took place. Our results thus suggest that c-Fos protein is involved in the induction of phenotypic expression of osteoblasts in the fracture calluses in vivo.

Fig. 3. Immunohistochemical detection of c-Fos protein in the ossifying fracture callus on day 14. In (a), the architecture of the fracture calluses is shown schematically. The framed area indicates the portion of the calluses shown in (b) and (c). In (b), the section was incubated with a polyclonal anti-c-Fos antibody at a final dilution of 1:600. In (c), a consecutive section was incubated with non-immune IgG. After thorough washing, bound antibody was detected by the avidin-biotin-peroxidase method. In (d), the framed area in (b) is shown at higher magnification. Arrows indicate the fracture site. Woven bone, cortical bone and cartilage are denoted by A, B and C, respectively.

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