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SEQUENTIAL ANALYSIS OF GENE EXPRESSION AFTER AN OSTEOGENIC STIMULUS: *C-FOS* EXPRESSION IS INDUCED IN OSTEOCYTES

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SUMMARY: We have recently developed an experimental model whereby mechanical stimulation induces osteogenesis in the caudal vertebrae of rats. We used this model to assess expression of genes induced by mechanical loading. Bulk preparations of mRNA extracted after loading did not show >2-fold increases in expression of mRNA for matrix proteins or growth factors in Northern blotting analysis. *c-jun* was undetectable. However, *c-fos* showed a 4-fold increase in expression within 60 mins of loading, before returning to control levels by 4 hrs. This increase was associated with intense signals in *in situ* hybridization, not seen in any nonloaded vertebrae, for *c-fos* over cortical osteocytes: thus osteocytes respond to mechanical loading with *c-fos* expression so strongly as to be visible even in the bulk RNA preparations. The results represent persuasive evidence for a role for osteocytes, and for *c-fos*, in the osteogenic response of bone to mechanical stimulation.

Although the overall shape of bones is determined genetically bones modify their structure in response to mechanical stimuli, so that bone structure remains optimal for the prevailing mechanical environment. The mechanisms underlying this mechanical adaptation are poorly understood. It has been suggested that osteocytes play a role (1). However, almost nothing is known of the sites and sequences of changes in gene expression which accompany the process.

We have recently developed an experimental model in which pins, inserted into the 7th and 9th caudal vertebrae of rats, are used to load the 8th caudal vertebra in compression (2). A single, 5 min application of external loads sufficient to cause dynamic strains within the physiological range stimulates bone formation in

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metaphyseal trabecular bone. We found that osteocytes express mRNA for IGF-I within 6 hrs and matrix protein gene expression is maximal 72 hrs after loading (2). Thus, the model provides an opportunity to identify genes whose expression is associated with osteogenesis. In this communication, we analyse the expression of 3 groups of genes after mechanical stimulation: early response genes, growth factor genes, and genes for matrix proteins.

MATERIALS AND METHODS

Northern analysis

Pins were inserted into the 7th and 9th caudal vertebrae of 13-week-old female Wistar rats. The 8th caudal vertebrae (C8) were then loaded in compression as previously described (2), by 300 cycles at 1 Hz using 150 N. At intervals after mechanical stimulation, groups of 9 rats per time point were killed. Vertebrae were removed and cleaned of adherent soft tissues. Epiphyses were removed and marrow flushed through with a water jet. The remaining bone was frozen and pooled into two groups (C6 and C8) (n = 9 per group) for each time point. The frozen bone was crushed, transferred to the lysis/binding buffer of a Dynabeads mRNA DIRECT kit (DYNAL) and homogenized for 2 mins by a Polytron homogenizer. Direct extraction of poly(A)$^+$ RNA from the homogenate was carried out according to the kit protocol.

One hundred and fifty nanograms of each isolated poly(A)$^+$ RNA was separated on formaldehyde-agarose gel electrophoresis and transferred to a Biodyne A transfer membrane (Pall). Probes were radiolabeled with a rediprime DNA labeling system (Amersham) using [α-^{32}P]dCTP. Hybridization was performed by the standard procedure (3). The blots were washed twice with 2x SSC, 0.1% SDS at room temperature for 20 mins and twice with 0.2x SSC 0.1% SDS for 20 mins at 50°C or 60°C. cDNAs used as probes were as follows: rat alkaline phosphatase (4), rat α_1 (I) collagen (5), rat osteocalcin (6), rat osteopontin (7), rat bFGF (8), human BMP-2 (9), rat IGF-I (10), rat IGF-II (11), rat TGF-$ß_1$ (12), mouse *c-fos* (13), mouse *c-jun* (14) and rat ß-actin (15). The cDNAs of alkaline phosphatase and osteopontin were kindly provided by Dr G Rodan, Merck Sharp and Dohme Research Laboratories, Philadelphia. The cDNAs of bFGF and BMP-2 were generous gifts from Professor N Itoh, Kyoto University, Kyoto and Dr Y Maruoka, Tokyo Medical and Dental University, Tokyo, respectively.

In Situ Hybridization

One and 6 hrs after mechanical stimulation, groups of 6 animals were killed after intracardiac perfusion fixation with 4% paraformaldehyde for 15 mins. C6 and C8 were removed, postfixed in 4% paraformaldehyde at 4°C for a further 24 hrs, decalcified in 10% EDTA (Sigma) and embedded in paraffin wax. Rat *c-fos* cDNA(16) was subcloned into pSP72 (Promega, Southampton, UK) using standard procedures (3). The vector was linearised with BamHI and transcribed with T7 polymerase to generate a 2kb antisense strand. The sense probe was generated by linearising with EcoRV and transcribing with Sp6. The cRNA probes were labelled with [^{35}S]UTP using a Sp6-T7 transcription kit (Boehringer Mannheim, Mannheim, Germany) to a specific activity of 1x10^8 cpm/µg.

Sections were cut and mounted onto glass slides coated with 3-aminopropyltriethoxysilane (Sigma). Prehybridization was performed as previously described (2). Sections were then hybridized for 16 hrs at 45°C against radiolabelled probe (specific activity 5 x 10^4 cpm/µl).

Sections were then rinsed with 2x SSC twice at room temperature, 1x SSC/50% formamide once at 50°C, 2x SSC once at room temperature and incubated for 30 mins in 50 µg/ml RNAse A (Sigma) in 2x SSC at 37°C. Sections were then given two more 1x SSC washes at room temperature before a final wash in 0.5x SSC.

For autoradiography, sections were coated with nuclear emulsion (K5) (Ilford, Ilford, UK), stored for 28 days at 4°C, developed and counterstained.

RESULTS

Each caudal vertebra, from which connective tissues, epiphyses and marrow had been carefully removed, yielded 0.7-0.9 µg of poly(A)$^+$ RNA by direct extraction with oligo(dT)-magnetic beads. Northern blotting gave strong signals when probed for genes directly involved in matrix deposition (α_1 (I) collagen, osteocalcin, osteopontin and alkaline phosphatase), confirming a major contribution of bone cells to the extracted poly(A)$^+$ RNA. None of the mRNA preparations from loaded vertebrae (C8) 1-120 hrs after the loading showed a > 2-fold change in mRNA expression levels of bone matrix proteins (Fig.1). The vertebrae (C6) anterior to the anterior loading pins, which represented control vertebrae, showed a similar lack of significant change in gene expression of bone matrix proteins after loading.

Expression of IGF-I, IGF-II and TGF-β_1 was detected in caudal vertebrae by the Northern blotting analysis. No (> 2-fold) change in expression of these growth factors was detected in the mechanically-stimulated vertebrae (Fig.1), while expression of bFGF and BMP-2 were undetectable regardless of the loading. There was, however, an early, rapid and substantial increase in *c-fos* expression in C8, but not C6, to reach 4 times control levels within 1 hr after loading (Fig.2). The increase

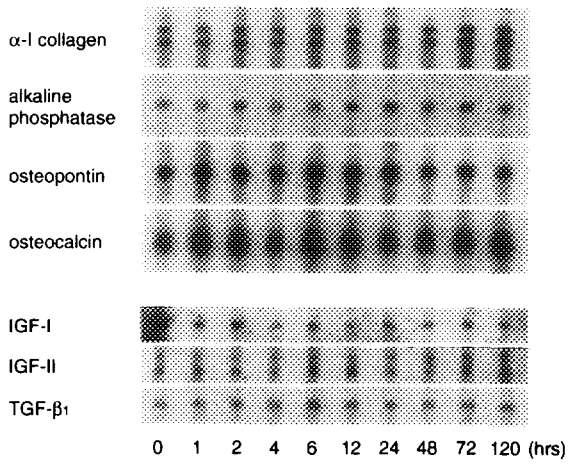

Figure 1. Sequential analysis of gene expression of bone matrix proteins and growth factors by Northern blotting after mechanical stimulation of caudal vertebrae. Poly(A)$^+$ RNA was isolated from loaded vertebra 0-120 hrs after mechanical stimulation.

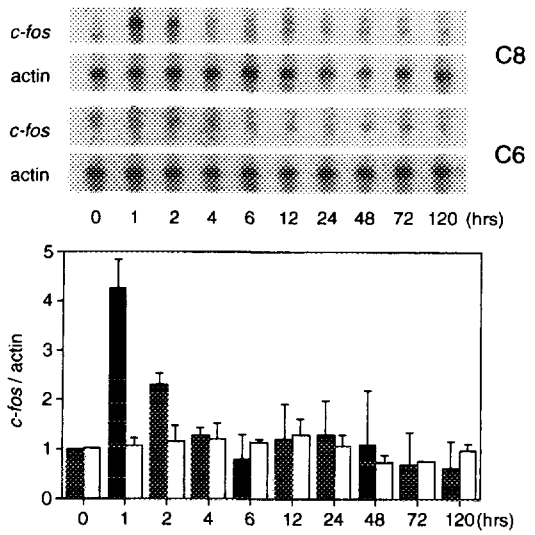

Figure 2. Transient mRNA expression of *c-fos* induced by 5 mins mechanical stimulation in loaded vertebrae (C8) and absence of *c-fos* induction in control vertebrae (C6). Signal intensities of the Northern blots were analyzed quantitatively with a BAS-2000 Bio-imaging Analyzer (Fuji film) and normalized with signals obtained for corresponding samples with a ß-actin cDNA probe. Normalized intensities (*c-fos*/actin) for C8 and C6 are represented in the lower panel as solid and open bars, respectively. Each data point is the mean of results obtained in three independent blotting experiments.

was transient and *c-fos* expression level returned to normal by 4 hrs after loading. *c-jun* was undetectable by the Northern blotting.

The increase in *c-fos* expression correlated with *in situ* hybridization indicating strong expression of mRNA for *c-fos* in osteocytes in the mid-diaphyseal cortex (Fig.3) in the C8 vertebrae from all of the animals killed 1 hr after mechanical stimulation. We noted no detectable expression of *c-fos* in osteocytes in any of the C6 vertebrae. *c-fos* expression was undetectable in osteocytes in any of the C6 or C8 vertebrae from rats killed 6 hrs after mechanical stimulation. Sections labeled with sense riboprobes showed no hybridization.

DISCUSSION

In previous studies in which rat caudal vertebrae are exposed to mechanical stimulation, we have found increased expression of bone matrix genes on cancellous bone surfaces (2). We have not detected such substantial increases in the bulk poly(A)$^+$ RNA preparations by Northern analysis. It is likely that residual paracortical areas of epiphyseal plates and primary spongiosa contribute a high baseline of bone matrix gene expression. Moreover, some of the cancellous trabeculae, and/or the cancellous bone surface cells, upon/within which much of the

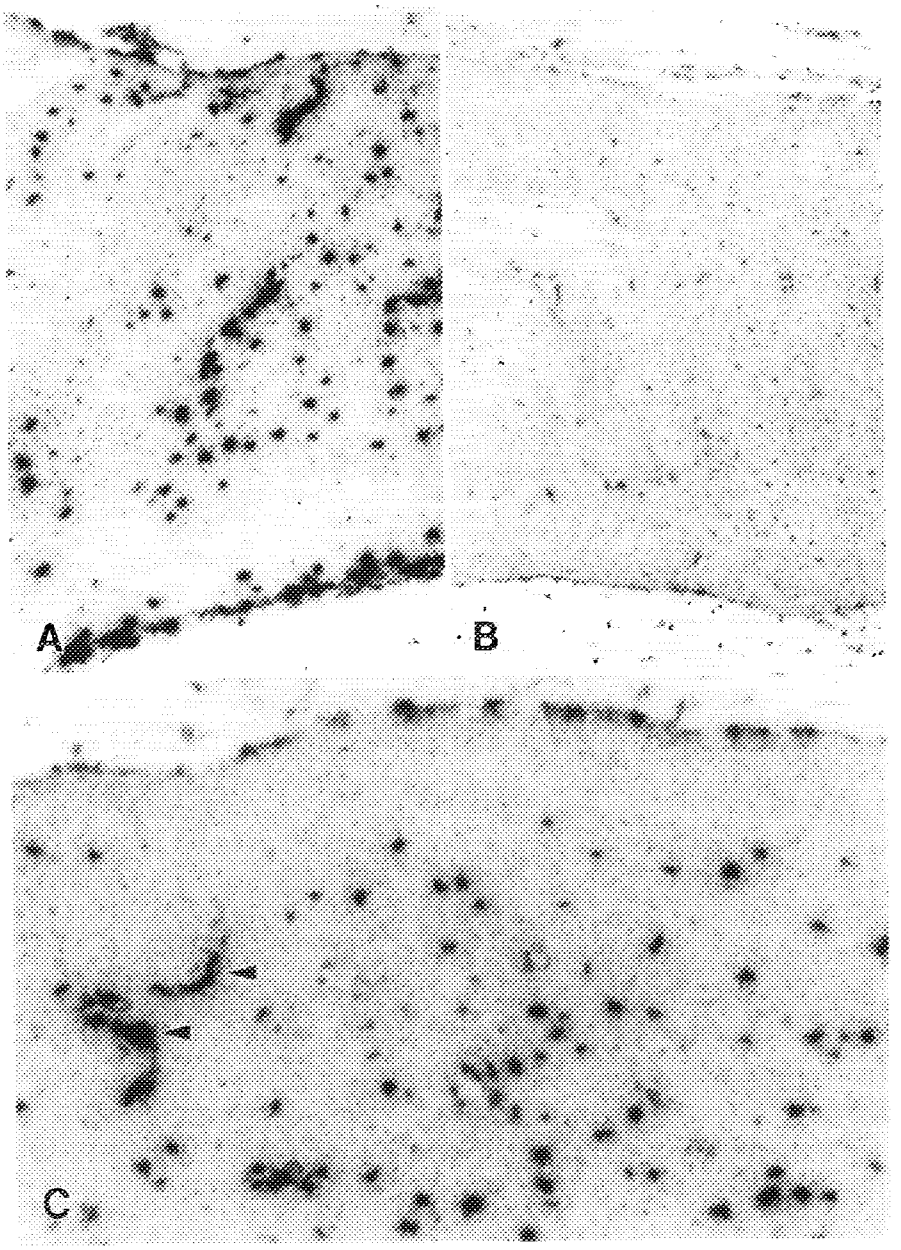

Figure 3. *In situ* hybridization for *c-fos* mRNA. A: many osteocytes in cortex of mechanically stimulated vertebra show strong hybridization. B: nonloaded control vertebra shows no hybridization signal. C: higher magnification of *c-fos*-expressing cortical osteocytes. Note some cells on periosteal surface in both A and C show hybridization, as do cells lining small blood vessels in cortex (arrowheads). A and B x140; C x350.

increase in bone formation occurs, are likely to be lost during removal of the marrow from the vertebrae prior to RNA extraction.

However, we noted a substantial increase in *c-fos* expression by Northern analysis. This increase was associated with intense hybridization over osteocytes, by

in situ hybridization. Increases in expression relative to baseline levels are inherently likely to be greater for transient mRNA species than for species such as matrix protein genes which are expressed for more prolonged periods. Our results are consistent with those of Raab-Cullen *et al* (17), who found increased expression of *c-fos* in tissue scraped from the periosteal surface of rat long bones after mechanical stimulation.

Osteocytes have long been thought likely to play a crucial role in the mechanisms by which bone adapts its structure to the prevailing mechanical environment (1). However, largely due to their inaccessibility, they remain poorly understood. Others have found that mechanical loading increased osteocyte RNA synthesis after 24 hrs *in vivo* (18); we have recently found increased IGF-I mRNA antedates mechanical stimulus-induced bone matrix protein expression (2). The present data identifies strong expression of *c-fos* in osteocytes within 1 hr of mechanical stimulation. The finding of an early response gene in osteocytes, so soon after loading, represents strong evidence for a role for osteocytes in the primary response to mechanical stimulation.

It is of interest that *c-fos* was increased in the bones after loading, since *c-fos* expression has been specifically linked to bone biology. The *v-fos* oncogene was first detected as the transforming gene of mouse osteosarcomas (19); during embryogenesis *c-fos* is expressed specifically in the central nervous system and bone (20-22); osteoblasts are target cells for transformation in *c-fos* transgenic mice (23); and *c-fos* expression precedes bone formation in organ culture and during fracture healing (24-26). Although the role of *c-fos* in osteogenesis is not understood, the clear temporal relationship between the mechanical stimulus, induction of *c-fos*, and subsequent osteogenesis in the present model may enable analysis of the molecular mechanisms by which *c-fos* expression is implicated in bone formation. Thus our results represent strong evidence for a role for osteocytes, and for *c-fos*, in the osteogenic response of bone to mechanical stimulation.

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