c-fos Expression Precedes Osteogenic Differentiation of Cartilage Cells In Vitro

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Abstract. We have investigated the temporal pattern of expression of c-fos in cartilage cells in mouse mandibular condyles. During in vitro cultivation, the progenitor cells in this organ differentiate to osteoblasts, and hypertrophic chondrocytes start to show features indicative of osteogenic differentiation. Prior to these processes we observed two distinct patterns of c-fos expression. High, transient c-fos expression was found in the entire tissue within 30 min of culture. This type of c-fos expression appeared to result from mechanical forces applied during dissection. The second type of c-fos expression appeared in individual cells in the zone of hypertrophic chondrocytes. A varying number of formerly quiescent chondrocytes expressed high levels of c-fos mRNA after between 30 min and 10 d in culture, with a peak in the number of cells between days 1 and 3. c-fos expression in these cartilage cells was followed by DNA replication and expression of genes typifying osteoblastic differentiation. After 7 d in culture, groups of cells with the typical ultrastructural features of osteoblasts, and surrounded by an osteoid-like matrix, were observed in single chondrocyte-type lacunae, suggesting division of chondrocytes and differentiation to osteoblasts. The data suggest that c-fos may play a crucial role in the perturbation of determined pathways of skeletoblast differentiation and in the regulation of endochondral bone formation.

T he protooncogene c-fos is a multifaceted gene which is expressed during growth, differentiation, and development in various experimental systems (for review see Müller and Verma, 1984; Verma and Graham, 1987). Much of the data on the role of the c-fos protein remains correlative, but recent studies have shown that c-fos can act as a transcriptional regulator (Setoyama et al., 1986; Distel et al., 1987; Chiu et al., 1988; Lech et al., 1988; Schönhal et al., 1988) and that it is required in cell proliferation (Holt et al., 1986; Nishikura and Murray, 1987; Riabowol et al., 1988). The potential of c-fos to interfere with normal bone development (Rüther et al., 1987) and to induce bone tumors in c-fos transgenic mice (Rüther et al., 1989), and the detection of c-fos expression in mesodermal web tissue, growth plate of fetal bone, and the developing hip (Done and Gruss, 1987; Sandberg et al., 1988; Togni et al., 1988; Heckl and Wagner, 1989), indicate a specific role of c-fos in the regulation and differentiation of skeletal cells.

The mandibular condyle of the newborn mouse is a powerful model for studying skeletoblast differentiation in vitro. It is composed of distinct zones containing cartilage cells at different stages of differentiation. An outer layer of mesenchyme-like cells, the perichondrium, surrounds a zone of progenitor cells comprising an apical and lateral sheath, which in turn encompasses an area of young chondroblasts and hypertrophic chondrocytes. During in vitro cultivation, cells in the progenitor zone undergo osteogenic differentiation and form new bone (Silbermann et al., 1983, 1986). In vitro infection with the v-fos bearing Finkel-Biskis-Reilly murine osteosarcoma virus (FBR MSV) results in the formation of an osteosarcoma-like lesion with a morphological resemblance to FBR MSV-induced osteosarcomas in mice (Schmidt et al., 1986, 1989; Silbermann et al., 1987; Closs, 1989).

Recent studies have shown that during culture of mandibular condyles not only do cells in the progenitor zone differentiate to osteoblasts, but also that mature cartilage cells start to express genes typifying bone cell differentiation (Strauss et al., 1990). In the following sections we describe the temporal pattern of c-fos expression which precedes the expression of bone cell characteristic genes, cell proliferation, and the appearance of osteoblast-like cells in the zone of hypertrophic chondrocytes.

Materials and Methods

Tissue Culture

Mandibular condyles were dissected from the mandibles of newborn NMRI mice (from the breeding colony of the GSF). The explants were freed from adherent muscle, ligaments, and mandibular bone, transferred onto collagen...
Effect of Serum Factors and Mechanical Stress

Experiments were performed to determine whether the peak level of c-fos expression at 30 min after start of the culture resulted from mechanical stress applied during dissection of the tissue, or stimulation by serum added to the culture medium. Serum effects were tested in condyles which were cultured in the absence of FCS for 6 or 18 h. Serum (10%) was added to the medium, and the cultures were kept for an additional 0.5-6 h. The condyles cultured in serum-free medium showed the same high c-fos expression after a 30-min incubation period as did condyles cultured in serum-containing medium. Addition of serum (10%) at 6 or 18 h after start of the serum-free cultures was not followed by any increase in c-fos expression within the next 2 h. Similarly, c-myc expression was not affected by the addition of serum to the culture medium (data not shown).

In another experiment, mechanical stress was applied to the condyles to simulate the process of dissection. After 6 h of culture in medium containing 10% FCS, i.e., at a time when c-fos RNA expression had returned to the low level present throughout the rest of the culture period (Fig. 3), individual condyles were carefully pinched with forceps several times, avoiding tissue injury. The condyles were cultured for a further 60 min before slot-blot analysis.

DNA Probes

The following probes were used for slot-blot analysis and in situ hybridization: pλ Hac-69A (human actin; Moos and Gallwitz, 1983), pH R28-I (human ribosomal RNA; Erickson et al., 1981), pλfos I (c-fos; FBS; Curran et al., 1982), and pSV c-myc I (murine c-myc; Land et al., 1983; Hf677 [human procollagen I collagen]; Chu et al., 1982). Only the probe-specific inserts were 32P labeled to a specific activity of 5 × 106 cpm/μg DNA using a random primer labeling kit (Amersham Buchler GMBH, Braunschweig, FRG). For in situ hybridizations, the length of the probes was determined by alcaline agarose gel electrophoresis. The average probe size was adjusted to ~500 bp by radiolytic degradation.

Slot-Blot Analysis

Frozen condyles from the same time points were pooled and homogenized in the presence of guanidinium isothiocyanate buffer (Chirgwin et al., 1979) in liquid nitrogen. Total RNA was prepared by ultracentrifugation of thawed homogenate through a CsCl cushion (Chirgwin et al., 1979). Total RNA yield from one condyle was 5.0-5.5 μg. For slot-blot analysis, the RNA was denatured in 2.5 M formamide (0.1 M NaCl) for 15 min at 60°C. 5 μg of total RNA was applied to a Gene Screen Plus membrane (Du Pont Co., Wilmington, DE) using a Miniord II apparatus (Schleicher and Schuell, Dassel, FRG). The filters were hybridized as described (Schön et al., 1986). The amount of transferred RNA was estimated by hybridization of the filters with 32P-labeled human actin or rRNA sequences.

In Situ Hybridization

In situ hybridization was carried out by a modification of the procedures described by Hafen et al. (1983), Wolf et al. (1984), and Deschepper et al. (1986). 8-μm sections were cut on a cryostat at ~20°C and spread on pretreated slides (Brahic and Haase, 1978). The sections were dried on a hot plate for 5 min at 50°C, fixed in freshly prepared 4% formaldehyde (0.1 M phosphate buffer) for 30 min at room temperature, washed in PBS and dehydrated in ethanol, dried, and stored at ~80°C. Before hybridization, the sections were incubated in 0.2 M HCl at room temperature for 20 min, incubated in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate) for 30 min at 70°C, and treated with 1 mg/ml of proteinase K for 15 min at 37°C. The sections were fixed again in formaldehyde, dehydrated in ethanol, and air dried. Prehybridization was performed at 45°C for 3-4 h with 10 ml of 50% formamide, 0.6 M NaCl, 10 mM Tris HCl (pH 7.4), 1 mM EDTA, 1 mg/ml t-RNA, 0.1 mg/ml polyadenylate, 1 mg/ml salmon sperm DNA, and Denhard's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.1% BSA). Hybridization was performed in 10 ml of the same solution containing 1 × 106 cpm of 32P-labeled DNA (specific activity 5 × 108 cpm/μg DNA; the DNA was denatured by boiling for 10 min and chilled on ice) at 45°C for 24 h. After hybridization the slides were washed three times (5 h each) in formamide buffer (50% formamide, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) at room temperature followed by washing in 2×SSC and in 0.2×SSC for 30 min each at 45°C. The slides were rinsed with ethanol ammonium acetate (70-90%/0.3 M), and air dried.

Autoradiography was performed by covering thin sections mounted on nickel grids with a thin film of Ilford L.4 emulsion (Cheshire, UK) from a loop. Sections were exposed for 1-2 wk at 4°C in a dry chamber. The grids were developed with Kodak D-19 developer and fixed in Tetanal Superfix (Tetanal; Nordenstedt, FRG) for 20 min. The slides were counterstained with haematoxylin eosin.

[3H]Thymidine Labeling

Condyles were cultured in the presence of 2 μCi/ml of [3H]thymidine (specific activity 117 Ci/4.3 MBq/mMole; Amersham Buchler GMBH) for the last 2 h of the culture period. Thereafter, the cultures were prepared for EM (see below). 1-μm sections were cut and autoradiography was performed as described for in situ hybridization. Sections were counterstained with toluidine blue.

EM

Cultures were washed in PBS, fixed in 4% glutaraldehyde followed by 1% osmium, dehydrated in alcohol, and flat-embedded in epon. Thin sections were stained with uranyl acetate and lead citrate and viewed in a Zeiss EM 10 CR electron microscope.

Autoradiography was performed by covering thin sections mounted on nickel grids with a thin film of Ilford L.4 emulsion (Cheshire, UK) from a loop. Sections were exposed for 1-2 wk at 4°C in a dry chamber. The grids were developed with Kodak D-19 developer and fixed in Tetanal superfish. Prepared grids were contrasted and viewed as described above.
Results

Osteogenic Differentiation of Progenitor Cells and of Cells in the Zone of Hypertrophic Chondrocytes

Mandibular condyles from neonatal mice represent a cartilagenous tissue which contains distinct zones of cartilage cells at different stages of differentiation. The differentiation process of progenitor cells towards differentiated osteoblasts, and the formation of bone-like tissue within a 7-10-d cultivation period, has been documented previously (Silbermann et al., 1983, 1986). The major morphological changes observed histologically are summarized and depicted schematically in Fig. 1.

During prolonged cultivation, cells in the zone of hypertrophic chondrocytes underwent marked morphological changes particularly in condyles cultured for 7 d or longer. Ultrastructurally, some cells showed osteoblast-like features, i.e., a polygonal shape, eccentric nuclei, and large amounts of RER. Many cells, including those with a chondrocytic appearance, were partly or completely surrounded by an osteoid-like matrix containing collagen fibrils of varying thickness (arrows). Bar, 5 μm.

Temporal Pattern of c-fos and c-myc Expression

To investigate the expression of c-fos in mandibular condyles during a 9-d cultivation period, we first analyzed total RNA extracted from whole condyles. Slot-blot analysis showed high c-fos expression 30 min after start of the culture, followed by a decrease to low levels within 6 h (Fig. 3). Low levels of c-myc expression were found throughout the culture period. The elevated c-fos-expression in 30-min cultures was not followed by detectable elevation of c-myc-RNA expression (Fig. 3).
Expression of c-fos and c-myc. Total RNA was isolated from condyles cultured for the time indicated. RNA was bound to a filter by the slot-blot procedure and sequentially hybridized to actin-, fos-, and myc-specific probes. The probes were stripped from the filter completely after each hybridization.

We performed in situ hybridizations on sections of mandibular condyles to determine the localization of the c-fos and c-myc expressing cells. c-fos expression was not found in any cells in condyles that had been frozen immediately after dissection (Fig. 4, a and b). In contrast, a strong signal was found in condyles that had been cultured for 30 min (Fig. 4, c). The hybridization signal was distributed over the entire tissue. The intensity of the signal corresponded with the cell density in the different zones: i.e., it was highest in the area of progenitor cells. Individual cells expressing c-fos were not prominent.

Similar to the pattern observed by slot-blot analysis, the diffuse type of c-fos expression decreased after 2 h of culture and did not reappear during prolonged cultivation (Fig. 4, d–h). However, high levels of c-fos RNA were found in an increasing number of individually identifiable cells in the basal part of the condyle, i.e., the area of hypertrophic chondrocytes (Fig. 4, c–h). This type of c-fos expression was first observed in a few cells along the basal border as early as 30 min after start of the culture. The number of cells showing high expression peaked between 1 and 3 d, at a time when the diffuse type of c-fos expression was no longer detected (Fig. 4, d–h). With increased time of culture the area of c-fos expressing cells extended towards the zone of chondroblasts. After 3 d the number of hypertrophic chondrocytes expressing c-fos decreased, and only a few c-fos-positive cells were found after day 10.

Distinct c-myc expression was not found in fresh explants (Fig. 5, a and b) or in condyles cultured for 30 min (Fig. 5 c); it was first observed 2 h after start of the culture in a few cells located in the basal area (Fig. 5 d). During culture the number of c-myc-expressing cells increased and the area containing c-myc positive cells extended further into the zone of hypertrophic chondrocytes (Fig. 5 e). In contrast to c-fos, the highest number of c-myc expressing cells was observed between 16 and 24 h of culture (Fig. 5, e and f). At this time, c-myc expressing cells were also found in the perichondrium. No significant c-myc expression was found in the condylar tissue in the late phase of the cultures (Fig. 5, g and h). c-myc RNA could also be observed in some of the cells that had migrated into the underlying collagen sponge (data not shown).

FCS did not affect the level of c-fos or c-myc RNA (see Materials and Methods). However, c-fos expression was observed after manipulation of condyles in culture simulating mechanical stress applied to the condyles in the process of dissection. Slot-blot analysis showed the same high c-fos expression 1 h after mechanical manipulation as observed 1 h after dissection (Fig. 6).

c-fos Expression Is Followed by Cell Proliferation and the Expression of Osteoblast Marker Genes

To examine whether c-fos activation in hypertrophic chondrocytes was followed by DNA replication and cell proliferation, we determined [3H]thymidine incorporation into cellular DNA by autoradiography (Fig. 7). After incubation of freshly obtained explants with [3H]thymidine for 2 h, labeled nuclei were found in cells in the progenitor zone but not in the zone of hypertrophic chondrocytes (Fig. 7, a and e). After 24 h, labeled cells were found in the zone of hypertrophic chondrocytes (Fig. 7 f) as well as in the perichondrium and progenitor zone (Fig. 7 b). The number of labeled cells in the zone of hypertrophic chondrocytes increased further by day 2 (Fig. 7 g). The number of labeled cells decreased in the hypertrophic zone after prolonged culture, whereas the number of labeled cells in the progenitor zone remained constant (Fig. 7, d and h). A consistent finding in 7-d cultures was the appearance of label in cells that had migrated into the underlying sponge (Fig. 7 h). Electron microscope autoradiography confirmed the uptake of [3H]thymidine by the nuclei of mature chondrocytes in areas showing signs of early calcification (Fig. 8).

One of the earliest marker genes expressed at high levels during differentiation of osteoblasts (Yoon et al., 1988) is collagen type I; it is also expressed in chondroprogenitor cells (Castagnola et al., 1988). In situ hybridization on sections of freshly obtained condyles showed expression of collagen type I in cells in the progenitor zone but not in hypertrophic chondrocytes (Fig. 9 a). In cells of the hypertrophic zone, collagen type I expression was first found in the basal area of the condyle after 2 h in culture (Fig. 9 b). After 3 d of culture, collagen type I expression was found throughout the area of hypertrophic chondrocytes (Fig. 9 c).

**Discussion**

The mandibular condyles of newborn mice contain undifferentiated progenitor cells, young chondroblasts, and mature
Figure 4. Localization of c-fos expressing cells. Autoradiographs of sections processed for in situ hybridization with a fos-specific probe and photographed with bright-field (a) or dark-field (b-d), (f) or shadow-cast (e). The areas of progenitor cells (PR), chondroblasts (CB), and hypertrophic chondrocytes (HC) are indicated. Bar, 100 μm.
Figure 5. Localization of c-myc-expressing cells. Autoradiographs of sections processed for in situ hybridization with a c-myc-specific probe. c-myc-expressing cells are observed at 6 h (a and b) and at 24 h (c and d) after treatment with 20 ng/ml EGF. The different zones of cells are indicated in Fig. 3. Bar, 100 μm.
et al., 1990), indicating a switch from chondrogenic to osteogenic differentiation. We have now investigated the expression of c-fos and c-myc protooncogenes which precedes the transient c-fos expression typically expressed during bone cell differentiation (Strauss et al., 1983; Kaczmarek et al., 1985; Colletta et al., 1986). (b) c-fos expression preceded hypertrophic chondrocytes. The transient c-fos expression was not observed in the perichondrium except at the start of culture as a result of mechanical stress. The transient peak of c-myc expressing hypertrophic chondrocytes was larger than that of c-myc expressing cells, and c-fos expression was observed for a longer period of time. We have shown that in this zone of hypertrophic chondrocytes start to express de novo collagen type I osteonectin, alkaline phosphatase, and bone gla protein, genes typical of cells of the osteogenic lineage, within the first 3 d of culture (this study, and Strauss et al., 1990). Both c-fos expression and expression of bone marker genes were seen at a time when the zone of hypertrophic chondrocytes contains a homogeneous population of mature cartilage cells in characteristic lacunae formed by a variably calcified matrix. Neither we nor other investigators have observed other cell types by light or EM within this zone within the first 3 d of culture (Lewinson and Silbermann, 1982; Silbermann et al., 1983, 1986). Taken together these results indicate that the expression of c-fos in the hypertrophic chondrocytes was associated with the subsequent change in gene expression.

The de novo expression of genes typifying cells of the osteogenic lineage, which follows the high expression of c-fos in hypertrophic chondrocytes, suggests that c-fos may control the lineage specificity of skeletal cells. Support for this hypothesis comes from experimental induction of chondroosseous neoplasms by v-fos or c-fos. Newborn mice infected with FBR MSV and FBJ MSV, both carrying the fos oncogene, as well as mice transplanted with in vitro FBR MSV-infected mandibular condyles, develop sarcomas which are characterized by various degrees of osseochondrous differentiation (Finkel and Biskis, 1966; Finkel et al., 1973; Ward and Young, 1976; Schmidt et al., 1986; Silbermann et al., 1987). Furthermore, c-fos transgenic mice develop primarily slowly progressing benign skeletal lesions (Rüther et al., 1987) and later osteochondrosarcomas (Rüther et al., 1989). These heterogeneous tumours comprise populations of cells of both the chondrogenic and osteogenic lineages. In both these cases the v-fos oncogene and the c-fos protooncogene interfere specifically with the differentiation pathway of skeletoblasts.

c-fos expression was not observed in the perichondrium and progenitor zone except at the start of culture as a result of mechanical stress. The transient peak of c-myc expressing cells found in the perichondrium on day 1 coincided with the beginning of enhanced proliferation activity of these cells.
Figure 7. [3H]thymidine uptake in mandibular condyles. Autoradiographs of mandibular condyles (semithin epon sections) pulse-labeled with [3H]thymidine for the last 2 h of the culture period. (a and e) Fresh explant; (b and f) condyles cultured for 24 h; (c and g) for 2 d; (d and h) for 7 d. (a-d) Zone of apical progenitor cells; (e-h) zone of hypertrophic chondrocytes. Bar, 100 μm.
which grew out into the underlying collagen sponge. Presumably, continuously proliferating cells need much lower levels of c-fos product to maintain cell division and undergo differentiation (Nishikura and Murray, 1987; Riabowol et al., 1988). Thus, it is not surprising that c-fos expression could not be detected by in situ hybridization in progenitor cells, although they proliferated and underwent osteoblastic differentiation, since the procedure has a relatively low sensitivity. Low signals of c-fos and c-myc were observed by slot-blot analysis throughout the culture period, indicating a permanent low level of expression. In addition, cells in the progenitor zone already expressed collagen type I at the time of dissection. At this stage they may be highly susceptible to factors that influence the pathway of differentiation even if they are present at low concentration. Indeed, previous studies have reported on expression of c-fos in the perichondrial region of the growth plate of human and mouse long bones (Dony and Gruss, 1987; Sandberg et al., 1988; Heckl and Wagner, 1989), and in rat chondroblasts (Togni et al., 1988).

Our findings indicate that c-fos may play a role in endochondral bone formation in vivo. It has been suggested that cartilage cells may be actively involved in bone formation (for review see Cowell et al., 1987) and might differentiate to osteoblasts (Crelin and Koch, 1967; Silbermann and Frommer, 1974; Kahn and Simmons, 1977; Yoshioka et al., 1988). Our data show that chondrocytes can reenter the cell cycle and express genes de novo that are characteristic for osteogenic differentiation. The ultrastructural observations also indicate the onset of cell division and possible osteoblastic differentiation of hypertrophic chondrocytes. It is tempting to assume that c-fos may have an inductive role in these processes; interestingly, c-fos expression has also been found in bone forming cells in the zone of endochondral ossifica-

Figure 8. [3H]thymidine uptake in chondrocytes. Autoradiograph of an electron microscopic section through chondrocyte-like cells in the hypertrophic zone of a mandibular condyle cultured for 24 h, showing early signs of cartilage calcification. Note the heavily labeled nucleus (arrow). (double-headed arrows) Foci of calcification. Bar, 5 μm.

Figure 9. Expression of collagen type I in chondroprogenitor cells and hypertrophic chondrocytes. Autoradiographs of sections processed for in situ hybridization with a collagen type I-specific probe. Dark-field illumination. Lateral-basal parts of mandibular condyles of (a) fresh explant; (b) explant cultured for 2 h; and (c) explant cultured for 3 d. In fresh explants the signal was confined to the cells in the progenitor zone. Hypertrophic chondrocytes also expressed collagen type I RNA with increased time in culture. Bar, 100 μm.
tion (Heckl and Wagner, 1989). Further investigations are in progress to clarify the regulatory effects of c-fos on bone formation.

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