軟骨細胞の増殖，分化における細胞内カルシウムの役割とシグナル伝達系の解析

（研究課題番号　12470309）

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はしがき

関節硝子軟骨は自己修復能に著しく乏しい組織であり，ひとたび損傷されると硝子軟骨での修復は困難である．生じた軟骨損傷は力学的負荷により進行し，最終的には変形性関節症を惹起する．軟骨欠損において修復機転が働かない理由のひとつは軟骨細胞が厚い細胞外基質で取り囲まれているために細胞同士が接触できず，軟骨細胞に与えられた機械的・化学的刺激に対する情報（シグナル）が他の軟骨細胞に伝達されにくいことが考えられる．すなわち，他の組織と異なりこのシグナル伝達系が不良で，修復機転に働くべき他の細胞反応が緩慢であるために軟骨の変性過程が進行すると考えられる．

軟骨細胞の機械的刺激に対するシグナル伝達系とそれによる細胞の増殖，分化過程が明らかになれば，これを調節することによってこれまで困難とされていた軟骨修復の新しい治療手段を確立できるかもしれない．

本研究では軟骨修復に大きく関与するといわれる c-fos 遺伝子の発現と細胞内カルシウム濃度による調節機構に関し研究を行った．

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Involvement of ATP, Increase of Intracellular Calcium and the Early Expression of *c-fos* in the Repair of Rat Fetal Articular Cartilage

Abstract

To compare the potential of adult and fetal animals to repair articular cartilage, we investigated the early process after creating superficial defects in the femoral knee cartilage using rat models. In fetuses at 19 days of gestation, both chondrocytes and the extracellular matrix responded remarkably by 48 hours after artificial injury of superficial defect. Staining patterns with safranin O revealed that by 1 hour after injury, some components of the extracellular matrix around the wound were modified, and the change spread from the limited region to the entire knee cartilage in 24 hours. The chondrocytes in the area surrounding the wound transiently expressed increased level of *c-fos* from 1 to 6 hours. The wound remained 1 day after birth, i.e., 72 hours after injury, but was completely repaired 10 days after birth. In contrast, neither visible responses nor transient *c-fos* expression was observed in 12-week-old adult articular cartilage 48 hours after injury. Then, we examined the relationships between intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the induction of *c-fos* expression in the cartilage. Applications of ATP or Ca^{2+} ionophore A23187 that increase $[Ca^{2+}]_i$ induced immediate expression of *c-fos* in primary cultured chondrocytes. One μM ATP elicited the increase of $[Ca^{2+}]_i$ in chondrocytes in fetal but 1 mM was required in adult cartilage slices. Our findings showed that there is a signaling pathway, apparently active in the repair of fetal but not adult articular cartilage, that involves intercellular transfer of ATP, increase of $[Ca^{2+}]_i$, and expression of *c-fos* in the cartilage.

Introduction

Articular cartilage plays important roles in the smooth movement of joints by buffering shearing force and reducing friction. It consists of abundant extracellular matrix and sparsely arranged chondrocytes. It lacks blood supply and nerve control, and its nutrition depends on diffusion from the joint fluid. There are two types of cartilage defect. In injury of the superficial layer of the cartilage, the wound is limited to the cartilage layer and does not extend to the underlying subchondral cancellous bone. Repair of the defect is not spontaneous, and early change in osteoarthritis involves such injuries (Buckwalter 1992). In the other type of defect, the injury penetrates the cartilage and reaches to the subchondral bone marrow, which is rich in many kinds of progenitor cells including hematopoietic and other types of cells, and blood vessels. Although it is partially repaired, the wounds become filled mainly with fibrous cartilage that does not function normally (Shapiro et al. 1993). In 1743, W. Hunter, a pioneer of cartilage research, stated, "From Hippocrates to the present age it is universally allowed that ulcerated cartilage is a troublesome thing and that, once destroyed, is not repaired" (Hunter 1743). Despite many attempts to overcome the problem, there are currently no effective ways to cure articular cartilage defects, which eventually result in joint dysfunction and pain (Zimmermann 1989).

Fetal tissues, including skin, palate, tendon, and bone, demonstrate remarkable regenerative capacity during embryonic development (Longaker et al. 1990; Longaker et al. 1992; al-Qattan et al. 1993; Stone 2000). For example, fetal skin wounds heal rapidly and perfectly, although this ability appears to diminish as development proceeds (Nodder and Martin 1997). Concerning regeneration in fetal articular cartilage, there are a few reports: spontaneous repair of superficial defects in a fetal lamb model (Namba et al. 1998); healing responses of chick embryonic rib hyaline cartilage *in vitro* (Waker et al. 2000).

To clarify the differences of healing between fetal and adult articular cartilage, we carefully compared early responses to wounding, and studied the involvement of

c-fos expression in the process. The product of the protooncogene *c-fos*, which composes a nuclear transcription factor that regulates expression of target genes in various cells, (Angel and Karin 1991) has been shown to play a functional role in wound healing. In a confluent culture of NIH 3T3 fibroblasts, monolayer scratch-wounding immediately induced the transient expression of *c-fos* in cells at the wound edges (Verrier et al. 1986). *In vivo*, the expression of *c-fos* was induced in cells at the epidermal wound margin in rat embryo (Martin and Nobes 1992) and in rat liver after partial hepatectomy (Thompson et al. 1986). The early expression of *c-fos* after injury may be a key step in the early repair process of superficial defects of articular cartilage.

Localized communication between the damaged cells and the surrounding cells could also be important in tissue repair (Sammak et al. 1997). Adenosine triphosphate (ATP) has been shown to be a paracrine signaling molecule (Dubyak and El-Moatassim 1993; Abbracchio and Burnstock 1994; Chen et al. 1995; Lewis et al. 1995; Sugioka et al. 1996). Chondrocytes secrete ATP in a response to stimuli such as mechanical stress (Graff et al. 2000) and communicate with each other through the extracellular matrix using ATP (D'Andrea and Vittur 1996; Koolpe and Benton 1997; Elfervig et al. 2001). Additionally, ATP has been shown to inhibit the breakdown and stimulates the synthesis of proteoglycans, which are important components of the extracellular matrix of cartilage (Brown et al. 1997). ATP-mediated intercellular communication and signaling pathway might also be important in the repair process of damaged cartilage. The presence or absence of these processes may underlie the disparity between fetal and adult repair capacities. With these concepts in mind, we compared the patterns of *c-fos* gene expression in rat fetal and adult articular cartilage after injury, and investigated the involvement of extracellular ATP in the process.

Materials and Methods

Experimental animals

On the 19th day of gestation (E19), 1–7 fetuses each of 57 pregnant, 12-week-old, 300–360 g Wistar rats were operated on to injure their knees. The number of fetuses in a mother was usually 12. The period of pregnancy of Wistar rats was 20 days and the delivery was on the 21st day. At E19, the primordial structure including cartilage of the knee is formed, and primary ossification in the center of the long bone is present. We intensively examined the knees of operated fetuses up to 48 hours after injury before their birth. We also observed the repair process 1 and 10 days after the birth, i.e., 72 hours and 12 days after injury, respectively. However, only a few fetuses were observed after the birth because the operated fetuses were difficult to deliver and their mothers frequently ate them when they found unusual marks on the newborn rats. Twenty-six fetuses were used for RT-PCR. The number of fetuses used for histology and *in situ* hybridization is summarized in Table 1. Twelve were used for preparing cartilage slices.

For the adult experiments, 50 male Wistar rats, 12-weeks-old and weighing 300–360 g, were used. Twenty-five were used for histological observation of injury in the articular cartilage and *in situ* hybridization, 21 for RT-PCR, and 4 for preparing cartilage slices. Since the articular cartilage of the 12-week old rats was structurally mature, we refer to the tissue as "adult" cartilage.

For isolation of chondrocytes, fifteen 5-week-old males, weighing 100–150 g, were used because it was technically difficult to obtain enough cells from adult or fetal cartilage.

The animals were anesthetized with an intraperitoneal injection of 0.5 mg/kg Nembutal® (Pentobarbital Sodium, Dainippon Pharmaceutical Co., LTD. Osaka, Japan). All animals were treated and sacrificed according to the guidelines for the use of experimental animals of Shimane University School of Medicine.

Surgery

Fetal and adult rats were operated as follows.

Fetal rats: The uterus of each pregnant rat was incised, and a hind leg of a fetus was carefully exposed from the uterus under a binocular microscope. A 4 mm-long incision was made in the anterolateral aspect of the knee and the trochlear surface of the articular cartilage of the distal aspect of femur was exposed. Transverse incisions of 150 to 200 μm-deep were made in the cartilage on the medial and lateral sides of the trochlear groove using a specially designed instrument (Meira Co. Ltd., Nagoya, Japan). The other knee of the fetus was not operated as a non-treated control. As a sham control, the trochlear articular cartilage of another fetus was exposed without creating defects. Treated fetuses were marked either by cutting the tip of the tail or leaving a piece of thread in the knees and ankles to distinguish operated ones after birth. Then, the fetal skin was sutured by 8-0 nylon thread. The fetus was returned to the uterus. Warm sterile saline solution was injected into the uterus to restore amniotic volume. The uterus was then sutured and the abdomen was closed.

Adult rats: On the surface of the trochlear articular cartilage of the right knee joint, approximately 150 μm-deep defects were created using the procedure above. The other knees, exposed without creating a defect, were used as controls. The wounds were closed and the animals were allowed to move freely.

Histology

The distal femurs were dissected at 0, 1, 3, 6, 12, 24, 48 and 72 hours after the operation, fixed in 4% paraformaldehyde for 24 hours, and decalcified in 0.2 M EDTA, pH 7.5 at 4°C for 1 to 3 and 21 days for fetuses and adults, respectively. The specimens were embedded in paraffin and cut into 5 to 7.5 μm-thick sections. The specimens were examined by light microscopy after being stained with safranin O and fast green using conventional methods.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from adult articular cartilage or fetal knee joint

cartilage using the RNeasy Mini Kit (QIAGEN KK, Tokyo, Japan). RT-PCR was performed using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, UK). The PCR was carried out for 25-33 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec. Primers were designed from rat *c-fos* and glyceraldehyde-3-phosphate dehydrogenase gene (*G3PDH*) mRNA sequences of GenBank accession number X06769 and AB017801, respectively. Primer sequences of *c-fos* were CAG AAG GGG CAA AGT AGA GC and GAG AAG AGG CAG GGT GAA GG, and of *G3PDH* were GCC AAA AGG GTC ATC ATC TC and GCC TGC TTC ACC ACC TTC TT. Each experiment was repeated 3 times.

***In situ* hybridization**

Digoxigenin-labeled RNA probes were prepared using DIG-labeling mix (Roche, Basel, Switzerland) with the cloned RT-PCR fragment of *c-fos* as a template. Sections were treated with 12 μg/ml proteinase K for 15 min at room temperature, acetylated, and prehybridized in 50% formamide in 2X SSC buffer (0.3 M NaCl, 30 mM Na citrate, pH 7.0) for 30 min at 48°C. Hybridization was carried out for 12 hours at 48°C in a buffer composed of 50% formamide, 2X SSC, 1 mg/ml tRNA, 0.2 mg/ml ssDNA, 10% dextran sulfate, and 1X Denhardt's solution. The sections were washed twice with 50% formamide in 2X SSC and twice with 0.1X SSC, each for 30 min, at 48°C. The hybridized probes were detected with Anti-Digoxigenin-POD Fab fragments (Roche) and colorized using a TMB (3,3',5,5'-tetramethylbenzidine) Substrate Kit (VECTOR LABORATORIES, Burlingame, CA, USA).

Effects of intracellular Ca^{2+} in expression of *c-fos* in primary cultured chondrocytes

Chondrocytes were isolated from knee, shoulder and hip joint articular cartilage of 5-week-old rats, using a conventional protocol (Tsukazaki et al. 1994), and cultured in DMEM with 10% fetal bovine serum in 24-well tissue culture plates for 5 to 7 days. Before reaching the confluence, some portions of cells were exposed to 0 (control), 100 μM ATP, or 20 μM Hoechst A23187 (Behring Diagnostics, La Jolla, CA,

USA), for 10 min at 37°C. Then cells were washed twice and incubated in the fresh culture medium for a further 20 min. Remaining cells were preincubated in the presence of 10 μM BAPTA-AM (Dojindo Co. Ltd., Kumamoto, Japan) for 30 min and then treated with either 0 or 100 μM ATP as above and incubated in the fresh culture medium for a further 20 min. All treatments above were done in DMEM either with or without fetal bovine serum. The total RNA was extracted from each culture, and the expression of *c-fos* was analyzed by RT-PCR.

Change of intracellular Ca^{2+} in ATP-stimulated fetal and adult articular cartilage slices

Thin slices of cartilage were prepared from medial and lateral articular condyles of fetal or adult knees. DMEM supplemented with 10% fetal bovine serum was used throughout the experiments. The cartilage slices were immediately immersed in the medium in a flask, and incubated for 60 min with bubbling of 95% O_2 and 5% CO_2 at 37°C as described for recovery of brain slices (Sakmann and Stuart 1995). A flow rate of the air was carefully controlled to avoid foaming of the medium during the incubation. This procedure was applied for recovery of cartilage slices from the preparation damage and for dilution of factors that might be secreted from injured chondrocytes. It was also to minimize influences of serum on the experiments. Then, the cartilage slices were incubated in the presence of 2 μM fura-2 AM (Dojindo) and 0.1% cremophore (Sigma, St. Louis, Missouri, USA) for 40-60 min. After loading of fura-2, the slices were placed in a recording chamber as described (Sakmann and Stuart 1995). Individual cells were observed using an epifluorescence microscope with an excitation wavelength of 340 nm (Olympus IMT-2). We only used specimens that showed fluorescence enough to distinguish the shape of each cell in the slices. ATP, 1 μM to 1 mM, was applied to the specimen, and without washing, the intracellular calcium concentration ($[Ca^{2+}]_i$)-dependent fluorescence changes were monitored using an image processing system (DVS-3000, Hamamatsu Photonics, Japan) and recorded on video tape. A continuous image-subtraction method (i.e., real-time subtraction of an

image acquired at one moment, referenced image, from live images) was used to obtain the time courses of the changes in $[Ca^{2+}]_i$ (Furuya and Enomoto 1990). This method is suitable to measure changes of $[Ca^{2+}]_i$ at the video rate, although absolute values of $[Ca^{2+}]_i$ were not measurable. The time course of the relative changes of fluorescence intensity in individual cells was obtained from the recorded images using the AG-5 image processor board and macro command of Scion Image (Scion Corporation, Frederick, MD, USA).

Results

Repair process of cartilage injury in fetuses

The time course of recovery from defects in fetal femoral cartilage was observed for 72 hours after injury (Table 1). We operated on 99 fetuses, and 53 specimens were successfully examined. Up to 48 hours after the operation, fetuses were taken from the uterus, and more than half of the operated fetuses were available for analysis and showed the repair of the wound. The healing process continued after 72 hours although only 2 specimens could be examined. In addition, two specimens were obtained 12 days after injury (10 days after the birth).

Fig. 1 shows typical results. There were no differences in results of both non-treated and sham-operated controls in which the knee cartilage could be exposed to blood. Plates A to G in Fig. 1 show intact knee cartilage (the top row) while H1 to N4 (the second to fourth rows) show the damaged cartilage on the other leg, from 0 to 48 hours after injury. The V-shaped cleft in the fetal epiphysial cartilage, immediately after its creation, is shown in plates H1 and H2. In the normal tissue, the extracellular matrix of the cartilage was strongly stained with safranin O (Fig. 1, A to G). Safranin O stains negative charges in glycosaminoglycans, such as chondroitin sulfate and keratan sulfate, in the extracellular matrix of the cartilage (Rosenberg 1971). From 1 hour after the injury, the safranin O-staining became weak in the limited area around the wound, while no changes were observed in other areas of the cartilage (Fig. 1, I1 and I2). After 3 to 6 hours (Fig. 1, J1, K1, J2, K2 and J3), many nuclei of chondrocytes in the safranin O-less-stained area (indicated with lines in J2) were condensed (red arrows in J3). This suggested these chondrocytes were dying. After 12 hours, the defects were filled with matrix poorly stained with safranin O (Fig. 1, L1, L2 and L3).

After 24 hours, the staining pattern of the injured cartilage by safranin O dramatically changed. The safranin O-negative area spread into the entire region of the knee cartilage, even into the opposite tibia side of the joint (Fig. 1, M1). This suggested that there was some change or modification in the cartilage extracellular

matrix. The wound region was filled with the acellular matrix, and only traces of dead cells remained (Fig. 1, M2 and M3). After 48 hours, the entire knee cartilage was still safranin O-negative (Fig. 1, N1, N2, N3 and N4). Since the other side of the femoral cartilage from the wound, i.e., the hip joint and ankle cartilage, was stained as in intact controls (Fig. 1, N4), it was not a staining artifact. The superficial part of the wound was repopulated with chondrocytes presumably by migration of cells from the surrounding area (Fig. 1, N2 and N3). The deeper part of the wound was still acellular.

After 72 hours, the wound remained acellular and surrounded densely by chondrocytes (Fig. 1, P1 and P2). Some cells seemed to migrate into the wound from the superficial layer of the cartilage or from the synovium. Cell density in the surrounding area as well as in the whole cartilage remarkably increased. This was due to intensive growth of the tissue because it was also observed in the control knee (Fig. 1, O). The extracellular matrix of whole knee had not recovered yet, as indicated by the lack of staining with safranin O.

Since the survival ratio of operated fetuses was low, and operated newborn rats were killed and removed by their mothers, we could obtain only two specimens to observe later stages of the repair process. Ten days after birth, both specimens showed the same degree of recovery and growth. The control knee had grown bigger (diameter of joint: 4 mm) and the secondary ossification had taken place in the center of the joint cartilage of femur and tibia (Fig. 1, Q1 and Q2). Although we examined sections every 60 μm from medial to lateral aspect of the operated knee, the wound was not observed throughout the specimen. The wound seemed to be almost repaired (Fig. 1, R1 and R2).

Expression of *c-fos* in injured fetal cartilage

We examined the time course of *c-fos* expression in the cartilage of the fetal knee joint after injury, by RT-PCR (Fig. 2, A). The joint region of the fetal femur excluding the proliferative zone, of which diameter and length was about 2 mm each, was carefully taken and total RNA was extracted. Since RNA from the injured region

was diluted with that of the intact majority of the cartilage, the analysis had to be qualitative. As a control, RNA was extracted from the same region of both the non-treated knees and sham controls. In the injured fetal knees, the level of *c-fos* expression immediately after the operation was the same as that of the control, in which only a trace of *c-fos* was detected. However, the expression increased by 1 hour. The increased levels of *c-fos* expression were present until 6 hours, but decreased to the baseline by 12 hours (Fig. 2, A).

The spatial expression of *c-fos* was observed by *in situ* hybridization (Fig. 3). With non-treated, intact knees of E19 fetuses, the expression of *c-fos* was evident in bone marrow including osteoclasts, muscles and skin (Fig. 3, A to D, blue signals in cellular cytoplasm). It has been reported that *c-fos* is expressed in chondrocytes in the epiphysial cartilage and osteoclasts in the developing bone marrow in the human articular cartilage (Sandberg et al. 1988). However, the expression was undetectable in chondrocytes in the epiphysis of rat E19 (Fig. 3, E and I) to E21 fetuses (data not shown). With operated fetuses, it was also undetectable in the joint region immediately after injury (data not shown). One hour after the injury, chondrocytes in the area surrounding the injury expressed *c-fos* mRNA (Fig. 3, F and J). Three hours after, the expression of *c-fos* was evident in the chondrocytes under the superficial layer of the cartilage near the wound (Fig. 3, G and K). The expression continued until 6 hours (Fig. 3, H and L), but became undetectable at 12 hours after the injury (data not shown).

Repair and expression of *c-fos* in injured adult cartilage

Mature rat articular cartilage has a thickness of 150 to 300 μm and a layered structure with much lower cell density than that found in the fetal tissue. It is rich in extracellular matrix that stains positive with safranin O (Fig. 4, A). We created defects of about 150 μm-deep that did not reach to the subchondral bone or violate any blood vessels in the cancellous bone. One hour after injury, no changes were observed (Fig. 4, B). Even forty-eight hours after the injury, only a few dead chondrocytes were

observed adjacent to the defect, and staining with safranin O was weak in the matrix immediately surrounding the cleft. Otherwise the wound was unchanged (Fig. 4, C). These findings demonstrated that the cartilage defects in the adult articular cartilage were not repaired and the clefts remained over the observation period.

Expression of *c-fos* examined by RT-PCR indicated that in the injured knees, the expression was very weak as in the control, and there were no significant changes during the 48 hours after the injury (Fig. 2, B). By *in situ* hybridization, *c-fos* mRNA was undetectable in chondrocytes around the defect over the observation period of 48 hours (Fig. 4, E, F, H, I). This was similar to observations made in intact adult cartilage (Fig. 4, D, G).

Control of *c-fos* gene expression by intracellular Ca^{2+} in primary cultured chondrocytes

We examined the effect of increases in $[Ca^{2+}]_i$ on the early expression of *c-fos* in primary cultured chondrocytes using reagents that affect $[Ca^{2+}]_i$. Chondrocytes show fibroblast-like growth in monolayer culture regardless of the age of the donor animals (Aulthouse et al., 1989; Archer et al., 1990; Bonaventure et al., 1994), but recover the chondrocyte phenotype when they are cultured three-dimensionally (Bassleer et al. 1986; Delbruck et al. 1986; Aulthouse et al. 1989; Archer et al. 1990; Bonaventure et al. 1994; Häuselmann et al. 1994). The primary chondrocyte cultures are useful for investigating some characteristics of chondrocytes.

After the application of 100 μM ATP, *c-fos* expression was increased in the chondrocytes (Fig. 5, lanes 1 and 2). Application of UTP also induced the expression of *c-fos*, and the effect of 10 μM UTP was approximately equal to that of 100 μM ATP (data not shown). Both ATP and UTP are the agonist of a purinergic receptor P_{2Y2}. We first detected the presence of P_{2Y2} receptor in both fetal and adult cartilages by RT-PCR, and by sensitivity to suramin, a specific antagonist of P_{2Y2} (data not shown). The addition of 20 μM Hoechst A23187, a Ca^{2+} ionophore that works as a Ca^{2+} channel on the surface, also induced the expression of *c-fos* (Fig. 5, lane 3). These results

suggested that an increase of $[Ca^{2+}]_i$ was coupled with the expression of *c-fos*. This theory was supported by the fact that incubation of the cells in the presence of BAPTA-AM, a calcium-chelating reagent that inhibits and delays Ca^{2+} concentration changes, inhibited the ATP-induced expression of *c-fos* (Fig. 5, lanes 4 and 5). In addition, the presence or absence of fetal bovine serum in the treatment medium did not affect the results in the reaction (data not shown).

ATP induced increase of $[Ca^{2+}]_i$ and expansion of Ca^{2+} signaling in fetal and adult articular cartilage

Effects of ATP on the $[Ca^{2+}]_i$ in chondrocytes in cartilage slices were measured. In real-time subtracted images of fura-2 fluorescence, bright and dark spots showed the increase and decrease of $[Ca^{2+}]_i$, respectively, in each chondrocyte at the same moment relative to a reference (background) image acquired at the start of subtraction (Fig. 6). In fetal cartilage, periodic spontaneous change of $[Ca^{2+}]_i$ (oscillation) was observed in a small number of chondrocytes before application of ATP (data not shown). Ratio of cells with oscillating $[Ca^{2+}]_i$ to cells with non-oscillating $[Ca^{2+}]_i$ varied among cartilage slices, most by less than 1%. After application of either 1 or 100 μM ATP, $[Ca^{2+}]_i$ transiently increased in some cells with non-oscillating $[Ca^{2+}]_i$ (Fig. 6, A1 to A5 and B1 to B5). Although numbers of ATP-responding cells varied among specimens, they were larger than that of cells with oscillating $[Ca^{2+}]_i$. In adult articular cartilage, oscillation of $[Ca^{2+}]_i$ was also observed but rare (data not shown). The application of either 1 μM (data not shown) or 100 μM ATP (Fig. 6, C1 to C5) did not induce an increase in $[Ca^{2+}]_i$ in any cells which had not previously manifested oscillating $[Ca^{2+}]_i$. To observe the response in some cells, 1 mM ATP was necessary (Fig. 6, D1 to D5). Both in fetal and adult cartilage, not all cells responded to ATP. Some cells responded to ATP and increased $[Ca^{2+}]_i$ so quickly before the background subtraction was reset that they showed apparent decrease of fluorescence (dark spots in Fig. 6, D3 to D5). Influence of ATP application on the cells with oscillating $[Ca^{2+}]_i$ both in fetal and adult cartilage was obscured because of their rare occurrence. In addition, ATP did not

induce oscillation in other cells.

Time course of relative change of $[Ca^{2+}]_i$ after the application of ATP in some individual cells was compared in fetal and adult cartilage (Fig. 7A, B). In a fetal cartilage slice (Fig. 7A), there were cells that did not show obvious response to 100 μM ATP (Fig. 7A, cell 1 and 2), as well as cells that first showed increase of $[Ca^{2+}]_i$ and then return to basal levels (Fig. 7A, cell 3, 5 and 6). Some cells began responding within 10 sec after the application of ATP, and their fluorescence apparently decreased below the basal level after background subtraction was reset because background $[Ca^{2+}]_i$ at the time of subtraction had already increased (Fig. 7A, cell 4). The pre-onset time, duration, and amplitude of the response varied from cell to cell. The cells in adult cartilage slices showed such responses to 1 mM ATP (Fig. 7B). There were cells that showed increase of $[Ca^{2+}]_i$ after the application of ATP (Fig. 7B, cell 5 and 6) and cells that did not (Fig. 7B, cell 1 to 4). Strength of the response also varied among cells (Fig. 7B, cell 5 and 6). In the specimen, the response took more than 1 min to start after application of the ATP. This might be partly due to the rate of ATP diffusion into the cartilage, and to locations of cells in a slice. Cells located near the surface of a slice might be exposed to ATP soon after the application and might increase $[Ca^{2+}]_i$ immediately, whereas cells buried deep in the cartilage matrix might respond later.

Discussion

The dramatic change of fetal cartilage after injury

Repair responses to articular cartilage defects were remarkably different between fetuses and adults. Injuries in fetal articular cartilage were completely repaired by 10 days after the birth. The presence of the healing process within fetal cartilage defects has been previously reported (Namba et al. 1998; Waker et al. 2000). We observed cell death at wounds, change of the cartilage matrix concluded by safranin O-staining, and transient increase of *c-fos* expression within 48 hours in fetuses (Figs. 1, 2 and 3) but not in adults (Fig. 4). This is the first description of the changes in fetal cartilage after injury *in vivo*.

Since *c-fos* regulates the expression of the matrix metalloproteinase (MMP) family that hydrolyzes proteoglycans (Quinones et al. 1994), it might have induced the cartilage extracellular matrix modification that caused reduction of safranin O staining after the injury. *c-fos* might promote the repair of damaged cartilage by inducing the activation of proliferation or cellular migration of chondrocytes, and the change of the cartilage matrix might allow the migration of chondrocytes to the defect in fetuses.

Involvement of transient expression of *c-fos* in repair of damaged fetal cartilage

In the early processes of tissue repair, *c-fos* activates transcription of downstream genes to stimulate cellular proliferation and movement into the wound (Thompson et al. 1986; Verrier et al. 1986). It has also been shown that cellular injury elevates *c-fos* expression in fetal skin. Since this lesion undergoes complete recovery of epithelia within 24 hours, the rapid induction of *c-fos* after injury in this model supports a role for *c-fos* in regulating expression of downstream genes involved in the repair (Martin and Nobes 1992). A similar elevation in *c-fos* expression also occurs in injury of fibroblasts (Verrier et al. 1986), endothelial cells (Feldman et al. 1992), and intestinal epithelial cells (Dieckgraefe et al. 1997). *c-fos* also affects cartilage formation by playing crucial roles in proliferation and differentiation of chondrocytes (Wang et al. 1991; Watanabe et al. 1997).

On the basis of this information, we presumed that the immediate expression of *c-fos* controlled the expression of downstream genes involved in migration, differentiation and proliferation of chondrocytes in early phases of cartilage repair. On the other hand, since *c-fos* also has degenerative roles in rheumatic arthritic cartilage (Tsuji et al. 1996; Tsuji et al. 2000), it is possible that unregulated expression of *c-fos* can cause destruction of the cartilage.

ATP induced the increase of *c-fos* expression

The expression of *c-fos* is induced transiently as an immediate early response to many extracellular signals such as mitogenic factors, differentiation factors, and neurotransmitters (Cochran et al. 1984; Greenberg and Ziff 1984; Kruijer et al. 1984; Müller et al. 1984; Müller and Wagner 1984; Hunt et al. 1987). We found that either ATP or UTP, or A23187, all of which increase $[Ca^{2+}]_i$, elicited the immediate early expression of *c-fos*. BAPTA-AM, which decreases $[Ca^{2+}]_i$, reduced this expression (Fig. 5).

When the cartilage is damaged *in vivo*, ATP could leak from the cytoplasm of damaged or dead chondrocytes and diffuse into the extracellular space, because concentration of ATP in cytoplasm (on the order of mM) is much higher than that in extracellular matrix or in serum (on the order of μM) (Born and Kratzer 1984). The rat model of postoperative pain suggested a role for ATP that leaked from the cytoplasm of damaged cells (Tsuda et al., 2001). ATP is also actively secreted from chondrocytes in a response to stimuli such as mechanical stress (Graff et al., 2000). These facts suggest the existence of relatively high local concentrations of extracellular ATP at wound sites of cartilage injury.

ATP and UTP bind to P_{2Y2} purinoreceptors on the cell membrane (Leong et al. 1994), activate phosphatidyl inositol turnover that induces transient increases of $[Ca^{2+}]_i$ by the release of Ca^{2+} from intracellular stores, and thereby stimulate Ca^{2+}-dependent signal transduction pathways in cells (Dubyak and El-Moatassim 1993; Sanderson et al. 1994; Boeynaems et al. 1996; Sammak et al. 1997), including chondrocytes (D' Andrea and

Vittur, 1996). ATP also activates other pathways in chondrocytes (Berenbaum et al. 2003). Ca^{2+}-dependent signal transduction pathways induce the expression of *c-fos* in many types of cells such as neuronal and endothelial cells (Hsieh et al. 1993; Yamamoto et al. 1993; Ghosh et al. 1994). We observed increase of $[Ca^{2+}]_i$ after the application of ATP to the cartilage both in fetuses and adults, although chondrocytes in tissue showed heterogeneous responsiveness to ATP (Fig. 6 and 7). In addition, we previously found that stimulation of cultured chondrocytes with UTP induced the secretion of ATP (our unpublished results). Therefore ATP or UTP released from injured chondrocytes, can be one of the paracrine factors controlling the induction of *c-fos* in adjacent chondrocytes in cartilage repair *via* Ca^{2+}-dependent signal transduction pathways.

Differences in cartilage repair between fetuses and adults

What causes the difference between fetus and adult responses? There are two possibilities. First is the efficiency of cellular communication with paracrine factors. In adult cartilage, cellular density is low, and the extracellular matrix predominates, which might prevent the signal transduction with ATP. Second is the cellular responsiveness to extracellular signals, as shown by the sensitivity and responsiveness of chondrocytes in cartilage slices to extracellular ATP (Fig. 6 and 7). The sensitivity to ATP was three magnitudes lower in adults than in fetuses, which can be influenced by the number and activity of ATP receptors. Thus the injury signals might not spread far enough to reach surrounding cells and thereby trigger wound repair in the adult cartilage.

In conclusion, spontaneous repair of superficial defects of articular cartilage was demonstrated in fetal rat cartilage, and was associated with the *c-fos* gene expression in the chondrocytes. This did not occur in adult cartilage. The *c-fos* expression patterns indicated high and low repair potentials of fetal and adult articular cartilage. ATP and $[Ca^{2+}]_i$ were key control factors in the induction of *c-fos* gene expression. Further investigations into the molecular mechanisms of the healing process in the fetal cartilage defect may help in the development of treatments for adult cartilage defects and osteoarthritis in humans in the future.

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Figure legends

Fig. 1. The repair process of superficial defects in fetal articular cartilage of the femoral condyle.

Sections were stained with safranin O and fast green. The cartilage extracellular matrix was stained red with safranin O, while nuclei were counterstained dark green with fast green. The 1st (top) to the 3rd rows (A to N2) show the time course of recovery from injury. A to G, control; H1 to N1, injured; H2 to N2, higher magnification of H1 to N1. From the left, 0 (immediately after injury), 1, 3, 6, 12, 24 and 48 hours after the injury. The specimen in each figure is arranged as follows: the femur is on top, the tibia is on the bottom, the anterior aspect of the knee is to the left, and the posterior to the right. In the 4th row, J3, L3, M3 and N3 have the same magnification as J2, L2, M2 and N2, respectively, where the superficial layer of the cartilage is toward the top. N4 shows another section of the hind leg 48 hours after the injury. The anterior is on the top and the femur is to the left. The inset in N4 shows the ankle. The last row, O, P1 and P2 are cartilage 72 hours after the injury (1 day after the birth). O: control. P1 and P2 are images of the injured cartilage at low and high magnifications, respectively. Q1 and Q2 are images of the control cartilage 10 days after birth at low and high magnifications, respectively. R1 and R2 are images of injured knees 12 days after the injury, i.e., 10 days after birth, at low and high magnifications, respectively. The center of secondary ossification had been forming in the knee cartilage. The orientation of figures Q1 to R2 is the same as that of A to N2. Black arrowheads in H1 to N1 and N4 indicate the defects. White lines in J2 show an area that stained poorly with safranin O (safranin O-less-stained area). Red arrows in J3 indicate shrunken nuclei of chondrocytes in the injured region. Red asterisks in N3 show the superficial region of the wound that was repopulated with chondrocytes. Blue arrows in Q1 and R1 indicate the area where blood vessels were invading the cartilage into the center of secondary ossification. a: ankle; f: femur; lg: ligament; t: tibia. Scale bars: 0.5 mm (A to N1 and N4), 100 μm (H2 to N2, O, P1,

Q2 and R2) and 40 μm (J3, L3, M3, N3 and P2), 1 mm (Q1 and R1).

Fig. 2. Time course of *c-fos* expression in control and injured articular cartilage analyzed by RT-PCR. A: fetuses; B: adults. PCR products amplified after 26 cycles of reaction were compared. The upper and lower rows, in A and B, show the expression of *c-fos* and a housekeeping gene *G3PDH*, respectively, in the cartilages taken from each knee 0, 1, 3, 6, 12, 24 and 48 hours after the operation as described in Materials and Methods. Left (control) and right (injured) lanes show non-treated and injured knees, respectively. PCR products of *G3PDH* reached a plateau after 30 cycles of reaction (#). *c-fos*: 361 bp; *G3PDH* : 448 bp.

Fig. 3. Expression of *c-fos* in the repair process of superficial defects in fetal articular cartilage of the femoral condyle. *c-fos* mRNA was detected by *in situ* hybridization with a sense probe (A, B and E to H), or with an antisense probe (C, D and I to L). A to E and I are tissue sections of intact cartilage. Magnification of regions enclosed in rectangles b and e in A are shown in B and E, respectively. Magnification of regions enclosed in rectangles d and i in C are shown in D and I, respectively. F and J, G and K, and H and L are sections of femoral epiphysial cartilage 1, 3 and 6 hours after the injury, respectively. In G and K, a magnified view of a part of the cartilage was inserted. *c-fos*-positive signals (blue) were present in the cytoplasm of chondrocytes in J, K and L. In controls, only background staining was seen in the extracellular space (A, B, E and I). Black arrowheads in F to H and J to L indicate the injury. ac: articular cartilage; bm: bone marrow; hc: zone of hypertrophic chondrocytes; o: osteoclasts (black arrows); p: perichondrium; and s: skin. Scale bars: 0.5 mm (A and C), 100 μm (B, D and E to L).

Fig. 4. The repair process of superficial defects in adult articular cartilage of the femoral condyle. A to C: sections stained with safranin O and fast green. A: a

non-treated control. B and C: 1 and 48 hours after the injury, respectively. The defect exhibited almost no change for 48 hours. D to I: expression of *c-fos* analyzed by *in situ* hybridization with a sense probe for negative controls (D, E and F) or with an antisense probe (G, H and I). Expression of *c-fos* was negative in intact cartilage (D and G), and also in cartilage at 1 hour (E and H) and 48 hours (F and I) after injury. Dark blue at the surface of cartilage (I) was background staining. Scale bars: 200 μm (A to C); 100 μm (D to I).

Fig. 5. Effects of reagents that change $[Ca^{2+}]_i$ on expression of *c-fos* in primary cultured chondrocytes analyzed by RT-PCR. The upper and lower rows show the expression of *c-fos* after 23 cycles and *G3PDH* after 21 cycles of reaction, respectively. Chondrocytes were treated as indicated below, washed and incubated in the fresh culture medium for 20 min. Treatments were as follows: 1, in the medium for 10 min (control); 2, in 100 μM ATP for 10 min; 3, in 20 μM Hoechst A23187 for 10 min; 4, in 10 μM BAPTA-AM for 30 min, then in 10 μM BAPTA-AM for 10 min; 5, in 10 μM BAPTA-AM for 30 min, then in 10 μM BAPTA-AM and 100 μM ATP for 10 min. PCR products of *G3PDH* reached a plateau after 25 cycles of reaction (#). *c-fos*: 361 bp; *G3PDH* : 448 bp.

Fig. 6. ATP-induced Ca^{2+} responses in fetal and adult cartilage slices. ATP was applied to fura-2-loaded cartilage slices, then focus of the live images was manually adjusted, and recording of background-subtracted fluorescent images was started. The period between ATP application and resetting of the subtraction varied among experiments. Images in each row were acquired from the same field of a specimen. The first column shows live images of fura-2 fluorescence in individual chondrocytes in fetal (A1 and B1) and adult cartilage slices (C1 and D1). The 2^{nd} to 5^{th} columns show subtracted images after ATP application and resetting of the subtraction at 10-sec intervals. There was only noise immediately after resetting the subtraction (A2, B2,

C2 and D2). Bright and dark spots in subtracted images show the increase and decrease, respectively, of $[Ca^{2+}]_i$ in cells (A3 to A5, B3 to B5 and D3 to D5). A2 to A5 show effects of 1 μM ATP on $[Ca^{2+}]_i$ of fetal cartilage. In this case, image subtraction was reset at 38 sec after the application of ATP. A2 to A5 were images 13 to 43 sec after the reset. Small dark spots appearing in A2 to A5 were not cells but dust. B2 to B5 show effects of 100 μM ATP on fetal cartilage 1 to 31 sec after resetting the subtraction. The reset was 15 sec after the application of ATP. C2 to C5 show effects of 100 μM ATP on adult cartilage 2 to 32 sec after resetting the subtraction. The reset was 30 sec after the application of ATP. D2 to D5 show effects of 1 mM ATP on adult cartilage 2 to 32 sec after resetting the subtraction. The reset was 30 sec after the application of ATP. Scale bars: 100 μm.

Fig. 7. Time course of change of fura-2 fluorescence that reflects change of $[Ca^{2+}]_i$ in chondrocytes of fetal and adult cartilage slices. Panel A: fetal chondrocytes before and after the application of 100 μM ATP (cells 1 to 6). Panel B: adult chondrocytes before and after the application of 1 mM ATP (cells 1 to 6). Fluorescence intensity in several individual cells before and after ATP application was obtained by data acquisition from subtracted video images. Since the fluorescent intensity of the cells at the start of the recording was subtracted from that at each second, the amplitude of fluorescence began from the same value (set as 0). Owing to disturbance of the images by the application of ATP, subtraction was repeated after adjusting focus to correct the fluorescent intensity of real images of the cells at the time of reset. The time of ATP application and resetting of fluorescence image subtraction (set as time 0) were indicated by arrows. Since the values between times of ATP application and of reset subtraction were taken from disturbed images, they did not reflect cellular responses (open circles).

Table 1. Number of fetal specimens operated for the observation of the repair process.

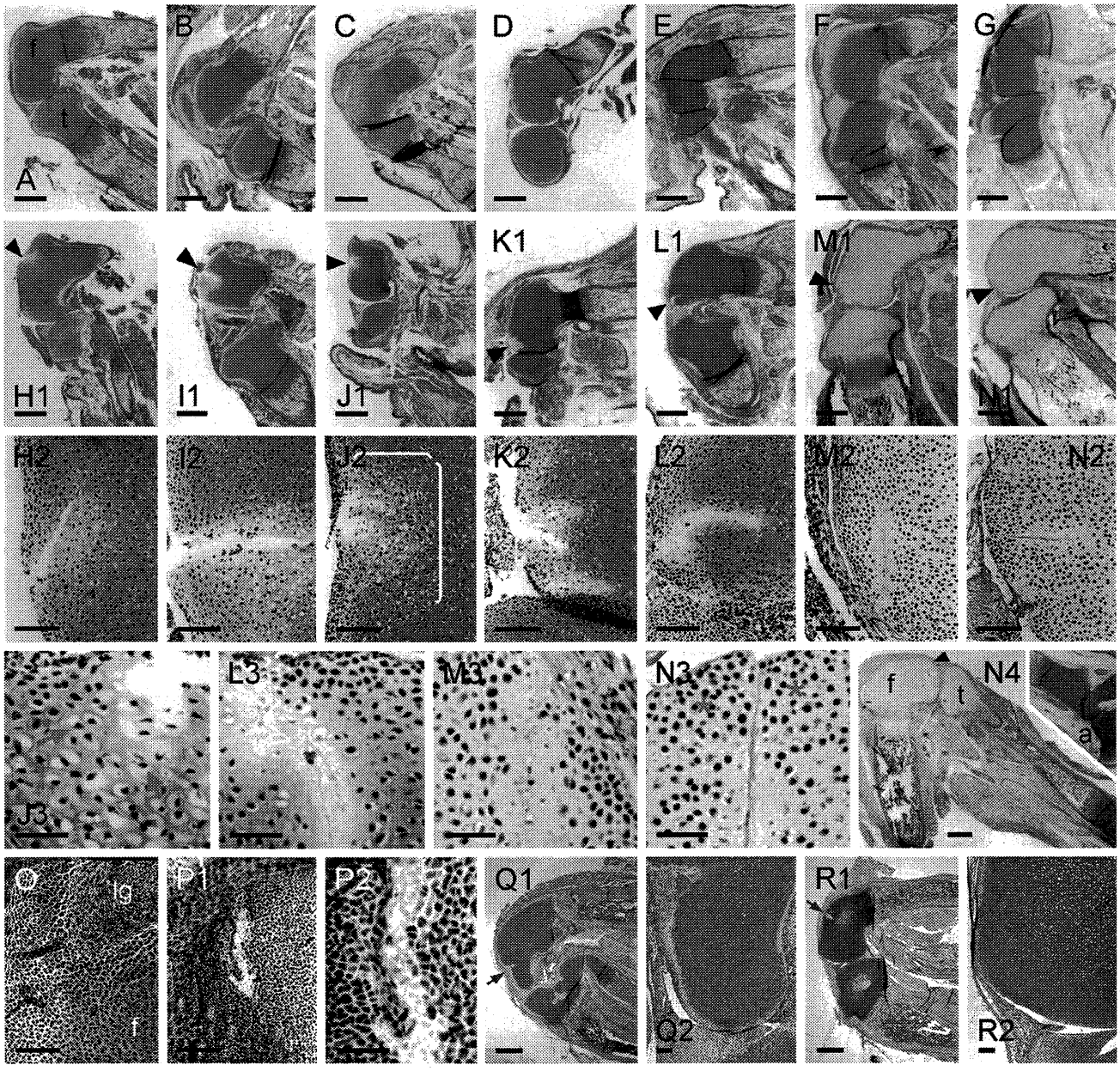
Operated fetuses	Time after injury (hours) 0	1	3	6	12	24	48	72	Total
Total	9	10	10	10	13	15	18	14	99
Dead[a]	0	1	1	2	4	5	6	8[d]	27
Failed[b]	1	2	2	2	3	2	3	4	19
Good[c]	8 (3)	7 (3)	7 (3)	6 (3)	6 (3)	8 (5)	9 (6)	2	53 (26)

[a] Fetuses died due to the procedure or death of their mothers after the operation.

[b] Wound was not found in the operated knee probably due to failures in introduction of injury in the cartilage, so that the repair process was not observed.

[c] Fetuses were alive until the time of observation, and wound was successfully observed. Numbers in the parentheses were fetuses used for the RT-PCR for *c-fos*. The rest of the fetuses were used for histological analysis and in situ hybridization.

[d] Operated infants were consumed by mothers after the birth before the observation.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M1
N1
H2
I2
J2
K2
L2
M2
N2
J3
L3
M3
N3
f
t
N4
a
O
lg
f
P1
P2
Q1
Q2
R1
R2

Fig. 1

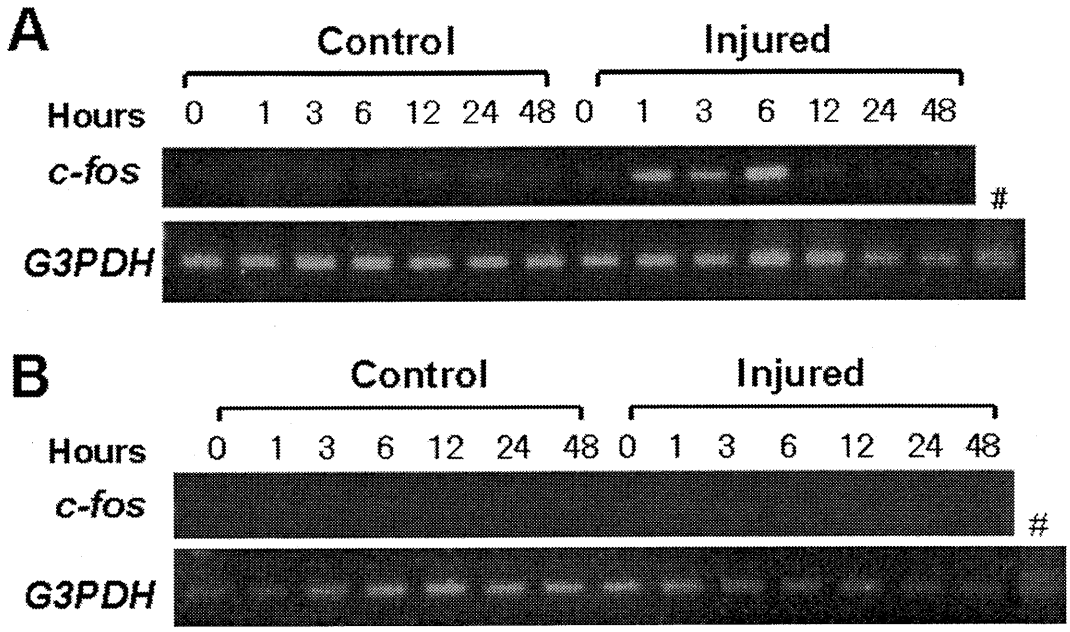
A
Control
Injured
Hours
0 1 3 6 12 24 48 0 1 3 6 12 24 48
c-fos
#
G3PDH
B
Control
Injured
Hours
0 1 3 6 12 24 48 0 1 3 6 12 24 48
c-fos
#
G3PDH

Fig. 2

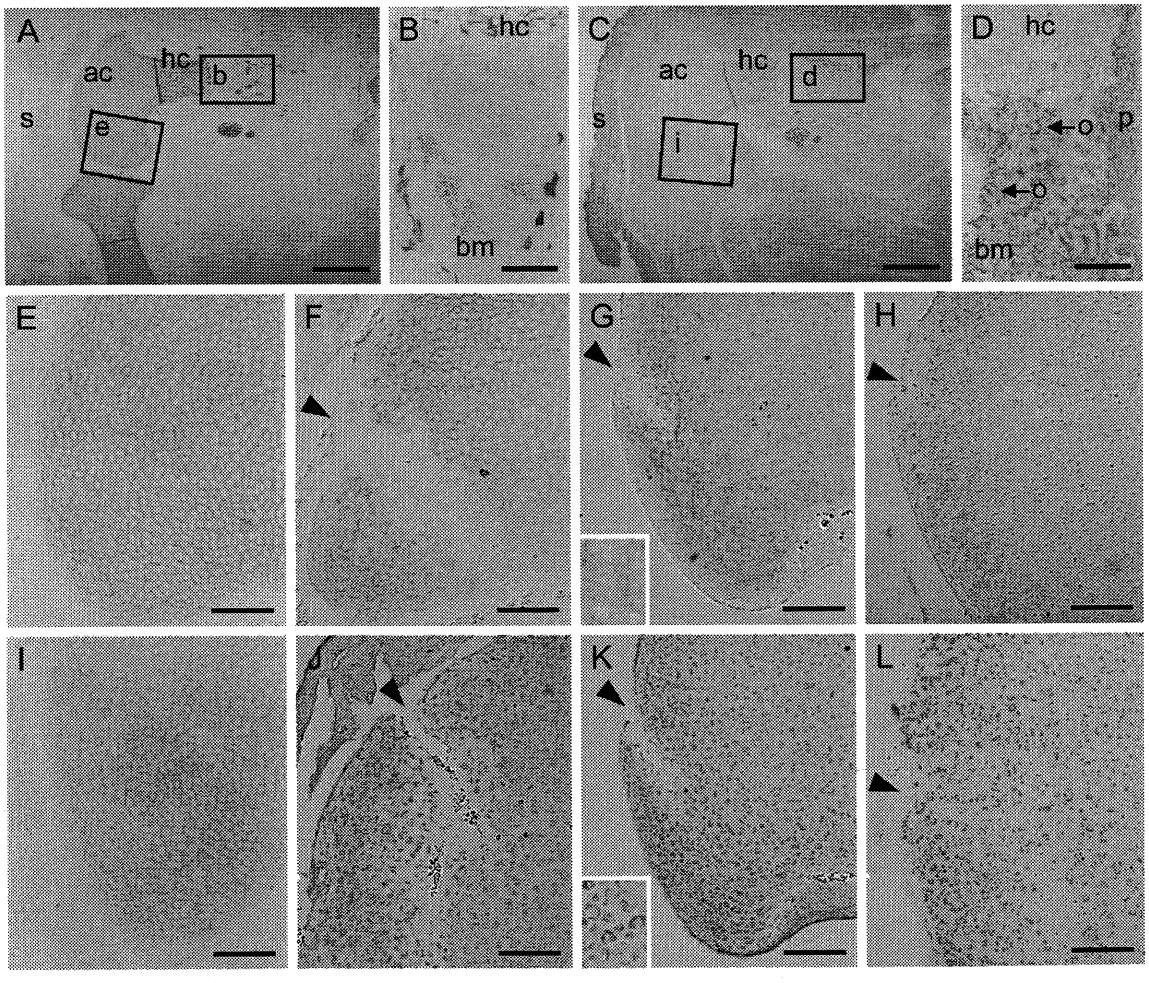
A
ac
hc
b
s
e
B
hc
bm
C
ac
hc
d
s
i
D
hc
o
p
o
bm
E
F
G
H
I
K
L

Fig. 3

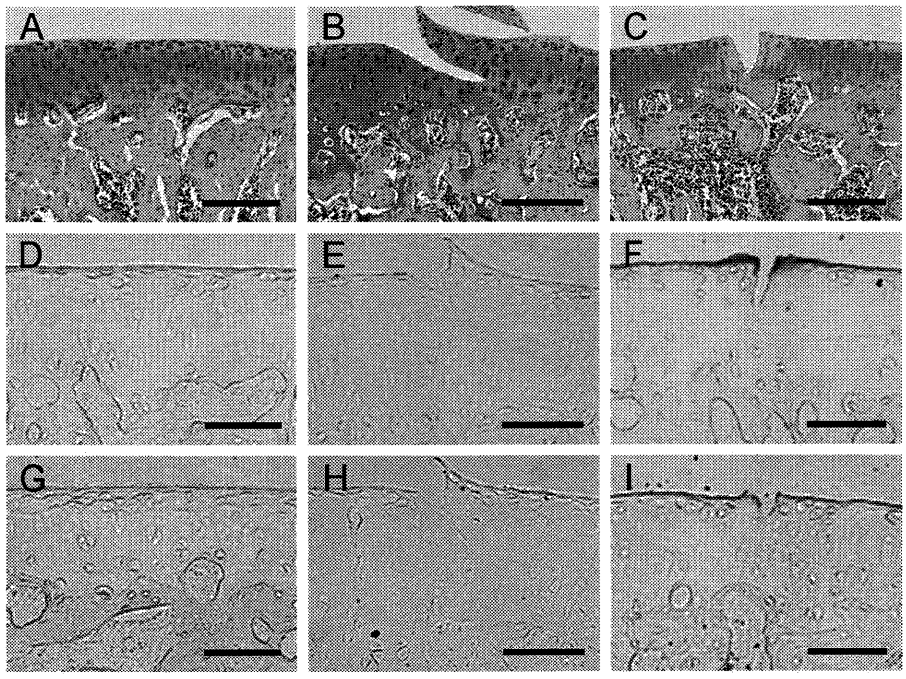

Fig. 4

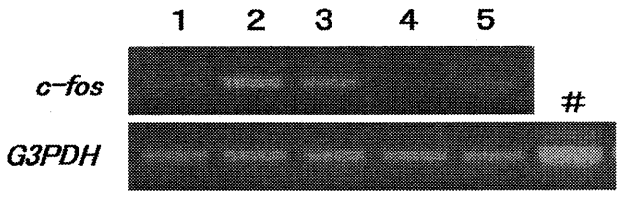

Fig. 5

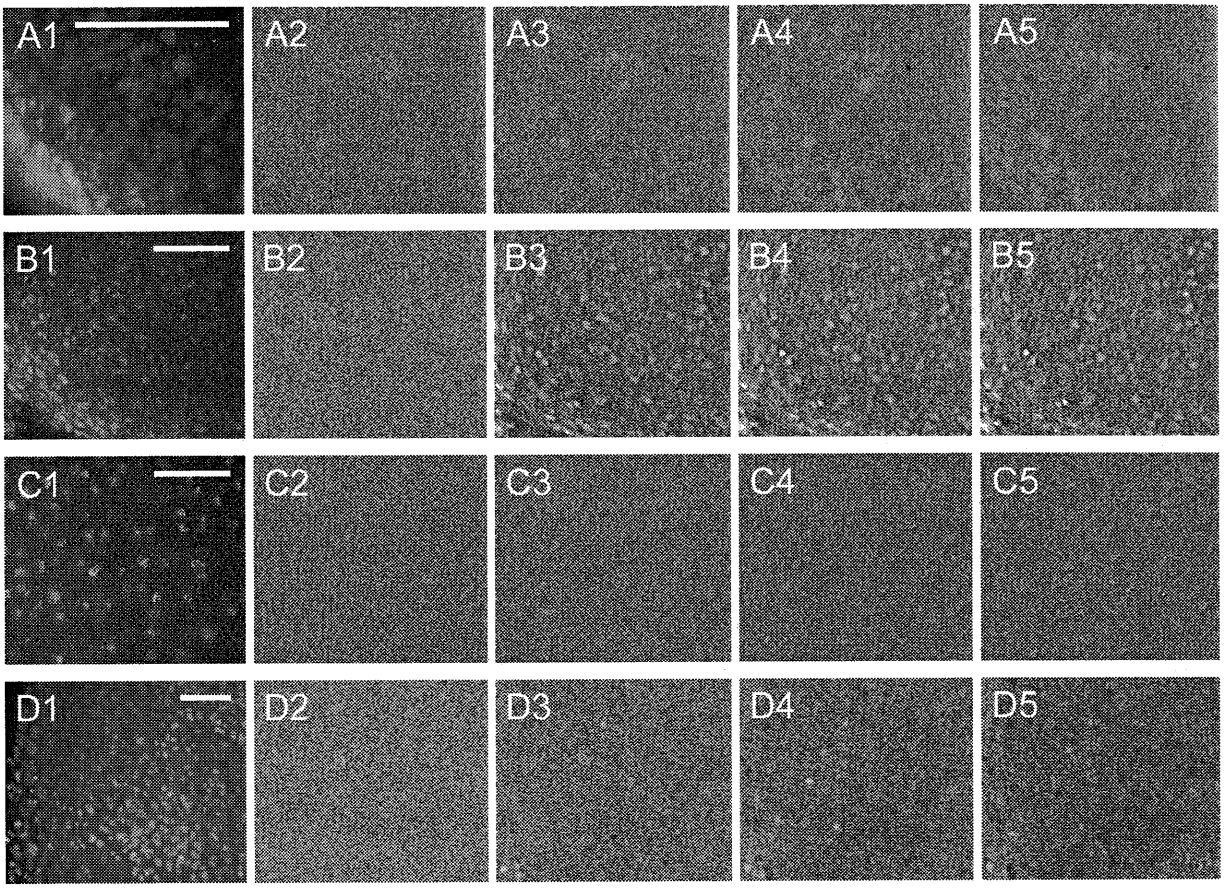
A1
A2
A3
A4
A5
B1
B2
B3
B4
B5
C1
C2
C3
C4
C5
D1
D2
D3
D4
D5

Fig. 6

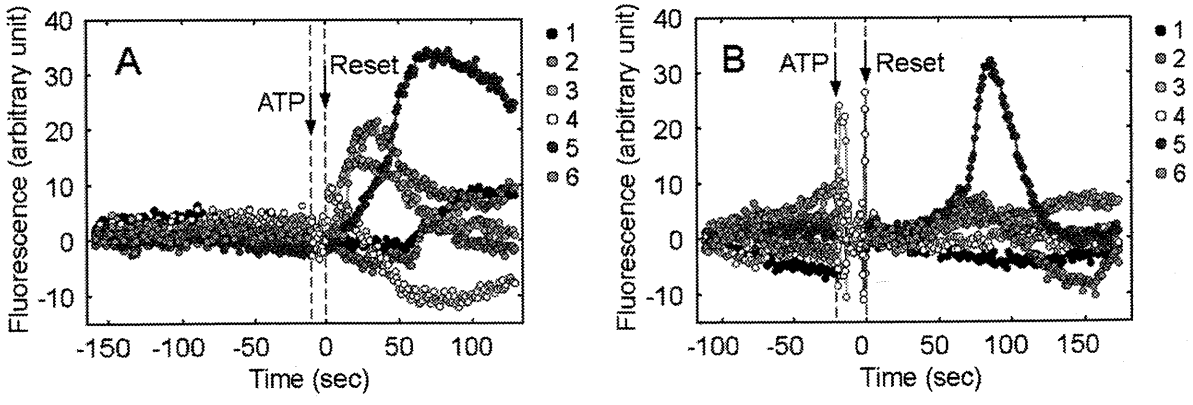
A
ATP
Reset
Fluorescence (arbitrary unit)
Time (sec)
40
30
20
10
0
-10
-150
-100
-50
0
50
100
1
2
3
4
5
6
B
ATP
Reset
Fluorescence (arbitrary unit)
Time (sec)
-100
-50
0
50
100
150

Fig. 7