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原　著

Expression of Heat-Shock-Proteins in the Differentiation Process of Chondrocytes

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Abstract

To know the role of HSP70 in chondrocytes, HSP70 expressions under heat stress or in a non-stress condition were examined by using electrophoresis, immunohistochemistry, and *in situ* hybridization. As a result, HSP70 was observed in proliferating chondrocytes in the micro-mass cultures without heat stress. In this culture, chondrocytes maintained the terminal differentiation potency. On the other hand, HSP70 did not appear in the chondrocytes in the logarithmic growth phase of the monolayer culture. In growth plates *in vivo*, HSP70 expressions in the chondrocytes located in the resting and hypertrophic zones were observed with immunohistochemistry. Appearance of HSP70 mRNA was also confirmed by *in situ* hybridization in the proliferating zone of growth plate. HSP70 can be expressed not only in chondrocytes under heat stress but also in the cells without stress, and the expression would be related to the terminal differentiation of chondrocytes. HSP70 is surmised to promote hypertrophy and calcification by stopping protein synthesis in chondrocytes which possess terminal differentiation potency.

Introduction

Heat-shock-proteins (HSP) are induced in a cell in response to heat stress (1, 2). HSP expression has been observed in a wide range of species, including plants, microorganisms, and mammals (2, 3), and the structure of HSP has been well maintained in the evolution process (3, 5). Therefore, they have been considered to be basic and important proteins for living creatures. HSP have also been called "stress proteins" because they are expressed not only by heat but also by other stresses such as inflammation, virus infections and exposure to heavy metals (6–9).

Concerning the HSP inductions in chondrocytes, Madreperla in 1985 (10) reported that HSP70 was induced proportionally to the strength of heat stress. Kubo et al. (11) studied chondrocytes of os-

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Key words: chondrocyte, heat shock protein (HSP), differentiation, micro-mass culture, growth plate

teoarthritis (OA) patients, and demonstrated that HSP70 was induced proportionally to the severity of OA. Several studies have also showed HSP expressions in experimental arthritis, rheumatoid arthritis, and rheumatic diseases (12–18). Consequently, HSP could be closely related to tissues which consist of chondrocytes, and it will be important to elucidate the biological role of HSP in chondrocytes.

The present study examined biological effect of HSP in normal rat chondrocytes to elucidate expression sites of HSP70, i.e., the major HSP in chondrocytes, and its possible biological effects, by using cultures of growth plates and micro-mass cultures of chondrocytes separated from articular cartilage.

Materials and Methods

1. SDS-polyacrylamide gel electrophoresis

From ten 7-day-old Wistar male rats (purchased from Shimizu Experimental materials, Kyoto, Japan), chondrocytes of articular cartilage were sterily collected from the distal part of the femur and the proximal part of the tibia. The cells were cut into pieces in phosphate-buffered saline (PBS), and treated 4 hours at 37°C in 0.025% collagenase (collagenase S-1, Nitta Gelatin, Japan). Isolated chondrocytes were spin down and washed in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (20% FBS/DMEM) 3 times, then seeded on 6-well multi-plastic dishes at a density of 5×10^4 cells/3 ml, and cultured for 10 days in a humidified chamber in 5% CO_2/95% air environment at 37°C. Culture medium was changed every 3 days.

To give heat stresses, the plates of 10 days culture were immersed into an incubation cistern for 30 min, which temperature was maintained at 39, 41, 43, or 45°C. Then, medium was changed to 20% FBS/DMEM without methionine but containing 2.22 MBq/ml of ^{35}S-labeled amino acid, and maintained for 4 hours. Cell were then washed with PBS 3 times, removed by trypsin, washed 3 times, and stored at −80°C until used. These samples were dissolved with sample buffer in accordance with the method of Laemmli (19), their protein levels were adjusted, applied to a polyacrylamide slab gel with 7.5% sodium dodecyl sulfate, and electrophoresed (SDS-PAGE).

The control cells were cultured in an incubator at 37°C, and applied to SDS-PAGE in the same procedure.

2. Immunohistochemistry

(i) Micro-mass culture cells

Chondrocytes (1×10^5 cells/1 ml), which were obtained according to the above mentioned procedure, were seeded in a 15 ml tube, centrifuged at 1000 rpm, spin down for 5 min, and cultured (20). On Day 28 of the culture, the cells were fixed in 4% paraformaldehyde, embedded into paraffin, cut into 5 μm slices by using a microtome, and mounted on slides. After deparaffinized, cells were blocked, and incubated with the primary antibody, i.e., anti-HSP70 antibody (1 : 2000) (Amersham), for 30 min at room temperature. At this stage, a negative control was included using anti-rat IgG. Then, cultures were incubated for 30 min with the biotinylated 2nd antibody, and stained with avidin-biotinylated-peroxidase complex (ABC) method. Nuclei were stained with methylgreen.

To observe HSP expressions in the chondrocytes in the logarithmic growth phase under mono layer cultures, the cells were seeded in 6-well multi-plastic dishes, cultured 10 days, and applied to the above mentioned procedures.

(ii) Growth plate

From 1, 2, 3, 4, 5, 6, or 12 weeks old Wistar male rats (n=14 for each age group), the growth plate were collected from the distal part of the femur and the proximal part of the tibia, fixed in 4% paraformaldehyde solution at room temperature for 24 hours. When the collected samples contained bone tissues, they were decalcificated in PBS/EDTA at room temperature for one week. The samples were cut into 5 μm slices, mounted on slides, and stained immunohistochemically with the ABC method using anti-HSP70 antibody as the primary antibody and the biotinylated 2nd antibody, as indicated above. As a negative control, anti-rat IgG was used as the primary antibody. Nuclei were stained with methylgreen.

3. *In situ* hybridization

HSP70 DNA probe was prepared from the *Escherichia coli* built-in plasmid DNA which was a generous gift of Dr. R. I. Morimoto (21). The plasmid DNA was cut by using BamH I (Toyobo, Tokyo, Japan) and Hind III (Toyobo, Tokyo, Japan), and the segments containing HSP70 DNA were collected, purified, and biotin-labeled with nick translation method. Hybridization solution consisted of 22.5% formaldehyde, 2×SCC buffer, 1×Denhardt solution, 10% dextran sulfate, 250 μg/ml salmon sperm degeneration DNA, 20 mM Vanadyl ribonucleoside complex, 200 μ/ml of yeast tRNA, and 1 μg/ml of the probe DNA. The probe DNA and the salmon sperm DNA were single-stranded at 100°C for 3 min before the hybridization. In a negative control, plasmid DNA without containing HSP70 DNA was used.

Epiphyseal plates were collected from the distal part of the femur and the proximal part of the tibia of 1, 2, 3, 4, 5, 6 weeks old Wistar male rats (n=4 each). They were fixed inPBS containing 4% paraformaldehyde at room temperature for 4 hours, embedded into paraffin, and prepared into 5 μm sections. After removing paraffin, the samples were treated with 0.2 N HCl at room temperature for 10 min, then, with 10 μg/ml of protease K at 37°C for 10 min, and with PBS containing 4% paraformaldehyde at room temperature for 10 min. The samples were fixed, dehydrated in ethanol, dried, and pre-hybridized in 50% formaldehyde and 2×SCC buffer at room temperature for 30 min, dehydrated, and dried. The samples were then treated in the hybridization solution at 37°C for 24 hours.

After hybridization, samples were washed extensively, blocked, treated with streptoavidin for 30 min, washed, reacted with biotinylated alkaline phosphatase, stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate, and sealed with glycerol gel.

Results

1. Expression of HSPs in rat chondrocytes

Expressions of 3 HSPs, with 70, 90, and 110 kDa of molecular weight, were confirmed in cultures with 41 or 43°C heat stress for 30 min (Fig. 1). Among the 3HSPs, amount of 70 kDa HSP (HSP70) was the highest. Expressions of proteins other than HSP were suppressed in cultures with 43°C heat stress, and expressions of all proteins were markedly suppressed in cultures with 45°C heat stress.

2. Immunostaining of micro-mass culture cells and monolayer culture cells

Micro-mass culture cells, which did not receive heat stress, presented flat cells on the surface, fusiform or oval cells in the middle layer, and hypertrophic chondrocyte-like cells on the deep layer.

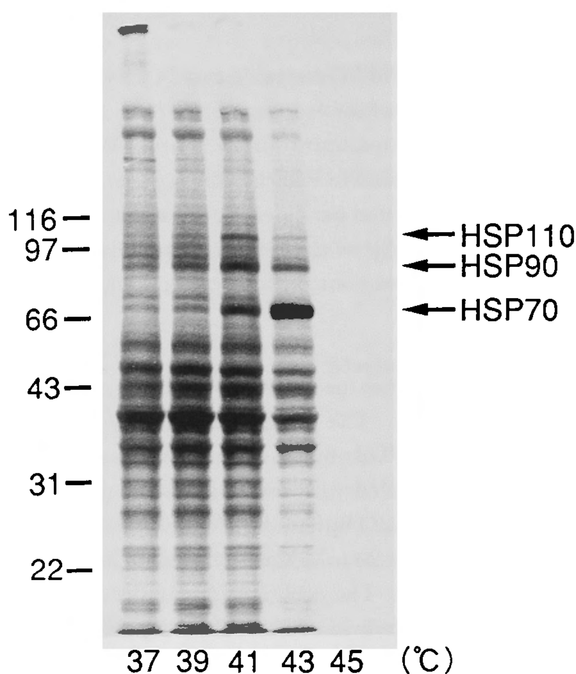

Fig. 1 SDS-polyacrylamide gel electrophoresis of control and heat-shocked chodrocytes. The cells were applied to 39°C (lane 39), 41°C (lane 41), 43°C (lane 43), or 45°C (lane 45) for 30 min, returned to the control temperature 37°C, and then labeled with [^{35}S] amino acids for 4 hours. In 39, 41 and 43 lanes, expressions of HSP70, HSP90, and HSP110 were identified. Lane 37 shows controls which were cultured at 37°C.

A part of cellular matrix was stained with calcium staining. Immunohistochemistry confirmed the presence of HSP70 in the fusiform or oval cells, but not in the flat cells on the surface nor the hypertrophic cells in the deep layers (Fig. 2). On the other hand, HSP70 was not detected in chondrocytes in the monolayer culture even if they were in the logarithmic growth phase (Fig. 3).

3. Immunostaining of growth plate

Immunohistochemistry for chondrocytes for the growth plate revealed that, regardless of rat age, some cells in a resting zone were slight positive to HSP70, and cells in the hypertrophic zone were strong positive (Fig. 4).

4. *In situ* hybridization of growth plate

In the cells of the growth plate, HSP70 mRNA was expressed on the cells in the proliferating and resting zones, whereas the expression in the hypertrophic zone was not detected (Fig. 5).

Discussion

In electrophoresis for detecting heat-induced HSP in rat chondrocytes, expressions of at least 3 kinds of HSP were confirmed, i.e., HSP70, HSP90, and HSP110. These are in agreement with previously confirmed HSPs in mammals (22). Among them, induced amount of HSP70 was the highest, indicating HSP70 is an important protein for chondrocytes. To date, HSP70 has been reported to take a basic protective role against heat stress in various cells (23, 24). Because articular

cartilage is a place where various physical stresses, such as weight and increased temperature (25), are loaded, HSP70 might protect the cartilage cells from physical stresses. We previously reported that, when HSP is induced by heat stimuli in chondrocytes, these cells will become resistant to the 2nd heat stress, and this resistance level relates positively to the previously induced HSP70 level (26).

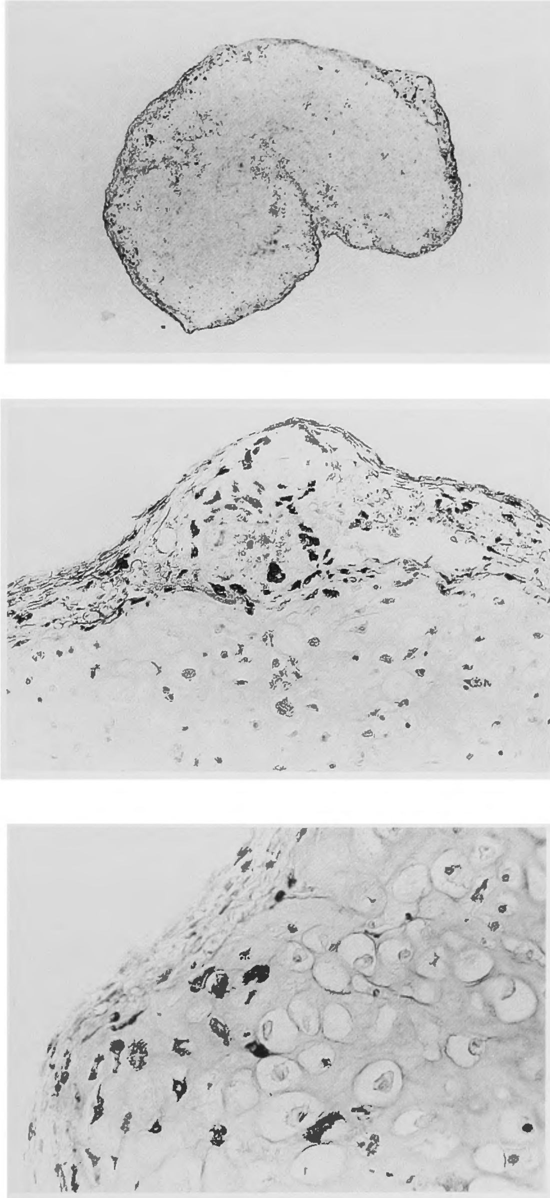

Fig. 2 Chondrocytes were seeded in 15 ml centrifuge tubes, centrifuged at 1000 rpm for 5 min, and maintained for 28 days. Sections for microscopy were stained with a monoclonal antibody to HSP70 (a, b, c). The cells surrounded by matrix show positive staining, but superficial cells and hypertrophic cells show negative staining. Original magnifications: (a)x16; (b)x100; and (c)x400.

In the micro-mass cultures, cells present morphology similar to young cartilaginous tissues, and could differentiate to the terminal stage, e.g., hypertrophy or calcification (27). The present study also showed appearance of hypertrophic chondrocyte-like cells, and calcium staining revealed the occurrence of calcification in the center of the cell gathering. This shows that cultured chondrocytes in

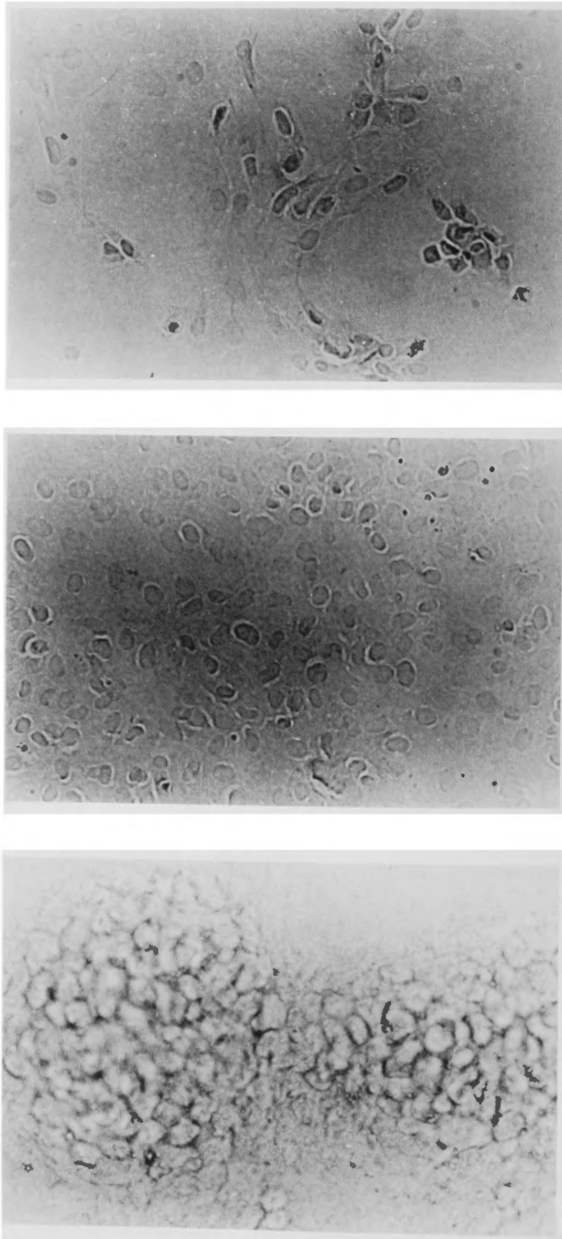

Fig. 3 Chondrocytes in the monolayer culture. (a) Cells were cultured for 10 days after seeding. (b) Cells were cultured for 14 days, reaching a plateau phase. (c) A culture, maintained for 21 days. Cells in all phases show negative staining.

this system have maintained their differentiation capability. HSP70 was expressed in some micromass culture cells even though these cells did not receive any physical stresses, such as heat and pressure. This indicates that HSP70 expression occurs regularly in some cells regardles of the presence or absence of physical stresses. In addition, all cells in the cultures which received heat stresses

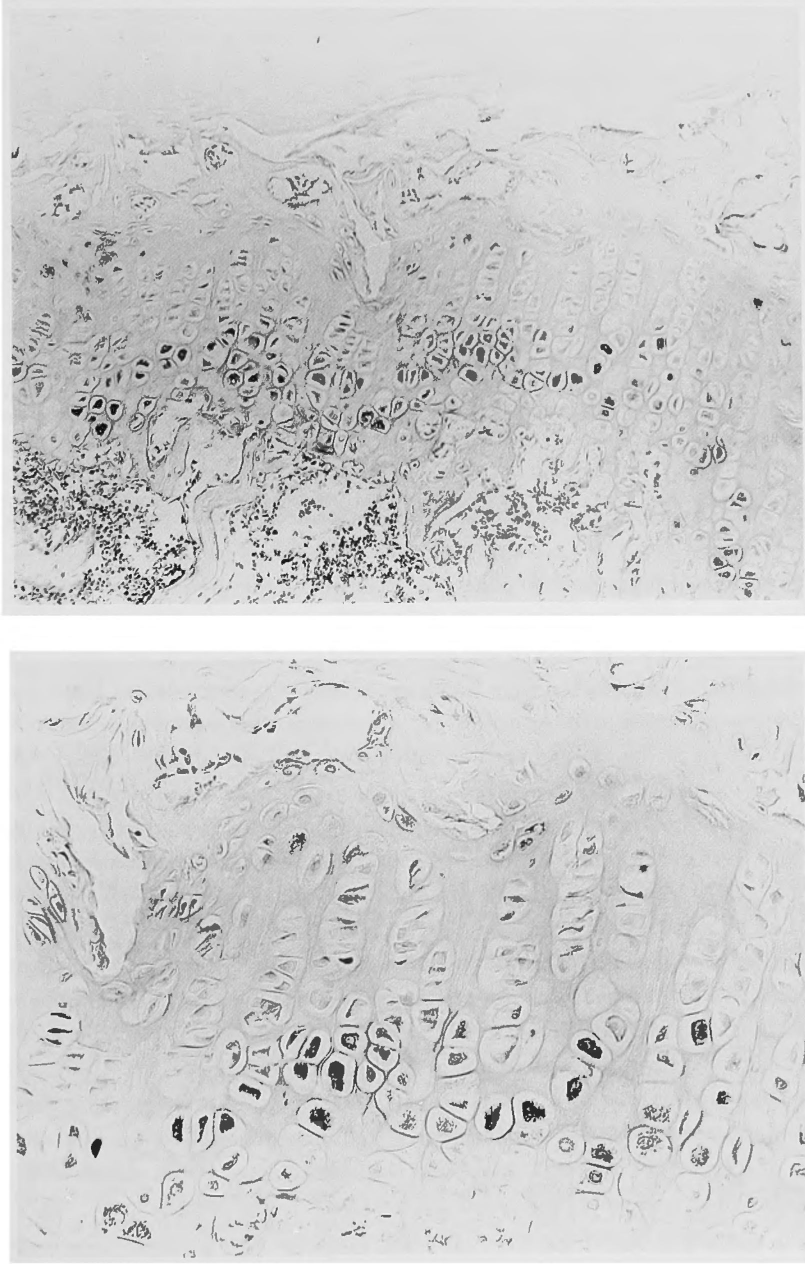

Fig. 4 Immunohistochemistry of growth plate. Cells in the hypertrophic zone of the growth plate were positive to HSP70. Some cells in the resting zone were weak positive. (a)x100. (b)x400.

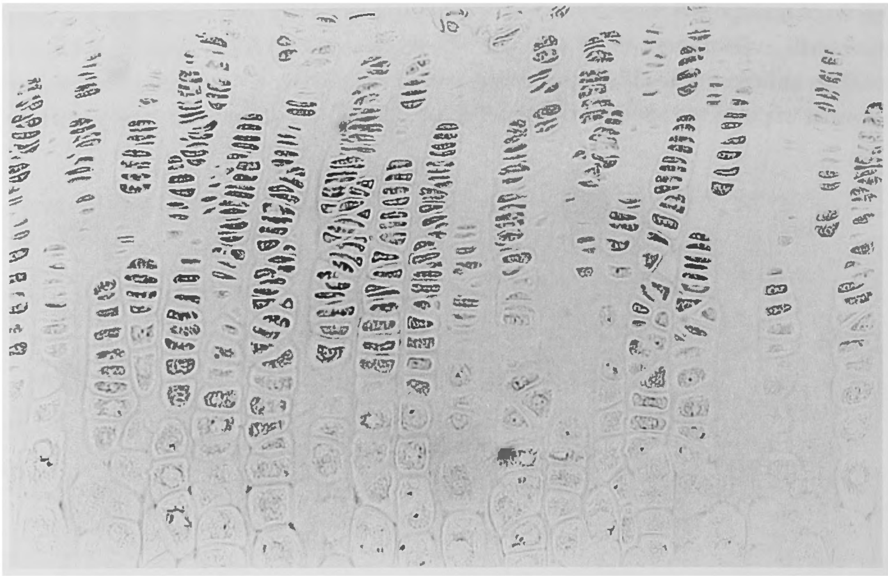

Fig. 5 *In situ* hybridization of growth plate. Chondrocytes in the resting and proliferating zones were positive to HSP70 mRNA (x200).

expressed HSP70. These findings show that HSP70 is induced by heat stress as well as via another mechanism.

HSP70 expression in the growth plate was positive in the resting and hypertrophic zones. These, however, were not in agreement with the HSP70 expressions obseved in the *in vitro* micro-mass culture chondrocytes where HSP70 was not detected in the hypertrophic chondrocyte-like cells, but in the proliferating cells. On the other hand, *in situ* hybridization detected HSP70 mRNA in the resting and proliferation zones of the growth plate, whereas there were no expressions in the hypertrophic zone cells. These *in situ* findings were in agreement with the findings of micro-mass cultures. A possible reason why *in vivo* HSP70 expressions did not agree with the *in vitro* and *in situ* findings is that differentiation and proliferation of the cells *in vitro* would occur very fast. Half life of induced HSP70 was 7–11 days in the cells without any proliferation, while it was 3–5 days in the cells with proliferation (11, 28). Expressed HSP70 would be consumed quickly; and only low amount of HSP70 would be accumulated *in vitro*.

It has been reported that HSP70 would be induced when a cell is in the G1/S gap of a cell cycle, or by certain proliferation factors/cytokines and cancer genes (21, 29, 30); and HSP70 appears in relation to generation and differentiation of a cell (3, 31–33). In this study, HSP70 was not detected in the monolayer culture cells in the logarithmic growth phase. Therefore, HSP70 in chondrocytes is unlikely to be expressed in relation to the G1/S gap. Relationship between cell differentiation and HSP70 has been reported on erythrocytes and sperm cells (31, 32). These 2 cells are at the terminal differentiation of a cell cycle, and will soon be in the resting stage of the cell activity. Therefore, HSP70 was surmised to inactivate the activated genes (34). In the present study, HSP70 expression was detected at increasing intensities as cell are differentiated into proliferated cells and then into calcified cells. This suggests that HSP70 expression could be related to the differentiation process of chondrocytes. Relationship between HSP70 expressions and growth factors/cytokines which act on

chondrocytes has not been elucidated, but they are most probably related to each other because growth factors/cytokines can regulate differentiation of chondrocytes.

In conclusion, the expression of HSP70 in chondrocytes could be related to the terminal differentiation of chondrocytes, because HSP70 was detected in proliferating cells which will be differentiated into hypertrophic or calcified cells. HSP70 is surmised to promote hypertrophy and calcification by stopping protein synthesis in chondrocytes which possess terminal differentiation potency.

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和文抄録

軟骨細胞の分化における HSP70 の発現

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軟骨細胞との関連が注目されている熱ショック蛋白質の発現を，遠沈管培養軟骨細胞と骨端軟骨板を用いて，*in vitro* および *in vivo* で検索した．温熱ストレス下のラット軟骨細胞では，少なくとも3種類の熱ショック蛋白質の発現がみとめられ，そのうちHSP70 の誘導量が最大であった．非ストレス下では，遠沈管培養軟骨細胞のうち肥大軟骨細胞様細胞への分化能を有すると考えられる細胞に HSP70 の発現が認められた．骨端軟骨板においては，肥大軟骨細胞で HSP70 が誘導されていた．また，HSP70 mRNA は肥大層に隣接する増殖層および静止層の軟骨細胞で観察された．このことは，肥大軟骨細胞への分化能を有する軟骨細胞では，すでに HSP70 mRNA が誘導されていることを示している．これらの結果から，HSP70 の発現が軟骨細胞の分化と関係していることが推察された．