Analysis of Cell Death in Meckel's Chondrocytes
In Vitro Induced by Heat-Shock Treatment

Miyayuki Kubo and Kiyoto Ishizeki

Department of Oral Anatomy, School of Dentistry, Iwate Medical University
(Chief: Prof. Tokio Nawa)
1-3-27 Chuo-dori, Morioka 020-8505, Japan
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Abstract: To clarify whether in vitro cell death in Meckel's chondrocytes induced by heat-stress treatment
involves apoptosis or necrosis, we performed morphological examinations using immunohistochemistry
including BrdU-incorporation, the TUNEL method, and light and electron microscopy. Cell death was
induced in Meckel's chondrocytes isolated enzymatically from 17-day embryonic mice, by exposure to
temperatures 40–60°C. On exposure to sublethal temperatures of 40–45°C, the incorporation of BrdU was
facilitated, and TUNEL-positive apoptotic cells appeared with high frequency. At the ultrastructural level,
apoptotic cell death was characterized by chromatin condensation, nuclear segmentation, and the formation
of apoptotic bodies. In contrast, cell death induced by lethal temperature (50–60°C) presented decreases in
TUNEL-positive cells and cell incorporating BrdU. High temperature-treated cells showed apparent disruption
of the membrane of cell organelles. Immunostaining for heat shock proteins (HSP) 27 and 70 revealed
that these proteins are secreted continuously at lower temperatures, but have a tendency toward decrease
on exposure to lethal temperatures. Our results suggest that Meckel's chondrocytes demonstrate apoptosis
at sublethal temperatures, but undergo necrosis at lethal temperatures.

抄録：本研究において、われわれは培養軟骨細胞の加温処理によって誘導される細胞死がアポトーシスかネクローシスによるものかをヒートショック（ストレス）タンパクの発現と BrdU 摂取細胞の免疫染色、TUNEL 法と光学および電子顕微鏡によって解析した。細胞死は、40–60°Cまでの加温処理によって胎生 17 日の培養マウスメッケル軟骨細胞に誘導された。40–45°Cまでの準致死的温度では BrdU の取込みが促進され、TUNEL 陽性細胞が高頻度に出現した。電子顕微鏡による観察では、アポトーシス様の細胞死は核クロマチンの濃縮、核分裂とアポトーシス小体の形成によって特微づけられた。一方、50–60°Cまでの致死的温度による細胞死では、BrdU の摂取細胞と TUNEL 陽性細胞の減少が認められた。高温処理を施した細胞は細胞小器官の膜構造の崩壊を伴っていった。ヒートショックタンパク 27 と 70 による免疫染色では、両者は低温度ではほぼ一定の強さで局在していたが、高温になるにつれて、その強さは減弱傾向を示した。本研究による形態的解析から、準致死的温度による細
胞死はアポトーシス様であったが、高温処理につれてネクローシスによる細胞死が誘導されることが示唆された。

Introduction

It has been generally accepted that cell death can be distinguished into apoptosis and necrosis by morphological and biochemical characteristics. Apoptosis is characterized by cell shrinkage, plasma membrane blebs and subsequent apoptotic body formation, condensation of the nuclear chromatin, and DNA fragmentation. Apoptosis often occurs during embryogenesis for the maintenance of correct cell numbers in various tissues. However, necrosis occurs
exclusively as a result of environmental disruption exceeding normal physiological conditions. It is characterized by swelling of the cell elements including several cell organelles, particularly mitochondria, disintegration of the plasma membrane, and random cleavage of the DNA strands. In contrast to cytoplasmic degeneration, the nuclei of the necrotic cells remain intact. Necrosis, therefore, may be seen in cells damaged by hypoxia, various toxins, and hyperthermia. These two processes for cell death, apoptosis and necrosis, have been clearly distinguished by ultrastructural appearances and by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method.

Recent data indicate that apoptosis occurs not only in programmed cell death, but also in accidental cell death induced by physical and chemical agents such as virus infection, UV-irradiation, and anti-cancer agents, in contrast to physiological apoptosis. There is some controversy as to whether heat-induced cell death is apoptosis or necrosis. Some reports indicate that apoptosis is enhanced by mild hyperthermia (a heat load on the order of 42 on 33°C for 30 min) both in certain normal tissues and in tumor cells. Hayashi, et al. reported that apoptosis was induced in NP 3 cells, a lymphoma cell line, by mild heating in contrast to necrosis which was induced by higher temperatures. Harmon, et al. reported that when cultures of mastocytoma (P-815 × 2.1 cells) were heated at temperatures ranging from 42 to 47°C for 30 min, marked apoptosis was induced after heating at 43, 43.5 and 44°C while only necrosis was produced at 46 and 47°C. Furthermore, some investigators suggested that apoptosis induced by heating was related closely to the expression of heat shock (or stress) proteins (HSPs). Liosis, et al. demonstrated that the overexpression of HSP-70 offers thermo-protection but enhances Fas-induced apoptotic cell death in the TCR/CD 3. They suggested that high molecular protein, e.g., HSP-70, regulates apoptosis in the Fas/Fas ligand, but HSP-27, the heat shock protein of lower molecules, inhibits rather than induces apoptotic cell death.

There have been many recent reports on apoptosis (programmed cell death) during embryonic mor-phogenesis, including our study demonstrating that apoptosis was irrelevant to the ultimate fate of Meckel's cartilage, but little is known about chondrocytes in culture, in particular, Meckel's chondrocytes after heat injury.

In the present study, therefore, we examined whether the cell death induced in Meckel's chondrocytes by heat-shock treatment is apoptosis or necrosis, and particular attention was paid to the expression of HSP-70 and -27 to analyze the involvement of HSPs in apoptosis.

Materials and Methods

1. Chondrocyte culture

Meckel's chondrocytes were obtained according to our previous reports. In brief, pregnant mice were killed with a lethal dose of CO₂ on the 17th day of gestation and embryos were obtained immediately. All animal experiments were performed according to the protocol filed with Iwate Medical University. Meckel's cartilage was dissected from the mandibles and chondrocytes were isolated enzymatically. The isolated chondrocytes were inoculated at a density of 1 × 10⁴ cells in Penicilinder-cups (P-cup: 0.28 cm²; Top Labo-ware, Osaka, Japan) placed at the center of a 35-mm dish (FALCON: Fukushima, Japan), and cultured in an alpha modified essential medium (α-MEM; Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal bovine serum (ICN Biomedicals Co., Ltd, Japan), 3 mM β-glycerophosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.03 mg/ml L-ascorbic acid phosphate magnesium salt n-hydrate (Wako), and 60 µg/ml kanamycin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) in a humidified incubator with a 5% CO₂ atmosphere. The medium was replaced every other day.

2. Heat-shock treatment

Confluent cultures after one week of culture were harvested for treatment at various temperatures. Cells were exposed to heat shock for 30 min at 40, 45, 50, and 60°C (±2, respectively) by water-bath (model: BM-81, Yamato, Tokyo, Japan), and were further
cultured for 24 h at 37°C with newly prepared α-MEM for use in the experiments as follows.

3. TUNEL methods

Apoptotic cells were detected immunohistochemically by the TdT-mediated biotinylated dUTP nick end-labeling (TUNEL) method. The TUNEL method was performed according to our previous detailed report26. In brief, cultures were washed with PBS, and fixed with cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS : pH 7.4) for 30 min, and intrinsic peroxidase activity was inhibited with 3% H2O2 in methanol for 10 min. Cells were washed with PBS that contained 0.1% Tween-20 (PBS-T : Kanto Chemical Co., Inc., Tokyo, Japan), and incubated with a reaction solution composed of 100 unit/ml TdT and 10 nmol/ml biotinylated 16-2'-dUTP (Boehringer-Mannheim-Yamanouchi, Osaka, Japan) in TdT buffer for 1 h at 37°C. After washing with PBS-T, specimens were incubated with peroxidase-conjugated streptavidin-biotin complex (1 : 1,000 : Boehringer-Mannheim) for 20 min at room temperature. For visualization of peroxidase activity, samples were developed for approximately 10 min with a Sigma Fast 3,3'-diaminobenzidine (DAB) peroxidase substrate kit (Sigma Chemical Company, St. Louis, MO, USA), stained with hematoxylin, and mounted with glycerol-PBS (9 : 1, v/v).

The number of TUNEL-positive cells from five different cultures was counted and significant differences were evaluated by Student's t-test. The findings are expressed as means±SD.

4. BrdU-incorporation

For detection of DNA-synthesizing cells, the incorporation of bromodeoxyuridine (BrdU) in normal cultures (control) and in heat-injured cells was analyzed morphologically and statistically.

For cell-labeling with BrdU, subconfluent cultures at one week in culture were treated with 0.5 mg/ml BrdU in α-MEM for 30 min at 37°C. Samples were washed with PBS, fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4), and detected immunohistochemically using a BrdU staining kit (Oncogene Research Products, Cambridge, MA, USA) according to the manufacturer's instructions. For visualization of peroxidase activity, the incorporated BrdU was developed with a DAB kit (Sigma) prior to the counterstaining with hematoxylin.

Statistical analysis of BrdU-labeled cells was evaluated at 5 points (1 mm², n=5 in 2 dishes).

5. Hoechst staining

For analysis of the nuclear morphology, the cultured cells were fixed with 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 30 min at room temperature, washed sufficiently with PBS and stained with hoechst 33258 (Molecular Probes, Eugene, Oregon, USA). Specimens were observed under a fluorescence microscope equipped with an ultraviolet filter (Carl Zeiss Co. Ltd., Oberkochen, Germany).

6. Immunohistochemistry for heat-shock proteins (HSPs)

The localization of HSPs in the cultures treated at 40, 45, 50, and 60°C was detected using indirect immunoperoxidase staining methods. Cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature, then washed thoroughly with PBS. Intrinsic peroxidase activity was inhibited with 3% H2O2 for 10 min. Cultures were further followed with blocking peptides (Santa Cruz Biotechnology, Inc., Delaware, CA, USA) for 10 min at a dilution of 1 : 100. After washing with PBS, they were incubated with anti-rabbit heat shock protein-27 (M-20 ; Santa Cruz) and -70 (K-20 ; Santa Cruz) at a dilution of 1 : 100 for 1.5 h at 37°C. HSP-27 is an affinity-purified goat polyclonal antibody raised against peptide mapping near the carboxy terminus of HSP-27 of mouse origin, while HSP-70 is a polyclonal antibody raised against a peptide corresponding to an amino acid sequence mapping at the carboxy terminal of the 70 kDa heat shock protein of human origin. Cultures were further incubated with horse radish peroxidase-conjugated (HRP-conjugated) rabbit antibodies against goat IgG (Cappel : Organon Teknika Corp., West Chester, PA, USA) at a dilution of 1 : 200 for 1 h at 37°C. After washing with PBS, positive immunoreactivity was visualized with a DAB-kit (Sigma) for 10 min and samples were lightly
counterstained with hematoxylin.
Control specimens were incubated directly with second antibodies without treatment by primary antibodies or heat-untreated cultures, and then processed as outlined above. No immunoreactivity was apparent in any of the controls.

7. Light- and electron-microscopy
For electron microscopy, intact chondrocytes at the nodule-forming stage and heat-exposed cells were fixed in a cold solution of 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 2 h and then post-fixed in a 1% solution (pH 7.2) of osmium tetroxide. The specimens were dehydrated through a graded ethanol series and embedded in Epon 812 according to conventional procedures. Ultrathin sections were cut with a diamond knife and double-stained with uranyl acetate and lead citrate prior to observation under a transmission electron microscope (H-7100: Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 100 kV.

For light microscopic observation, semithin sections (1 μm) that were obtained from Epoxy-resin samples for electron microscopy were stained with 0.1% toluidine blue (pH 3.0).

Results
1. Intact Meckel's chondrocytes
1) Phase-contrast microscopy and toluidine blue staining
Although it was difficult to detect apoptotic chondrocytes in normal cultures of Meckel's cartilage by phase-contrast microscopy (data not shown), they were distinguished easily by morphological characteristics using toluidine blue staining.

In the toluidine blue staining, apoptotic cells have chromatin-concentrated nuclei and apoptotic bodies, and were distinguished from intact cells containing prominent nucleoli (Fig. 1 A).

2) TUNEL method
Apoptosis occurred with a higher frequency in the typical mature chondrocytes than in undifferentiated cells. TUNEL-positive cells showing apoptotic cell death appeared sequentially in cartilage nodules consisting of differentiated chondrocytes (Fig. 1 B).

3) Hoechst staining
When the nuclear changes by apoptosis were analyzed by hoechst staining, nuclear debris was stained positively (Fig. 1 C). Such structures were identified as apoptosis in typical chondrocytes.

4) Electron microscopy
At the ultrastructural level, apoptosis occurring in intact chondrocytes was characterized as cells containing electron-dense heterogeneous nuclear debris (Fig. 1 D). In many cases, such apoptotic bodies were phagocytosed by neighboring chondrocytes. The reaction-positive elements as shown by the TUNEL method and hoechst staining were consistent with phagocytosed debris.

5) Statistical analysis of apoptosis in normal chondrocytes
The frequencies of the appearance of apoptotic cells in relation to increased duration of culture and with developmental stages in chondrocytes were analyzed statistically. During 5 to 20 days of culture, apoptotic cells appeared to increase after 5 days, but showed a tendency to decrease at 10 days of culture. However, statistical analysis did not show any significant difference with culture time (Fig. 2 A).

When the frequency in stage-specific apoptosis was compared between cultures at 5 and 15 days of culture, apoptosis occurred at all stages of chondrocytes (Fig. 2 B). These results therefore indicated that apoptosis in Meckel's chondrocytes takes place during specific culture days and cell stages.

2. 40-45°C-shock treatment
1) Phase-contrast microscopy
When the confluent cultures were treated by heating at 40°C, dynamic morphological changes occurred in the cultures: cells became irregularly shaped, and typical round chondrocytes disappeared from the cultures (Fig. 3 A).

2) Toluidine blue staining
Apoptotic chondrocytes could be detected easily by toluidine blue staining (Fig. 3 B). The cell shapes were irregular and nuclei were stained more densely than intact chondrocytes. Such cells contained many vacuoles and projected numerous cytoplasmic proces-
Fig. 1 Apoptosis in intact Meckel’s chondrocytes in vitro.

(A) Toluidine blue staining shows apoptotic cells with densely stained chromatin-concentrated nuclei (arrows) and apoptotic bodies (arrowheads) in early cartilage-nodules. (B) Apoptosis in chondrocytes shown by TUNEL method. Note the TUNEL-positive apoptotic bodies and nuclei. (C) Several apoptotic chondrocytes (arrows) appearing in the cartilage nodules shown by hoechst staining. (D) An electron-microscopic photograph showed that many apoptotic bodies (arrows) were phagocytosed by nodule-forming chondrocytes. (A, B) ×200, (C) ×150, (D) ×2,000.

3) TUNEL method

It was demonstrated clearly by the TUNEL method that the cells as observed by phase-contrast microscopy and toluidine blue staining revealed apoptosis-positive reactivity. Cells contained TUNEL-positive nuclei and apoptotic bodies consisting of nuclear debris (Fig. 3 C). Such cells were present in considerable numbers during exposure at 40–45°C (also, see...
3. **50—60°C-shock treatment**

When confluent cultures were treated at 50°C, both necrotic and apoptotic cells were detected. Since the morphological changes in apoptosis were similar to those of 40—45°C-treated cells, all findings described below, except those of the ultrastructures at 50°C-exposure, were obtained from the cultures treated at a lethal temperature of 60°C.

1) **Phase-contrast microscopy**

It was difficult to detect apoptosis by phase-contrast microscopy after heating at 60°C (Fig. 4 A). Cells injured at 60°C showed fewer morphological changes than those treated at 50°C. Nuclei were well-preserved and the extracellular matrix appeared to be organized by refractile materials consisting of proteoglycans.

2) **Toluidine blue staining**

On staining with toluidine blue, cells undergoing necrosis often contained vacuoles, but other morphological alterations were not remarkable (Fig. 4 B).

3) **TUNEL method**

Cultures that were exposed to the lethal temperature of 60°C showed a great decrease in TUNEL-positive cells showing nuclear fragmentation (Fig. 4 C). These statistical analyses are indicated in Fig. 6.

4) **Hoechst staining**

Hoechst staining showed that nuclei in the necrotic chondrocytes appeared to retain intact structures, and there were no nuclear changes such as those seen in apoptotic cell death (Fig. 4 D).

**Fig. 2**

(A) Frequency of apoptosis in chondrocytes during culture days. Note that there was no significant stage-specific relationship between apoptotic cells and culture days. (B) Frequencies of apoptosis in various stages of chondrocytes cultured for 5 and 15 days. The criteria for the cell morphology were based on the report of Ishizeki, et al. and each value was expressed as percent of 100 cells in apoptosis. Note that apoptosis occurs in all stages of chondrocyte differentiation.

**Fig. 6**.
5) Electron microscopy
At the electron microscopic level, it was identified that high temperature-exposed cells after heating at 50°C displayed cell death by both necrosis and apoptosis. In 50°C-treated cells showing typical apoptotic changes, the electron-dense cytoplasm was...
shrunken slightly but cell organelles were well-preserved (Fig. 4 E). The nuclei contained marginally condensed chromatin, and short cell processes like microvilli protruded into the pericellular spaces occupied by scattered cell debris. In particular, such cells were seen in typical chondrocytes forming cartilage nodules.

However, the chondrocytes damaged at 60°C revealed remarkable phenotypic changes toward necrosis. The morphological indications of necrosis are the formation of nuclear chromatin masses, the disruption of cell membranes and mitochondrial swelling. Nuclear membranes were dissolved partly, and heterochromatin was aggregated in tiger-spot like patterns (Fig. 4 F). The disturbances of the cell organelles at an early stage were accompanied with mitochondrial swelling and the thickening of the plasma membranes of rough endoplasmic reticulum (Fig. 4 G).

4. Statistical presentations of the BrdU-incorporated cells and the TUNEL-positive cells

The incorporation of BrdU showing DNA-synthesizing cells was increased slightly in the 40°C-heated cells compared with conventional cultures at 37°C (Fig. 5 A, B). However, during the heat treatment from 45°C to 60°C, BrdU-incorporated cells were decreased significantly following 60°C-treatment (Fig. 5 C, D, E). These statistical results are shown in Fig. 5 E.

TUNEL-positive cells were increased in number during treatment at 40°C to 50°C as compared with control cultures. Especially, 40°C-treatment presented a significant increase of positive cells (Fig. 6). However, in the cultures of cells heated at 60°C, TUNEL-positive cells were significantly decreased in number (p<0.0001) in comparison with normal cultures.

5. Immunohistochemistry for HSP-27 and -70

Immunoreactivity for HSP-27 and -70 was not detected in intact Meckel's chondrocytes. Abundant immunoproduacts of HSP-27 were consistently localized in the nodule-forming cells throughout 40–60°C treatment (Fig. 7, upper).

In contrast, immunoreactivity for HSP-70 was localized intensively in 40°C-exposed cultures (Fig. 7, down) similar to that of HSP-27 at 40°C. In the cultures heated at 45–50°C, the localization of HSP-70 was seen sequentially in nodule-forming chondrocytes, but 60°C-treated cultures showed less intense immunoreactivity.

Discussion

In the present study, we examined whether cell death occurring in Meckel's chondrocytes in vitro after heating at various temperatures is apoptosis or necrosis. The present results indicated that apoptotic chondrocytes are induced in high frequency at sublethal temperatures of 40°C to 45°C, and also suggested that the increased number of apoptotic cells is associated with the expression of HSP-70, and HSP-70 may modulate apoptosis in chondrocytes. In contrast, the death of chondrocytes treated by the lethal temperature of 60°C was TUNEL-negative cell death; they appeared to be damaged by heat and to degenerate. These data indicate that accidental cell death under physical conditions such as heat-shock treatment induces both apoptosis and necrosis, but that these differences are affected greatly by the temperature.

There is some controversy as to whether terminal

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**Fig. 3** Meckel's cartilage cells treated at 40–45°C.

The appearance of many apoptotic cells was demonstrated by (A) phase-contrast microscopy, (B) toluidine blue staining, (C) TUNEL method and (D) hoechst staining. (E) At the ultrastructural level, early apoptotic cells obtained from 40°C-treatment had many cytoplasmic blebs protruding in the intercellular spaces. ER : endoplasmic reticulum, MT : mitochondria. (F) An apoptotic cell appearing in cultures treated at 45°C had several segmented nuclei (N) containing condensed chromatin, and heterogeneous dense bodies (DB). Note that several organelles including mitochondria (MT) and rough endoplasmic reticulum (ER) are well-retained in the center of the cell. (A, C, D) × 200, (B) × 400, (E) × 10,000, (F) × 8,400.
hypertrophic chondrocytes in vivo undergo apoptosis. Some investigators have reported that hypertrophic chondrocytes located in the lower zone of the growth plate die by apoptosis. Roach, et al. indicated that cells undergoing apoptosis were not confined to hypertrophic cells, and that apoptosis occurs in all stages of chondrocyte development. Recently, we also confirmed that apoptosis can occur in any chondrocyte during the development of Meckel's cartilage in vivo and is not involved directly in the disappearance of Meckel's cartilage itself. In the present cell culture system, it was observed by electron microscopy and hoechst staining as well as by the TUNEL method that apoptosis can occur at any chondrocyte.

Fig. 5 Light micrographs showing incorporation of BrdU in (A) intact, (B) 40°C, (C) 50°C, and (D) 60°C-treated cultures. (E) Statistical analysis of the BrdU-incorporated cells. BrdU was slightly incorporated in 40°C-treated cells, but this decreased markedly after heating at higher temperatures. ×80 (identical for all)

Fig. 4 Meckel's cartilage cells treated at 50–60°C.
(A) The cultures appear to retain the intact morphology by phase-contrast microscopy. (B) The cells stained with toluidine blue appear to have a more intact morphology than those at the sublethal temperatures. (C) TUNEL method did not show any apoptotic changes in the cytoplasm of the 60°C-treated cells. (D) Hoechst staining did not show any damage to nuclei by 60°C-treatment. (E) Electron micrograph of a 50°C-exposed cell. Note that this cell showing typical apoptotic characteristics has chromatins condensed to the nuclear margin and intact cell organelles such as rough endoplasmic reticulum (ER). DB : dense bodies, N : nucleus. (F) 60°C-treated cultures which were indicated at the electron microscopic level. Cell morphology was relatively preserved, but changes in heterochromatin that had accumulated in a tiger-spot manner were remarkable. N : nucleus. (G) Highly magnified cytoplasm of the 60°C-exposed cells. Many mitochondria (MT) were swollen, and thickening of the membranes of rough endoplasmic reticulum (ER) was seen. (A, C, D) ×200, (B) ×400, (E) ×7,000, (F) ×6,000, (G) ×9,500.
Fig. 6 Statistical analysis of the TUNEL-positive cells after treatment at varying temperatures.

TUNEL-positive cells showed a tendency to increase until 40–50°C (T-50), and in particular, there was a significant difference (p<0.0011) between intact cultures (I.T.) and 40°C-exposed cells (T-40). I.T. vs T-40 (p<0.0011). However, in the cultures after heating at 60°C (T-60), TUNEL-positive cells had decreased significantly (p<0.0001).

stage. If hypertrophic chondrocytes in vivo are programmed to die by apoptosis, cells under the in vitro condition should die preferentially by apoptosis. Indeed, apoptosis occurred at all stages of chondrocytes, not only at the stage of hypertrophic cells. Therefore, we infer that apoptosis occurring in intact Meckel's chondrocytes plays a role in the modulation of cell numbers in cultures.

Morphological differences between apoptosis and necrosis were distinguished distinctly in the heat-exposed cells. Apoptotic cells possessed segmented nuclei with condensed chromatin, and the morphology of the mitochondria was well-retained. These cells were seen numerously in nodule-forming chondrocytes after heating at 40–45°C. In contrast, necrotic cells were characterized as spot-like masses of heterochromatin, swollen mitochondria, and frequently vacuolated cytoplasm. Such cells increased with high temperature treatment, although the cells subjected to 50°C-heating underwent cell death by both necrosis and apoptosis. The difference between apoptosis and necrosis by temperature seems to be that under high temperature treatment necrosis occurs more rapidly than apoptosis, progressing with a certain cell cycle for the thermal degeneration of the proteins composing the cells. Roach, et al.23 and Roach and Clarke26 have reported that "paralyzed cells" with dark cytoplasm and worm-like inclusions appeared in the hypertrophic cartilage in vivo. However, in the present culture system, since such cells were not seen in cultures, death of heat-exposed cells seem to differ clearly from cell death occurring in chondrocytes in vivo. Based on the finding that cells of the same cultures exposed to heating underwent different types of cell death, we further suggest that chondrocytes may have different sensitivities to heat loads according to their development stage.

We further examined immunohistochemically whether the induction of apoptosis by heat treatment affects the expression of heat shock proteins (HSPs) as reported for other kinds of cells. HSPs have generally been considered to protect cells from lethal injuries24,25,27. However, there are several reports that high molecular heat shock proteins, such as HSP-70 and -90, facilitate apoptosis in jurkat cells, a human T-cell clone28. In contrast to high molecular heat shock proteins, since HSP-27, a small heat shock protein, inhibits the aggregation of nuclear proteins and recovers the synthesis of proteins inhibited by heat injury27, it has been suggested to act as a suppressor of apoptosis23. Therefore, we chose HSP-70 and -27 among the several families of HSPs and attempted to examine the immunolocalization of these proteins after heating. HSP-27 was synthesized sequentially during 40°C to 60°C-treatments and, in particular, it was localized intensely in cartilage nodules heated at 40°C. In contrast, HSP-70 was synthesized actively during 40–45°C injury, but was decreased remarkably at 60°C. High temperature-exposed cells incorporated BrdU poorly and inhibited cell differentiation to chondrocytes, while evidence of necrotic cell death was seen clearly by light and electron microscopy. Apoptotic cells were observed simultaneously in parallel with the localization of HSP-70. These results may support the concept that HSP-70 enhances apoptosis, as reported by some investigators24,25, but there was no evidence that
HSP-27 suppressed apoptosis\textsuperscript{33} in the present study.

In conclusion, our data proved that apoptosis in chondrocytes is induced by sublethal heating, but lethal heating at 50–60°C causes necrotic cell death. Cell death by thermal injury remains controversial but it was shown that the difference in cell death between apoptosis or necrosis depends on the degree of heat-stress. Judging from recent reports indicating that apoptosis is not always consistent with programmed cell deaths, it is necessary to further study the cell death of individual tissues or cells.

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