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Thermotolerance of Pulp Cells and Phagocytosis of Apoptotic Pulp Cells by Surviving Pulp Cells Following Heat Stress

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Abstract Apoptosis is known to be associated with wound healing and regeneration of dental pulp. We examined the effects of heat stress on clonal dental pulp cell line (RPC-C2A cells) to clarify the pulp wound healing process. RPC-C2A cells were exposed to heat stress at 43°C for 45 min. After several time intervals, the inhibition of cell proliferation and apoptosis induction were analyzed by cell viability assay, DNA gel electrophoresis, nuclear staining, and terminal deoxynucleotidyl transferase mediated labeling assay. RPC-C2A cells showed the thermotolerance following heat stress. We found that apoptosis was induced in some RPC-C2A cells, whereas others remained alive, and observed the engulfment of apoptotic cells by scavenger-like RPC-C2A cells following heat stress. We also analyzed the phagocytotic activity of RPC-C2A cells and found that they had an ability to engulf apoptotic RPC-C2A cells, which was stimulated by heat stress. These results suggest that heat stress induces apoptosis of RPC-C2A cells, which are phagocytosed by the surviving RPC-C2A cells. J. Cell. Biochem. 94: 826–834, 2005. © 2004 Wiley-Liss, Inc.

Key words: thermotolerance; apoptosis; phagocytosis; dental pulp

Apoptosis is the regulated cell death that is distinct from necrosis, which is an alternative mode of cell death resulting in cellular lysing and the release of cytoplasmic components into the surrounding environment [Raff, 1992]. Apoptosis plays an essential role in the control of various biological systems, including homeostasis in several diseases as well as embryonic development [Jacobson et al., 1997]. Apoptotic cells induced by several types of stress are subsequently engulfed by scavenger cells such as macrophages [Platt et al., 1998]. Recently, we demonstrated in vivo that apoptosis of dental pulp cells is induced during wound healing and regeneration, with heat stress related molecules playing important roles in pulp apoptosis [Kitamura et al., 2001, 2003].

Heat stress is known to induce death signals that lead to apoptosis, while heat stress produced by treatment of carious teeth is believed to be one of the major causes of damage to dental pulp [Mjör, 2002]. Pathological changes in dental pulp give rise to painful symptoms in teeth, and can adversely affect the response of the pulp to restorative procedures. Dental pulp consists of undifferentiated mesenchymal cells, odontoblasts, and immunocompetent cells including dendritic cells. Immunocompetent cells work when dental pulp is infected, especially dendritic cells in odontoblastic region play roles in capturing antigens, and presenting them to lymphocytes. We also found that apoptotic pulp

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cells, which were induced during the early phase of the pulp wound healing process, were engulfed by adjacent scavenger cells before the initiation of reparative dentin formation as part of the regeneration process [Kitamura et al., 2001]. However, it has not been clarified whether heat stress directly induces apoptosis in pulp cells, or how apoptotic pulp cells are eliminated by adjacent scavenger cells. Most studies regarding the phagocytosis of apoptotic cells were carried out using macrophages and monocytes [Brown et al., 2002; Hanayama et al., 2002; Lang et al., 2002]. During inflammatory reactions, apoptotic cells are known to be recognized and engulfed by macrophages before their degradation. Further, the engulfment of apoptotic cells by scavenger cells occurs in areas where macrophages do not infiltrate, such as the testes [Miething, 1992].

In the present study, we investigated the effects of heat stress on a clonal dental pulp cell line (RPC-C2A cells), which has the characteristics of dental pulp cells, and examined the process of elimination of the damaged RPC-C2A cells.

MATERIALS AND METHODS

Cell Cultures and Heat Stress

Rat clonal dental pulp cells (RPC-C2A) [Kasugai et al., 1988] and mouse 3T3-Swiss albino fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA) containing 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml of streptomycin, and 100 U/ml of penicillin, and then incubated in a humidified atmosphere of 5% CO_2 at 37°C. Twenty-four hours from the subculture, RPC-C2A cells and 3T3-Swiss albino fibroblasts were exposed to heat stress at 43°C for 45 min, and then incubated in a humidified atmosphere of 5% CO_2 at 37°C. Both cells just before the exposure to heat stress were used as non-treated cells. At several time points (non-treated, and 0, 6, 12, 24, and 48 h following heat stress), cell viability and apoptosis induction were analyzed.

Cell Viability Assay

Inhibition of cell proliferation following heat stress was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. RPC-C2A cells and 3T3-Swiss albino fibroblasts (1×10^4/well) were incubated in 96-well dishes in a humidified atmosphere of 5% CO_2 at 37°C for 24 h, and exposed to heat stress. In each time point following heat stress, MTT (Sigma-Aldrich Co., St. Louis, MO) (20 μl/well) added to each well, and the dishes were incubated at 37°C for 4 h. After acid-isopropanol (100 μl/well) was added and mixed thoroughly, the cell viability in each time point was analyzed. All experiments were performed in triplicate.

Detection of Apoptosis

In a qualitative DNA fragmentation assay, heat-stressed RPC-C2A cells (5×10^6) were treated with a lysis buffer containing 1% NP-40 (Biosciences, Inc., La Jolla, CA), 20 mM EDTA, 50 mM Tris-HCl, pH 7.5, on ice for 20 min. After centrifugation, the supernatant was incubated with RNase (Roche Molecular Biochemicals, Mannheim, Germany) solution (5 mg/ml) at 37°C for 1 h, and then proteinase K (Invitrogen Corp.) (20 μg/ml) at 37°C for 1 h, after which DNA was extracted using a phenol/chloroform/isoamylalcohol solution. The DNA was electrophoresed on a 2% agarose gel with a 100-bp ladder (Pharmacia Biotech, Piscataway, NJ) as the marker and stained with ethidium bromide [Moore and Matlashewski, 1994].

For nuclear staining, cells were first cultured on coverslips in DMEM containing 10% FCS in a humidified atmosphere of 5% CO_2 at 37°C for 24 h, and then exposed to heat stress. Non-treated and heat-stressed cells were fixed with 4% paraformaldehyde-phosphate buffer (PFA/PBS), pH 7.3 for 30 min, and washed with 1× PBS. Fixed cells were immersed in 1 mg/ml of bovine serum albumin (BSA) (Roche Molecular Biochemicals) in 1× PBS and permealized with L-α-phosphatidylcholine (Roche Molecular Biochemicals) at 4°C for 30 min. After rinsing with 1× PBS, cells were stained with 0.1 μg/ml of 4′-diamino-2-phenylindole (DAPI) (Roche Molecular Biochemicals) and 0.56 μg/ml of hydroethidine, and then observed under a fluorescence microscope.

To detect in situ DNA strand breaks at the 3′-hydroxyl ends, non-treated and heat-stressed RPC-C2A cells on coverslips were fixed with 4% PFA/PBS for 30 min, and then subjected to terminal deoxynucleotidyl transferase-mediated labeling (TUNEL) assay using In Situ Cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cells were immersed

in 1 mg/ml of BSA in 1× PBS and permealized with 0.05% Triton X-100 at room temperature for 15 min. Some samples of non-treated RPC-C2A cells were also treated with DNase I (Roche Molecular Biochemicals) (20 U/ml) as a positive control. After rinsing with 1× PBS, cells were treated with RNase A (1 mg/ml in 1× PBS) at 37°C for 1 h, then proteinase K (20 μg/ml in 1× PBS) at 37°C for 15 min, and finally incubated with a terminal deoxynucleotidyl transferase (TdT) mixture, which consisted of TdT, TdT buffer containing fluorescein-labeled nucleotide mixture, and 10% BSA, at 37°C for 1 h. Cells were then rinsed with 1× PBS, stained with propidium iodide (Nacalai Tesque, Inc., Kyoto, Japan) (1 mg/ml in 1× PBS), and observed under a fluorescence microscope.

The measurement of apoptotic RPC-C2A cells following heat stress was carried out using APO-BRDU™ Kit (Chemicon International, Inc., Temecula, CA) according to the manufacturer's instructions. Non-treated and heat-stressed cells (2×10^6) were fixed with 1% PFA, washed with 1× PBS, and then suspended in 70% ice-cold ethanol for 30 min. After rinsing, the cells were reacted with a DNA labeling solution containing TdT reaction buffer, TdT enzyme, and Br-dUTP at 37°C for 1 h, and then incubated with fluorescein labeled anti-brdU at room temperature for 30 min. Fluorescein-labeled cells were analyzed using FACScalibur flow cytometer EPICS XL (Beckman Coulter, Fullerton, CA).

Phagocytosis Assay

For the phagocytosis assay of microspheres by RPC-C2A cells, cells (4×10^5/well) were cultured on chamber slides in a humidified atmosphere of 5% CO_2 at 37°C for 24 h, and then exposed to heat stress. Just after the exposure to heat stress, fluorescein-labeled microspheres (1 μM in diameter, 0.025% final concentration) (Polysciences, Inc., Warrington, PA) were immediately added to non-treated and heat-stressed cells, and incubated at 37°C for 12 h. After the incubation, cells were fixed with 4% PFA/PBS, washed with 1× PBS, and stained with DAPI. Signals were observed under a fluorescence microscope.

The phagocytosis of apoptotic RPC-C2A cells by intact RPC-C2A cells was examined using a minor modification presented in a previous study [Shiratsuchi et al., 1997]. To prepare apoptotic cells, RPC-C2A cells (4×10^6) were cultured in a humidified atmosphere of 5% CO_2 at 37°C for 24 h, and then treated with 100 nM concanamycin A (Wako Pure Chemical Industries, Ltd., Osaka, Japan), which is an inducer of apoptosis [Nishihara et al., 1995], for 2 days. The optimal concentration of concanamycin A was determined by MTT assay. Recovered apoptotic RPC-C2A cells were labeled with biotin (EZ-Link™ NHS-LC-Biotin) (Pierce Biotechnology, Inc., Rockford, IL) at 37°C for 30 min. Intact RPC-C2A cells (4×10^5/well) were cultured in the chamber slide in a humidified atmosphere of 5% CO_2 at 37°C for 24 h. The pre-prepared biotin-labeled apoptotic cells (4×10^6/well) were added to non-treated or heat-stressed RPC-C2A cells, and then incubated at 37°C for 12 h. After fixation with 4% PFA, cells were incubated with fluorescein-avidin D (Vector Laboratories, Burlingame, CA), stained with DAPI, and observed under a fluorescence microscope.

To detect alteration of the phagocytotic activity, intact RPC-C2A cells (4×10^5/well) were cultured in 6-well dishes in a humidified atmosphere of 5% CO_2 at 37°C. After 24 h, RPC-C2A cells were heat stressed, and non-treated and heat-stressed RPC-C2A cells were incubated with pre-prepared biotin-labeled apoptotic RPC-C2A cells (4×10^6/well) in a humidified atmosphere of 5% CO_2 at 37°C for 12 h. For inhibition of the engulfment of labeled apoptotic cells by heat stressed RPC-C2A cells, 10 μM cytochalasin D (Sigma-Aldrich Co.) was added to heat-stressed cells before the addition of biotin-labeled apoptotic cells. After the incubation, cells were permealized with 0.05% Triton X-100 solution at room temperature for 5 min, recovered with centrifugation, and incubated with fluorescein-avidin D (Vector Laboratories) at room temperature for 20 min. The engulfment of fluorescein-labeled apoptotic RPC-C2A cells by RPC-C2A cells with or without heat stress was analyzed by flow cytometry.

RESULTS

Cell Viability of RPC-C2A Cells and 3T3-Swiss Albino Fibroblasts

To examine whether RPC-C2A cells have the thermotolerance, we compared the cell viability between RPC-C2A cells and 3T3-Swiss albino fibroblasts. Figure 1A shows representative phase-contrast microphotographs of RPC-C2A cells following heat stress. The shrinkage of

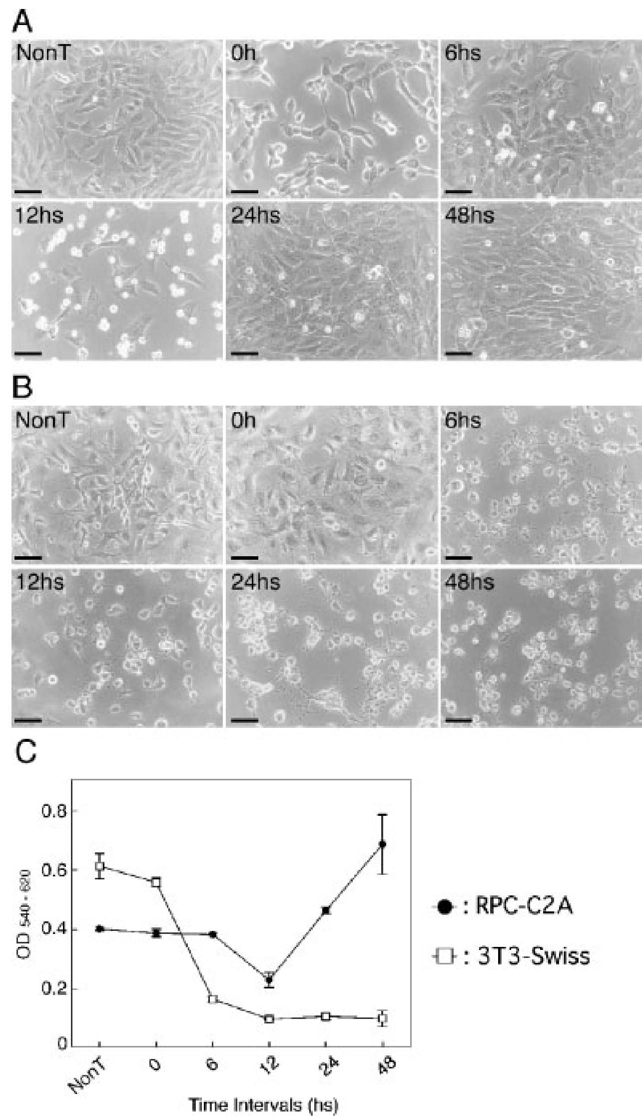

Fig. 1. Thermotolerance of RPC-C2A cells. Phase-contrast microphotographs of RPC-C2A cells (**A**), and 3T3-Swiss albino fibroblasts (**B**) following heat stress. Scale bars = 7.5 μM. **C**: MTT assay of RPC-C2A cells and 3T3-Swiss albino fibroblasts following heat stress. NonT, non-treated; 0 hs, 0 h after stress; 6 hs, 6 h after stress; 12 hs, 12 h after stress; 24 hs, 24 h after stress; 48 hs, 48 h after stress.

RPC-C2A cells at 0 h after heat stress, and the rounding and the detachment of some RPC-C2A cells at 12 h after heat stress, were observed. During 24 and 48 h culture periods, the proliferation of RPC-C2A cells was observed. All of 3T3-Swiss albino fibroblasts rounded and detached 6, 12, 24, and 48 h after heat stress, and no proliferation was observed (Fig. 1B). In Figure 1C, effects of heat stress on the cell viability both of RPC-C2A cells and 3T3-Swiss albino fibroblasts were shown. The viability of RPC-C2A cells decreased at 12 h after heat stress, thereafter the cell viability increased at 24 and 48 h. In contrast, heat stress showed great cytotoxic effect on the viability of 3T3-Swiss albino fibroblasts, and there were no recovery of the viability of 3T3-Swiss albino cells 6, 12, 24, and 48 h after heat stress.

Identification of Apoptotic RPC-C2A Cells

Figure 2A shows representative results of agarose gel electrophoresis of DNA extracted from non-treated and heat-stressed RPC-C2A cells. DNA ladders were detected in cells cultured for 12, 24, and 48 h after heat stress, however, not after 0 and 6 h, and in non-treated

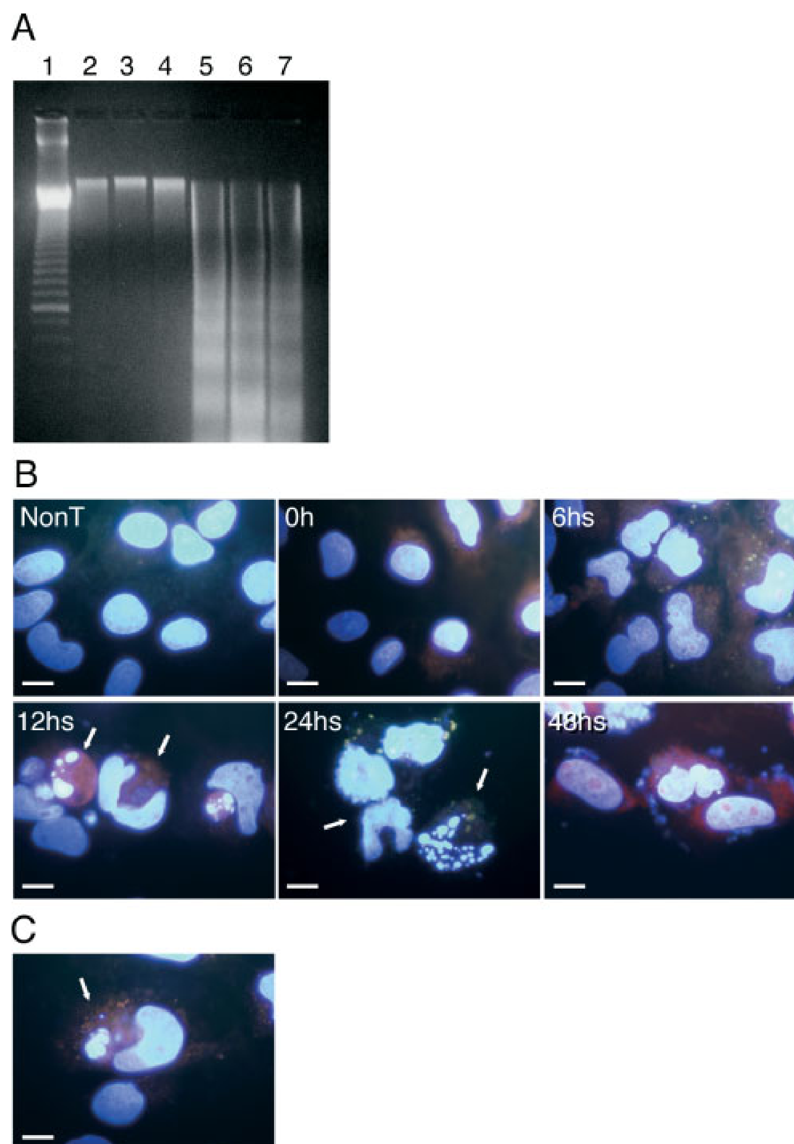

Fig. 2. **A**: Effect of heat stress on the DNA cleavage of RPC-C2A cells. After the exposure to heat stress, purified DNA by NP-40 was detected on agarose gel electrophoresis. **Lane 1**: 100-bp ladder; (**lane 2**) non-treated; (**lane 3**) 0 h after stress; (**lane 4**) 6 h after stress; (**lane 5**) 12 h after stress; (**lane 6**) 24 h after stress; (**lane 7**) 48 h after stress. **B**: Morphological changes of RPC-C2A cells following heat stress. Non-treated and heat-stressed cells were stained with DAPI. Chromatin condensation of nuclei was observed in 12 and 24 h following heat stress (arrows). NonT, non-treated; 0 hs, 0 h after stress; 6 hs, 6 h after stress; 12 hs, 12 h after stress; 24 hs, 24 h after stress; 48 hs, 48 h after stress. Scale bars = 20 μM. **C**: The engulfment of fragmented apoptotic bodies by intact RPC-C2A cells in the 12 h culture period (arrows). Scale bars = 20 μM.

cells. As for nuclear staining (Fig. 2B), morphological changes of nuclei were not observed in non-treated RPC-C2A cells. Morphological changes of nuclei in RPC-C2A cells were observed 0 and 6 h after heat stress, and the nuclear fragmentation was observed at 12, 24, and 48 h after heat stress. During these culture periods, we observed two different features of RPC-C2A cells, apoptotic cells showing the typical nuclear fragmentation, which decreased after 48 h, and surviving cells with normal nuclei. At 12 h after application of heat stress, a phagocytotic phenomenon of apoptotic cells by scavenger-like RPC-C2A cells were also observed (Fig. 2C).

Figure 3 shows the results of TUNEL assays of RPC-C2A cells following heat stress. In the histological analysis (Fig. 3A), TUNEL-positive

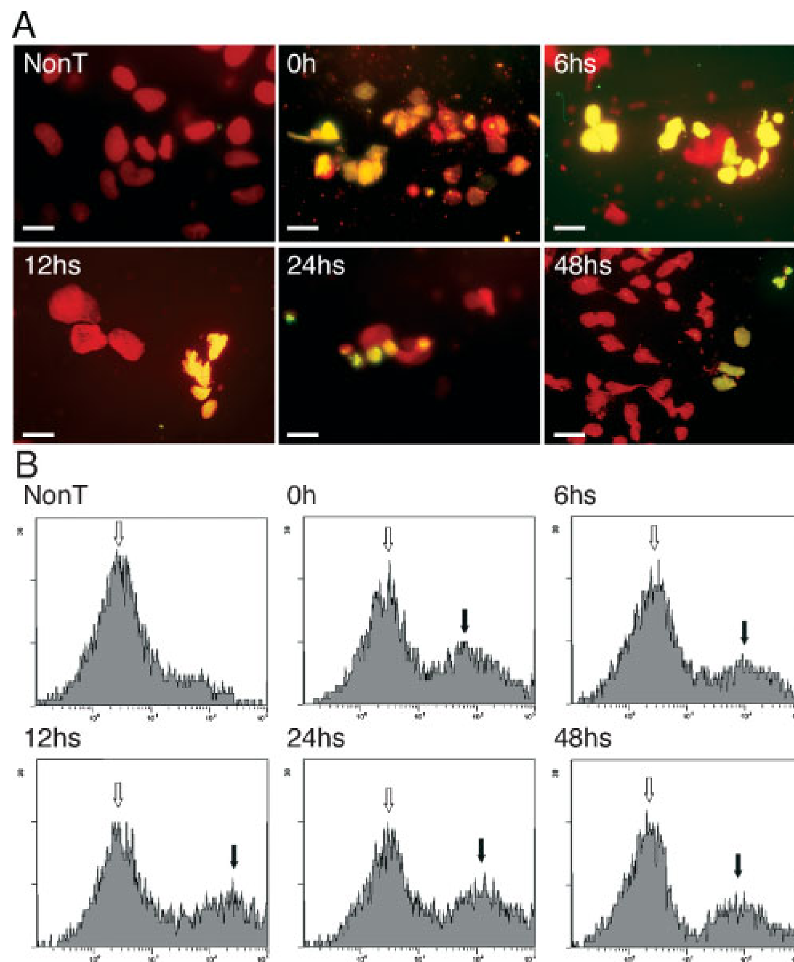

Fig. 3. **A**: In situ DNA fragmentation of RPC-C2A cells following heat stress by TUNEL assay. Red signals (propidium iodide positive) show intact RPC-C2A cells, and yellow signals (TUNEL and propidium iodide double positive) show apoptotic RPC-C2A cells. Scale bars = 10 μM. **B**: Flow cytometric analysis of TUNEL-positive RPC-C2A cells. TUNEL-negative population (white arrows) shows intact RPC-C2A cells. TUNEL-positive population (black arrows) shows apoptotic RPC-C2A cells. NonT, non-treated; 0 hs, 0 h after stress; 6 hs, 6 h after stress; 12 hs, 12 h after stress; 24 hs, 24 h after stress; 48 hs, 48 h after stress.

signals were not detected in non-treated RPC-C2A cells, however, TUNEL-positive RPC-C2A cells were observed at all time points after heat stress, although the number of TUNEL-positive cells in 48 h appeared to decrease. As the positive control, we treated RPC-C2A cells with DNase I before TUNEL assay, and all cells with fragmented DNAs were TUNEL-positive (data not shown). In the flow cytometric analysis (Fig. 3B), a TUNEL-positive population was not detected in non-treated cells, whereas we observed two distinct populations of RPC-C2A cells after heat stress, the TUNEL-negative population, which was identical to that in the non-treated cells, and the TUNEL-positive population.

Phagocytotic Activity of Heat Stressed RPC-C2A Cells

To examine whether RPC-C2A cells have a phagocytotic ability, fluorescein-labeled microspheres were added to non-treated and heat-stressed cells (Fig. 4A). Both types of RPC-C2A cells engulfed the fluorescein-labeled microspheres. We also found both types of RPC-C2A cells engulfed fluorescein-labeled apoptotic RPC-C2A cells treated with concanamycin A (Fig. 4B). In addition, some of non-treated cells (Fig. 4B, a and b) did not engulf fragments of the fluorescein-labeled apoptotic RPC-C2A cells. In contrast, nearly all of surviving cells following heat stress engulfed fragments of

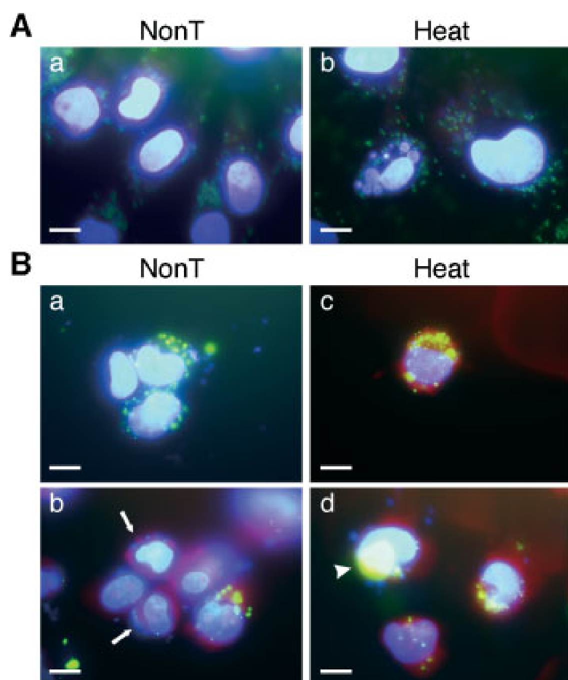

Fig. 4. Phagocytosis assay of RPC-C2A cells. **A**: The engulfment of fluorescein-labeled microspheres by non-treated and heat-stressed RPC-C2A cells. Microspheres (green signals) were engulfed by non-treated (**a**) and heat-stressed (**b**) cells. **B**: The engulfment of fluorescein-labeled apoptotic RPC-C2A cells by non-treated and heat-stressed cells. Apoptotic RPC-C2A cells were individually induced by concanamycin A. Fragments of apoptotic cells (small green signals) were engulfed in the cytoplasm both of non-treated (**a**, **b**) and heat-stressed (**c**, **d**) RPC-C2A cells. In non-treated cells (b), some of RPC-C2A cells did not engulf fragments of apoptotic cells (arrows). Non-fragmented apoptotic cells (arrowhead in d) attached to intact cells were also observed. Scale bars = 20 μM.

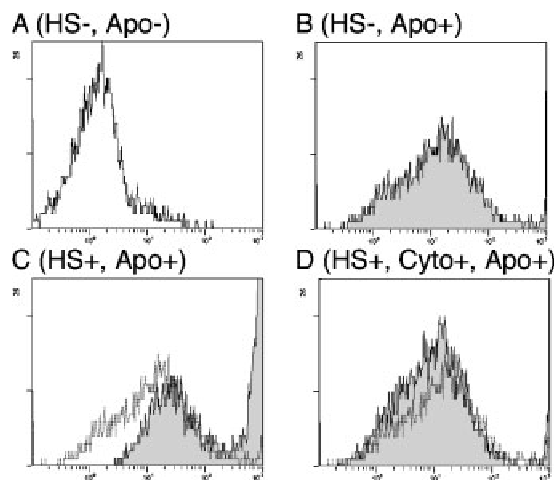

Fig. 5. Flow cytometric analysis of the phagocytotic activity. **A**: No positive peak was observed in RPC-C2A cells that were not exposed to heat stress (HS−) and not incubated with apoptotic cells (Apo−). **B**: Fluorescein-positive peak was observed in non-treated RPC-C2A cells that were not exposed to heat stress (HS−) and incubated with apoptotic cells (Apo+). **C**: Fluorescein-positive peak increased in heat stressed RPC-C2A cells (HS+) with apoptotic cells (Apo+). The engulfment of fragmented apoptotic cells increased after exposure of RPC-C2A cells to heat stress. **D**: Fluorescein-positive peak in heat stressed cells (HS+) with apoptotic cells (Apo+) in the presence of cytochalasin D (Cyto+) returned to the level in non-treated cells with apoptotic cells (HS−, Apo+). The phagocytotic activity of heat-stressed cells was inhibited by cytochalasin D. Dotted line shows the profile of (B). HS, heat stress; Apo, apoptotic cells; Cyto, cytochalasin D.

fluorescein-labeled apoptotic cells (Fig. 4B, c and d). We also observed that some of the non-fragmented, fluorescein-labeled apoptotic RPC-C2A cells adhered to non-treated and heat-stressed RPC-C2A cells.

We determined whether heat stress alters the phagocytotic activity of RPC-C2A cells by flow cytometric analysis (Fig. 5). The profile of non-treated RPC-C2A cells without both the exposure to heat stress and the addition of fluorescein-labeled apoptotic RPC-C2A cells indicated that there was no fluorescein-positive peak (Fig. 5A). We prepared fluorescein-labeled apoptotic RPC-C2A cells treated with concanamycin A, and added the pre-prepared apoptotic RPC-C2A cells into non-treated RPC-C2A cells. When non-treated RPC-C2A cells were incubated with fluorescein-labeled apoptotic cells, a fluorescein-positive peak was observed (Fig. 5B). In Figure 5C, we shows the profile of heat-stressed RPC-C2A cells incubated with the pre-prepared fluorescein-labeled apoptotic RPC-C2A cells. We found that the fluorescein-positive peak shifted to the right rather than the peak of non-treated RPC-C2A cells with fluorescein-labeled apoptotic cells (dotted line in Fig. 5C), indicating the increase of the phagocytotic activity of heat-stressed cells. Figure 5D shows the inhibition of the phagocytotic activity of heat-stressed cells by cytochalasin D, an inhibitor of phagocytosis. When pre-prepared fluorescein-labeled apoptotic cells were added to heat-stressed RPC-C2A cells treated with cytochalasin D, the phagocytotic activity of the heat-stressed cells returned to the level of non-treated cells (dotted line in Fig. 5D). These findings indicated that cytochalasin D inhibited the increase of the phagocytotic activity of RPC-C2A cells by heat stress.

DISCUSSION

Heat stress is known as the major impairment on dental pulp during the restorative procedures. The temperature of the pulp chamber is about 43–45°C during the restorative procedures, and it is known that increases in pulp temperature of more than about 5°C from physiological condition leads to damage on dental pulp [Mjör, 2002]. In previous reports, RPC-C2A cells, derived from rat dental pulp, partly have the characteristics of dental pulp cells [Kasugai et al., 1988; Yokota et al., 1992]. We first exposed RPC-C2A cells to heat stress, and examined whether RPC-C2A cells have the thermotolerance following heat stress. Compared with 3T3-Swiss albino fibroblasts, RPC-C2A cells showed the recovery of the cell viability following heat stress, indicating that RPC-C2A cells have the thermotolerance, although the reduction of the viability was induced in a specific cell type of RPC-C2A cells by heat stress. To clarify the reduction of the cell viability in this type of RPC-C2A cell by heat stress, we next qualified the apoptotic induction using different methods such as DNA gel electrophoresis, DAPI staining of nuclei, and TUNEL assay. DNA ladder formation (a hallmark of apoptosis), nuclear fragmentation (the typical morphological change of apoptotic cells), and DNA strand breaks at the 3′-hydroxyl ends (a characteristic event of apoptosis) were observed in RPC-C2A cells following heat stress. We also examined the induction of apoptosis on RPC-C2A cells by heat stress using flow cytometry and found that apoptosis was not induced in all RPC-C2A cells and the major population of RPC-C2A cells survived. These results indicate that the reduction of the cell viability induced in this specific cell type of the RPC-C2A cells following heat stress is the consequence of the apoptosis induction on RPC-C2A cells by heat stress. It was previously reported whether heat stress induces apoptosis or non-apoptotic events depends on the cell and tissue types [DeMeester et al., 2001]. Which molecular mechanisms decide the fate of RPC-C2A cells to undergo apoptosis or survive following heat stress will be the subject of future studies.

In DAPI staining following heat stress, the engulfment of apoptotic RPC-C2A cells by surviving RPC-C2A cells was observed (Fig. 2B, arrowhead). There were no reports about the phagocytosis of apoptotic pulp cells by the intact pulp cells. We hypothesized that the surviving RPC-C2A cells following heat stress recognize and engulf the apoptotic RPC-C2A cells. Phagocytosis assays with fluorescein-labeled microspheres and fluorescein-labeled apoptotic RPC-C2A cells showed that both non-treated and heat-stressed RPC-C2A cells engulfed microspheres and fragments of apoptotic RPC-C2A cells, indicating that RPC-C2A cells have an ability to phagocytose apoptotic cells. However, some non-treated RPC-C2A cells did not engulf apoptotic cell fragments.

We attempted to determine whether heat stress activates the phagocytotic activity of RPC-C2A cells using flow cytometry. The phagocytotic activity of RPC-C2A cells increased with heat stress, and the level of the phagocytotic activity returned to that in non-treated cells when heat-stressed cells were treated with cytochalasin D, an inhibitor of phagocytosis. There are no known reports regarding the effects of heat stress on the phagocytosis of apoptotic cells by scavenger cells, especially in dental pulp, though it has been reported that heat stress affected apoptosis induction under several conditions [O'Neill et al., 1998; Guzik et al., 1999; Sreedhar et al., 2000]. Our findings demonstrate that heat stress not only induces apoptosis in RPC-C2A cells, but also stimulates the phagocytosis of apoptotic RPC-C2A cells by surviving RPC-C2A cells. These are novel results about effects of heat stress on the apoptotic phenomenon.

It has also been reported that the subclone of RPC-C2A cells undergoes mineralization and possesses odontoblast-like characteristics [Ritchie et al., 2002], showing that this clonal pulp cell line may have the heterogeneity, and the co-presence of apoptotic cells and surviving cells acting as scavenger-like cells following heat stress in the present study might have been a result of this heterogeneity of RPC-C2A cells. Recently, Gronthos et al. [2000, 2002] demonstrated the presence of stem cells that differentiated into odontoblast-like cells in dental pulp tissue. RPC-C2A cells may also consist of several different populations such as a precursor population that differentiates into odontoblast-like cells, a regulator population that controls the differentiation of odontoblast-like cells, and a population of scavenger-like cells. The sensitivity to heat stress may be different among these populations. We are now recloning RPC-C2A cells to clarify this issue.

We also found non-fragmented apoptotic RPC-C2A cells adhered to intact RPC-C2A cells (Fig. 4B, d). As shown in Figure 5C, flow cytometric analysis findings revealed intense fluorescein-positive peaks, especially in heat-stressed cells. These results suggest that the intense fluorescein peak in flow cytometry might have been a results of fluorescein-labeled apoptotic cells that adhered to intact cells, not from fragmented apoptotic cells engulfed by intact RPC-C2A cells, and that the tight association of apoptotic cells with intact cells may occur for the subsequent phagocytosis. It has been reported that several molecules are responsible for the attachment and recognition of apoptotic cells [Savill et al., 1993; Luciani and Chimini, 1996; Brown et al., 2002; Hanayama et al., 2002]. We are now examining the mechanisms of recognition and phagocytosis of apoptotic RPC-C2A cells by surviving RPC-C2A cells.

In conclusion, we demonstrated that RPC-C2A cells have the thermotolerance, and that heat stress induces apoptosis in RPC-C2A cells, and apoptotic RPC-C2A cells induced by heat stress are targets for phagocytosis by sound and surviving RPC-C2A cells. We also found that this phagocytotic activity is potentiated by heat stress. These findings explain the in vivo cell death regulation of pulp cells [Kitamura et al., 2001, 2003], and suggest that the induction of apoptosis of pulp cells and phagocytosis of apoptotic pulp cells by other pulp cells play important roles in the cell-death process during pulp wound healing and regeneration.

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