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Apoptosis in stress-induced and spontaneously senescent human fibroblasts

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Abstract

Although apoptosis has been shown in vivo to be involved in the aging process, in vitro studies of age-dependent apoptosis are limited. In this study, apoptosis was examined in normal human fibroblasts exposed to H_2O_2 to induce premature senescence and in spontaneously senescent human fibroblasts. Although apoptosis was not observed for several days after exposure to H_2O_2, morphological changes indicating apoptosis were evident in about 5% of cells 7 days after exposure to 80 μM H_2O_2, concomitantly with expression of senescent phenotype. The apoptotic changes were preceded by caspase activation in majority of the exposed cells. As well as H_2O_2-induced senescent cells, spontaneously senescent human fibroblasts showed apoptotic changes in about 2% of cells and majority of the senescent cells also showed activation of caspases. The results indicate that the apoptosis pathway is activated in H_2O_2-induced and spontaneously senescent human fibroblasts in vitro.
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Keywords: Aging; Cellular senescence; Stress-induced premature senescence; Apoptosis; Caspases; Human fibroblasts

Recent studies suggest that apoptosis is involved in the aging process of various tissues. In the aged human brain, apoptotic-like changes [1] and an increased expression of Bak [2] have been reported. In peripheral blood lymphocytes from aged humans, activation of apoptosis pathway such as increased expression of Fas, Bax, and several caspases [3–5] as well as decreased expression of bcl-2 [3] have also been reported. In experimental animals, age-related activation of the apoptosis pathway in neuronal tissues [6,7], liver [8–10], lung and spleen [10], and arterial endothelial cells [11] has been demonstrated. All of these changes in the apoptosis pathway have been linked to age-related decline of physiological functions or age-related disorders, such as Alzheimer's disease or autoimmune diseases [12–14].

In vitro studies of age-associated apoptosis are limited. Although cultured normal human fibroblasts are widely used as an in vitro model of aging [15–17], studies about apoptosis in senescent human fibroblasts are scarce, and results from those studies are somewhat confusing [18–22]. It has been reported that senescent human fibroblasts are resistant to some apoptotic stimuli such as serum withdrawal [18], UV, and actinomycin D [19]. Other investigators have found, however, that senescent human fibroblasts undergo apoptosis in response to anti-Fas antibody plus cycloheximide [20], ceramide, okadaic acid, or TNF-α [21]. Another study reported that although spontaneous apoptosis was seen in approximately one-third of senescent human endothelial cells after serial passaging, no significant apoptosis was seen in human fibroblasts under the same conditions [22].

One of the characteristics of normal human fibroblasts in culture is that the cells show a senescent-like phenotype after exposure to sublethal stresses, such as

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H_2O_2 [23,24] or UV, a condition called stress-induced premature senescence [25]. Although several molecular changes observed in human fibroblasts after exposure to H_2O_2 have been reported [24,26,27], precise mechanisms for induction of senescence in these cells remain unclear. One particular feature of stress-induced premature senescence is that it takes from several days to one week after the stress to acquire the senescent phenotype. Although apoptotic response of normal human fibroblasts in several hours to one day after exposure to H_2O_2 has been reported [26,28,29], there is no report on apoptosis long after exposure to H_2O_2 when the cells would show senescent phenotype.

In this study, apoptosis was examined in normal human fibroblasts up to 2 weeks after exposure to mild concentrations of H_2O_2, which can induce premature senescence, and also in spontaneously senescent human fibroblasts. As a result, activation of the apoptosis pathway in H_2O_2-induced and spontaneously senescent human fibroblasts was suggested.

Materials and methods

Cell culture and treatment. Normal human fibroblasts designated HUC-F2 were obtained from RIKEN BioResource Center Cell Bank (Tsukuba, Japan) and grown in minimum essential medium with alpha modification (Sigma–Aldrich, St. Louis, MO) supplemented with heat-inactivated 10% fetal bovine serum. For caspase analysis, cells younger than 30 population doubling levels (PDLs) were seeded in several 60- or 100-mm dishes at a density of 2 or 5×10^5 cells per dish, respectively, and incubated overnight. Cells were then exposed to various concentrations of H_2O_2 diluted in PBS for 30 min, after which they were maintained in fresh media until analysis or reseeding. For morphological observations, cells were seeded in 35-mm dishes at a density of 5×10^4 cells per dish and treated similarly. Because it was confirmed that cells cultured at confluent density resulted in caspase activation and apoptotic cell death (unpublished results), cells were subcultured before they reached confluence and were maintained at medium to subconfluent density during the experimental period.

Histochemical detection of senescence-associated β-galactosidase. To evaluate cellular senescence, cells cultured on 35-mm dishes were fixed and stained for β-galactosidase using the Senescence Detection Kit (BioVision, USA). The number of β-galactosidase-positive cells per 1000 cells was scored after incubation with staining solution for 16–24 h at 37 °C.

Morphological identification of apoptosis. For morphological identification of apoptosis, cells cultured on 35-mm dishes were fixed with methanol for 10 min at room temperature and stained with 20 μg/ml acridine orange solution in PBS for 3 min. After staining, cells were washed with water and overlaid with 50% glycerol solution containing 5% 1,4-diazobicyclo-2,2,2-octane as anti-fade. Cells were then observed with an inverted fluorescent microscope (TE 300, Nikon, Japan) equipped with a blue excitation filter (B-2A).

Detection of caspase activation. Caspase activation was analyzed by flow cytometry using CaspGLOW Fluorescein Caspase Staining Kit (BioVision). Cells were harvested and incubated for 30 min at 37 °C in 300 μl medium containing fluorescein-labeled caspase inhibitor (FITC-VAD-FMK for pan-caspase, FITC-DEVD-FMK for caspase-3, FITC-IETD-FMK for caspase-8, and FITC-LEHD-FMK for caspase-9). After incubation, cells were washed with the kit-supplied Wash Buffer and analyzed with a flow cytometer (FACScan, Becton–Dickinson Biosciences, USA). Fluorescence was detected with an FL1 detector, and histogram data were generated with the CellQuest software.

Results

Spontaneous senescence and H_2O_2-induced premature senescence of normal human fibroblasts

HUC-F2 cells grew constantly until about the 17th passage, at which time cells reached a total of 35.5 PDL ($n = 8$). Thereafter, cells grew more slowly. Cell growth was arrested at approximately 40 PDL, by which time the cells showed typical senescent morphology, such as enlarged size or flattened shape. The mean doubling time was 36.4 h between the 7th and 17th passages ($n = 8$) and 95.1 h between the 18th and 22nd passages ($n = 3$). When cells reached a growth-arrested state, typically after the 22nd passage, more than 90% of cells had positive reactions for senescent-associated β-galactosidase, indicating acquisition of spontaneous senescence, which is referred to as replicative senescence as well (Fig. 1).

When non-senescent cells were exposed to 40 μM or higher concentrations of H_2O_2, cell growth was greatly suppressed (data not shown). While the number of β-galactosidase-positive cells increased slightly during the first 2 or 3 days after exposure to H_2O_2 (Fig. 1), cell morphology did not change greatly in these days. At 4 days after exposure to 80 μM H_2O_2, cells became a little larger, and the number of β-galactosidase-positive cells increased to about 20%. At 7 days after exposure to 80 μM H_2O_2, most of the cells showed senescent morphology similar to that of spontaneously senescent cells, and β-galactosidase-positive cells increased to about 80% (Fig. 1). A subset of

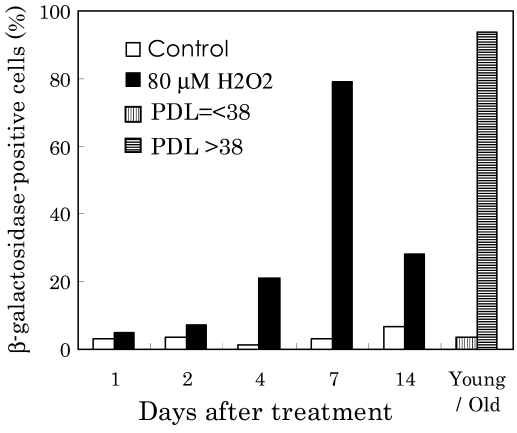

Fig. 1. Frequencies of β-galactosidase-positive cells in H_2O_2-induced and spontaneously senescent cells. β-Galactosidase-positive cells on culture dish were detected histochemically and scored as described in Materials and methods. Bars represent mean values from two experiments for H_2O_2-exposed cells, eight samples for young cells, and three samples for spontaneously senescent cells.

H_2O_2-exposed cells showed a tendency to recover from senescence between 7 and 14 days after exposure, and the numbers of β-galactosidase-positive cells decreased during this period.

Morphologically identified apoptosis in H_2O_2-induced and spontaneously senescent human fibroblasts

Cells undergoing apoptosis, which could be morphologically identified by fragmented nuclei or condensed chromatin in acridine orange-stained cells, were not observed for up to 4 days after exposure to H_2O_2. However, a dose-dependent number of cells showing apoptotic changes were observed 7 days after exposure to H_2O_2 (Figs. 2C and 3). At 80 μM H_2O_2, 5.3% of cells showed apoptosis at this time point ($n = 3$). The frequency of apoptotic cells decreased thereafter to about 1% at 14 days after exposure. Apoptotic changes similar to those observed in H_2O_2-induced senescent cells were also observed in 1.8% of spontaneously senescent cells after 38 PDL (Figs. 2D and 3).

Caspase activation in H_2O_2-induced and spontaneously senescent cells

Significant activation of caspases was recognized from 2 days after exposure to H_2O_2 by flow cytometric analysis. The activation intensified thereafter and showed about 4-fold increases compared with control cells at 4 and 7 days after exposure to 80 μM H_2O_2 (Figs. 4 and 5). This activation of caspases decreased during the second week after exposure. There were no notable differences among the 3 caspases analyzed. Like H_2O_2-induced senescent cells, spontaneously senescent cells showed caspase activation when compared to young cells (Figs. 4 and 5). The average mean

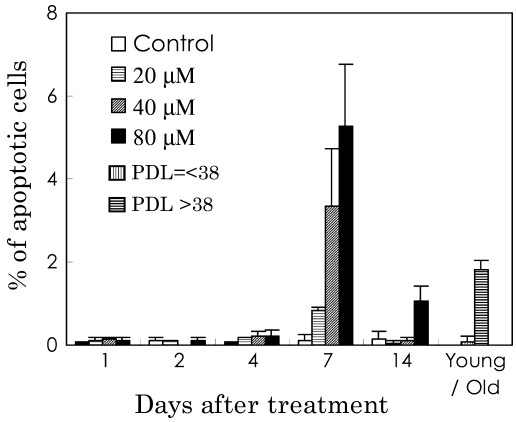

Fig. 3. Frequencies of morphologically identified apoptosis in H_2O_2-induced and spontaneously senescent cells. Cells were stained with acridine orange and scored for apoptosis as described in Materials and methods. Bars represent means ± SE from pooled results of six experiments for H_2O_2-exposed cells and from four samples each for young and spontaneously senescent cells.

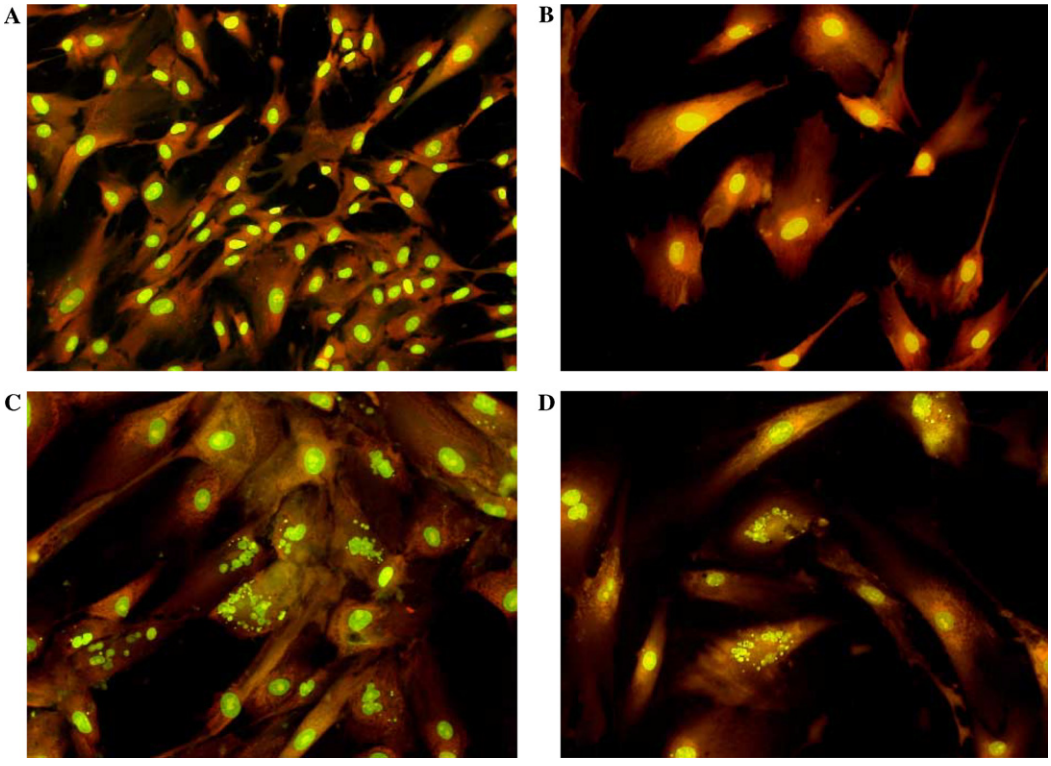

Fig. 2. Human fibroblasts (HUC-F2) stained with acridine orange. (A) Untreated young cells (34.0 PDL). (B) Cells showing slight enlargement at 4 days after exposure to 80 μM H_2O_2. (C) Cells showing fragmented nuclei at 7 days after exposure to 80 μM H_2O_2. (D) Cells showing fragmented nuclei in spontaneously senescent cells (43.0 PDL).

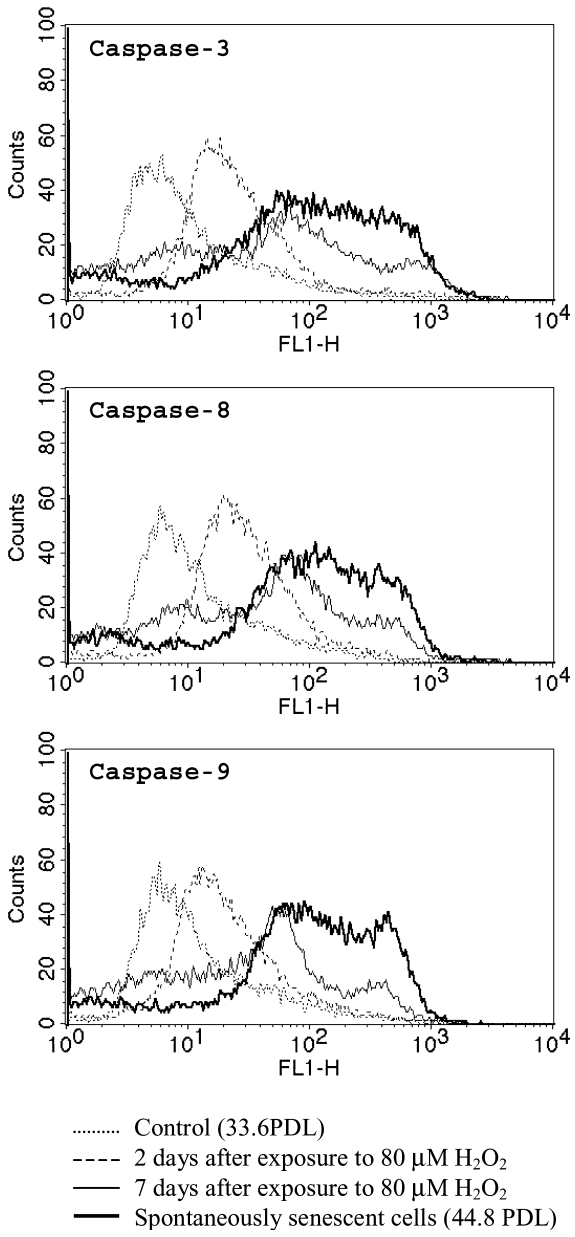

Fig. 4. Representative flow cytometry histograms showing activation of caspases in H_2O_2-induced and spontaneously senescent cells. Cells with activated caspases were labeled with fluorescein-labeled caspase inhibitors and analyzed by flow cytometer as described in Materials and methods.

fluorescence intensity in seven samples from old cells (PDL > 38) was 5.1-fold higher than in seven samples from young cells (PDL ⩽ 38) in flow cytometer analysis (Fig. 5).

Discussion

The results obtained in this study demonstrate that a small subset of normal human fibroblasts undergo apoptosis concomitantly with cellular senescence. Some

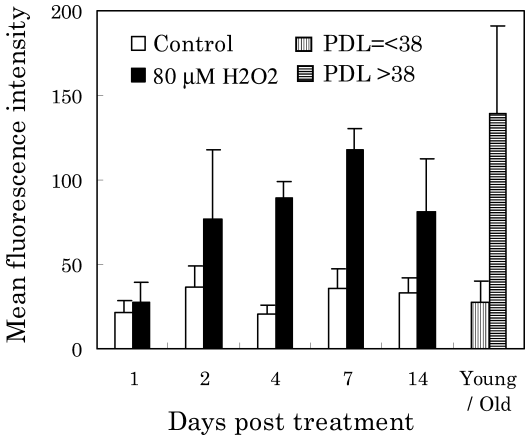

Fig. 5. Increase of pan-caspase activity in H_2O_2-induced and spontaneously senescent cells. Cells were labeled with a fluorescein-labeled pan-caspase inhibitor and analyzed by flow cytometer as described in Materials and methods. Values are expressed as mean fluorescent intensity in arbitrary units obtained by flow cytometer analysis. Bars represent means ± SE from three experiments for H_2O_2-exposed cells and seven samples each for young and spontaneously senescent cells.

investigators have suggested that age-dependent apoptosis might be a cell-type-specific phenomenon. Wagner et al. [22] reported that human fibroblasts are relatively resistant to spontaneous apoptosis in the senescent phase compared to human endothelial cells. In their data, however, DNA histograms of fibroblasts showed that cells in a sub-G1 peak increased from 0.5% in 23 PDL to 3.7% in 56 PDL. Yeo et al. [30] also showed that DNA fragmentation in cultured human fibroblasts increases to about 3% with serial passaging. These percentages are similar to the data based on morphological observation of apoptosis in H_2O_2-induced and spontaneously senescent cells in this study. Therefore, it seems that a small subset of normal human fibroblasts undergoes apoptosis with in vitro aging. The activation of caspases in H_2O_2-induced and spontaneously senescent human fibroblasts observed in this study also supports the idea that the apoptosis pathway is activated in senescent human fibroblasts. Nearly equal activation of the 3 caspases examined in this study suggest that both the death receptor pathway and the mitochondrial pathway are involved in senescent human fibroblast apoptosis.

Although H_2O_2 has widely been used to induce apoptosis in cell culture studies [28,31,32], apoptosis induction in those studies was an acute phenomenon that took place over several hours to 1 day after exposure to H_2O_2. It is unlikely that apoptosis that occurs 1 week after exposure to H_2O_2, as seen in this study, is a result of the direct effect of H_2O_2. Parallel changes in frequencies of cells with apoptotic changes, and senescent phenotype after a stress suggests that the apoptosis observed in this study is a result of cellular senescence.

However, there is a discrepancy between caspase activation and morphological observation of apoptosis in

H_2O_2-induced and spontaneously senescent cells. Although the majority of H_2O_2-induced senescent cells showed significant activation of caspases from 2 days after exposure to H_2O_2, only a small fraction of cells showed morphological changes indicating apoptosis at 1 week after exposure. Also, in spontaneously senescent cells, only a small fraction of cells showed apoptotic changes despite activation of caspases in a majority of cells. There are several possibilities that may account for these discrepancies. (1) Because morphological observation of apoptosis in this study was carried out on cells attached to dishes, cells detached by apoptosis were not scored. This might account for the low frequency of apoptosis in this study, but it cannot explain the time difference between caspase activation and morphologically identified apoptosis in H_2O_2-induced senescence. (2) Activation of caspases is not enough to cause apoptosis in the early phase of H_2O_2-induced senescence, but pronounced increases in caspase activities in a subset of fully senescent cells eventually result in apoptosis. The validity of this hypothesis is not clear, and more detailed examinations are necessary. (3) Apoptotic cell death in senescent cells is a slow process and proceeds in different timing in each cell despite activation of caspases in a majority of cells, so that only a small fraction of cells show morphological change indicating apoptosis at a given time. This hypothesis was suggested for aging neuronal tissue [7]. (4) Activation of caspases does not result in apoptosis immediately after activation, because other factors such as caspase inhibitors are regulating apoptosis and inhibition of these inhibitors is necessary for final execution of apoptosis. This point is becoming clear by recent research (reviewed in Liston et al. [33]) and might be related to hypotheses (2) and (3) as well. (5) Activation of caspases observed in this study is not related to apoptosis but to other physiological function(s). Although this point was also suggested in a recent report on caspase activation in Alzheimer's brain [34], further studies are necessary to validate this hypothesis.

Another point in this study relating H_2O_2-induced cellular senescence should also be discussed. Although cells exposed to H_2O_2 showed senescent phenotype by 1 week after exposure, these cells showed a tendency to recover from senescence thereafter, and the frequency of apoptotic cells decreased as well. Published reports regarding H_2O_2-induced premature senescence have suggested that the growth arrest caused by a sublethal dose of H_2O_2 may be irreversible [23,24]. In this study as well, growth arrest by exposure to 40 μM or higher concentrations of H_2O_2 seemed to be irreversible if cells were not subcultured after exposure (data not shown). However, when cells were subcultured several times during the experimental period to reduce the cell density of control cells, a subset of cells restarted to proliferate in the second week after exposure, and cells with senescent phenotype decreased. This recovery from senescence might be because the concentrations of H_2O_2 used in this study were not high enough to induce complete senescence in the cells. It is also possible that the senescent phenotype induced by H_2O_2 in this study does not represent authentic senescence. If the latter is the case, it is important and its validity should be confirmed.

Although much evidence indicates the apoptosis pathway is activated in aging tissues or cells, the role of apoptosis in the aging process is poorly understood. Further studies on mechanisms leading to age-associated apoptosis using in vitro senescent cells may contribute to our understanding of the role of apoptosis in the aging process.

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