Effect of oxygen radical on apoptosis in human submandibular gland cell lines

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Abstract

Oxygen radicals have been implicated as being involved in cellular damage, oxidation of proteins, membrane lipid peroxidation, inactivation of enzymes, and DNA chain breakage during the processes of aging, inflammation, and injury. Apoptosis is an essential mechanism for the selective elimination of cells that is associated with a variety of disorders and encompasses most cell systems.

We investigated the effects of extracellular oxygen radicals on apoptosis in a cultured human submandibular gland (HSG) cell line and HSG-AZA3 cells, using an acinar cell phenotype that was induced by treatment of HSG cells with 5-azacytidine. The induction of apoptosis was examined after the cells were exposed to an oxygen radical system composed of hypoxanthine (HX) and xanthine oxidase (XOD). The appearance of nuclei with DNA fragmentation was detected by a TUNEL method while the expression of active caspase-3 was found by immunocytochemistry. Apoptosis of HSG and HSG-AZA3 cells induced with HX/XOD was significantly greater, and active caspase-3 staining intensity was greater than in the control.

These findings suggest that caspase-3 expression may be regulated by oxygen radicals and involved in oxygen radical-induced apoptosis of HSG and HSG-AZA3 cells. Moreover, these established cell lines are useful as a model of aging in submandibular glands.

Introduction

It has been reported that salivary flow rates after stimulation is reduced by aging (1, 2), and Yeh et al. demonstrated significant reductions of both unstimulated and stimulated submandibular gland flow rates due to aging in an analysis of more than 1000 human subjects (3). Further, it has been reported that the synthesis rate of secretory proteins (4) and amylase synthesis decline with age (5) while the histologic structure of salivary glands also changes with age and shows a fairly linear loss of acinar cells, which fatty or connective tissues replace (6).

To maintain overall tissue and organ-size homeostasis, a balance between cellular proliferation and loss must be sustained. This balance is preserved by a complex system of growth factors and survival signals that are counterbalanced by an equally intricate system of cell death or apoptosis (7). Apoptosis is initiated either by ligand binding to cell surface receptors or by cell toxins via a pathway that targets mitochondria. In the receptor-mediated pathway, ligand binding to receptors of the TNF/Fas family leads to the activation of caspases, resulting in DNA fragmentation and cell death (8). Recently, Morimoto et al. (9) reported that treatment of HSG cells from a neoplastic epithelial duct cell line established from a human submandibular gland (10) with anti-Fas monoclonal antibody resulted in apoptosis.

The free radical theory of the aging process is based on the hypothesis that increasing age mutations of mitochondrial DNA will accumulate and lead to at least a loss of function along with a subsequent acceleration of apoptosis (11, 12). However, the mechanism of apoptosis caused by free radicals during the aging process in salivary gland cells has not been well elucidated. Therefore, we examined the effects of extracellular oxygen radicals on apoptosis in HSG cells using a hypoxanthine/xanthine oxidase system, as well as the oxidative changes in submandibular gland tissues in vivo.
Materials and methods

Cell culture

A human submandibular gland (HSG) cell line was derived from intercalated ductal cells (10) and cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (1:1 mixture) (Gibco, Grand Island, NY) while HSGAZA3 cells, HSG cell derivatives, with an acinar cell phenotype induced by treatment with 5-azacytidine, were cultured in Eagle’s Minimal Essential Medium (Gibco) (13). Both cell lines were provided by Dr. Mitsunobu Sato. The media were supplemented with 5% fetal calf serum (FCS). Cells were incubated at 37°C in a 5% CO2 atmosphere.

Animals

Female WKY rats at 6 and 31 months of age were obtained from Sankyo Labo Service Co. (Tokyo, Japan). They were bred under conventional conditions, housed at 23 ± 1°C, and allowed free access to food and water, with a light/dark cycle of 12 hours. After euthanasia, the submandibular glands were immediately excised and placed in 10% formalin and then fixed in an automatic tissue fixing machine. The tissues were carefully embedded in molten paraffin in metallic blocks, covered with flexible plastic molds, and kept under freezing plates to allow the paraffin to solidify. The metallic containers were then removed, and the specimens left embedded in paraffin on the plastic molds. Paraffin-embedded tissue blocks were cut into 4-μm sections, which were deparaffinized with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, 90%, 80%, and 70%). After washing, these were treated with 0.3% hydrogen peroxide in absolute methanol for 15 minutes at room temperature to block endogenous peroxidase activity before being used.

Treatment with HX/XOD system

HSG cells were seeded at 4 × 10⁴ cells/well and HSGAZA3 cells at 2 × 10⁴ cells/well in 8-well chamber slides (Nalge Nunc International, Naperville, IL). Confluent stage cells were treated with a hypoxanthine (HX, Sigma Chemical Co, St. Louis, Mo) and xanthine oxidase (XOD, Sigma Chemical Co.) system (14) for 10 minutes, the medium was removed, and the cells were washed twice with PBS. After washing, fresh medium was added, and the cells were incubated at 37°C in a 5% CO2 atmosphere. Finally, the cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes, then washed and used in the experiments.

Apoptosis assay

Apoptosis was determined by a TUNEL (TdT-mediated dUTP nick end labeling) reaction using an ApopTag® Peroxidase In Situ Apoptosis Detection kit (Intrtgen Co., Purchase, NY). Equilibration buffer was immediately applied directly onto the HSG and HSG-AZA3 cells and the rat submandibular glands on the chamber slides; they were incubated in a humidified chamber for 10 minutes at room temperature. The sections were blotted, and an appropriate amount of working strength TdT enzyme was immediately placed onto the slides with a pipette; they were incubated in a humidified chamber at 37°C for 1 hour, then with stop/washing buffer for 10 minutes at room temperature. After they were washed in 3 changes of PBS for 1 minute each, an appropriate amount of anti-digoxigenin peroxidase was applied to the slides, and they were incubated in a humidified chamber for 30 minutes at room temperature. After being washed again in PBS, the slides were stained with DAB substrate working solution for 3 minutes at room temperature and then counterstained with 0.5% methyl green for 10 minutes at room temperature. After being washed in distilled water, the slides were dehydrated, cleared, and mounted under a glass coverslip. Negative controls were performed with PBS in place of the TdT enzyme, and normal female rodent mammary gland tissue (supplied in the kit) was used as a positive control (results not shown). All images of HSG cells and HSG-AZA3 on the chamber slides were obtained using a 20 X objective with a 10 X eyepiece, which yielded a final magnification of 200 X. At least 300 cells were examined from more than 7 randomly selected images for each well. The average percentage of TUNEL-positive cells per well was determined from the results of 3 wells.

Immunohistochemistry for culture cells and tissue

HSG and HSG-AZA3 cells on chamber slides were incubated with a polyclonal antibody against active caspase-3 (1:250 dilution, Promega Co., Madison, WI) in a humidified chamber for 1 hour at room temperature. Rat submandibular gland tissues on slides were incubated with
a monoclonal anti-crotonaldehyde antibody (1:100 dilution, NOF Co., Tokyo, Japan) in a humidified chamber overnight at 4°C. Detection was accomplished with Histofine Simple Stain Rat MAX PO (MULTI) (NICHIREI Co. Tokyo, Japan). A universal immuno-peroxidase polymer was applied in a humidified chamber for 30 minutes at room temperature; the slides were stained with DAB substrate working solution for 3 minutes at room temperature, then counterstained with Mayer's haematoxylin, and dehydrated, cleared, and mounted under glass coverslips. Negative controls were performed with PBS in place of the primary antibodies. The average percentage of positive stained HSG and HSG-AZA3 cells was determined using the same method noted above for the TUNEL-positive rate.

Statistical analysis

Data are expressed as mean ± S.D. Differences between groups were analyzed using Student’s z-test.

![Graph of TUNEL-positive HSG cells percentage vs. XOD concentration](image1)

Fig. 1 Effect of XOD concentration

HSG cells were treated with HX (0.1 mg/ml) and varying concentrations of XOD for 10 minutes, the medium was removed, and the cells were washed twice with PBS. After washing, fresh medium was added, and the cells were cultured for 20 minutes. A significant increase in TUNEL-positive cells was recognized from 1 to 10 munit/ml of XOD in treatment with HX/XOD. Results are expressed as mean ± S.D. (n=3). *p < 0.05, compared with the control, in medium without HX/XOD.

![Graph of TUNEL-positive HSG cells percentage vs. HX/XOD stimulation time](image2)

Fig. 2 Effect of HX/XOD stimulation time

HSG cells were treated with or without HX (0.1 mg/ml) and XOD (5 munit/ml) for the various stimulation times noted, the medium was removed, and the cells were washed twice with PBS. After the cells were washed, fresh medium was added, and the cells were cultured for 20 minutes. A significant increase in TUNEL-positive cells was recognized from 10 to 40 minutes by HX/XOD. Results are expressed as the mean ± S.D. (n=3). *p < 0.05, compared with the control, in medium without HX/XOD.

![Images of HSG and HSG-AZA3 cells showing positive TUNEL labelled cells](image3)

Fig. 3 HSG and HSG-AZA3 cells showing positive TUNEL labelled cells

HSG and HSG-AZA3 cells were treated with HX (0.1 mg/ml) and XOD (5 munit/ml) for 10 minutes, the medium was then removed, and the cells were washed twice with PBS. After washing, the cells were cultured in fresh medium for 20 minutes. Several positive cells (arrows) were observed. Original magnification × 400. Scale bars indicate 25 μm.
Results

Effect of HX/XOD on expression of TUNEL-positive cells

HSG cells were treated with HX (0.1 mg/ml) and varying concentrations of XOD (1, 5, 10, and 25 munit/ml) for 10 minutes. The maximum number of TUNEL-positive cells was seen at 5 munit/ml of XOD (Fig. 1). Next, the effect of incubation time on the expression of TUNEL-positive cells treated with HX (0.1 mg/ml) and XOD (5 munit/ml) was examined. A significant increase in TUNEL-positive cells was recognized at 10 minutes, as shown in Figure 2.

Figure 3 shows representative HSG and HSG-AZA3 cells with positive labeling using the TUNEL method. Chromatin condensation and distribution to the edge of the nuclear membrane were also observed on TUNEL-positive cells. Table 1 summarizes the percentages of TUNEL-positive HSG and HSG-AZA3 cells treated with HX (0.1 mg/ml) and XOD (5 munit/ml) for 10 minutes.

Immunohistochemistry for active caspase-3 on HSG and HSG-AZA3 cells

HSG and HSG-AZA3 cells were treated with HX (0.1 mg/ml) and XOD (5 munit/ml) for 10 minutes. The number of active caspase-3 positive-stained cells (Fig. 4) was increased after treatment, as compared to the control (Table 2).

Immunohistochemistry for rat submandibular gland tissues

Immunohistochemistry was used to detect crotonaldehyde in paraffin sections of submandibular gland tissues taken from rats at 6 and 31 months of age. Antibodies specific for crotonaldehyde were found on the duct cells, and staining was generally stronger in the older rat specimens (Fig. 5).

Apoptosis was detected in submandibular glands from rats at both 6 and 31 months of age using the TUNEL method; however, while a number of positively stained cells were detected in those from rats at 31 months of age, those from 6-month old rats were nearly undetectable (Fig. 6).
Table 1. A percentage of the TUNEL-positive HSG and HSG-AZA3 cells (mean ± S.D.)

<table>
<thead>
<tr>
<th>Group (cells)</th>
<th>control</th>
<th>treated with HX/XOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSG cells</td>
<td>0.356±0.103</td>
<td>0.918±0.131*</td>
</tr>
<tr>
<td>HSG-AZA3 cells</td>
<td>0.352±0.086</td>
<td>0.824±0.168*</td>
</tr>
</tbody>
</table>

Table 1 Summary of the TUNEL-positive percentages on HSG and HSG-AZA3 cells. HSG and HSG-AZA3 cells treated with HX (0.1 mg/ml) and XOD (5munit/ml) for 10 minutes. After treatment, apoptosis was detected on these cells by TUNEL methods. A significant increase in TUNEL-positive cells that were treated with HX/XOD was recognized. *p < 0.05, compared with control in medium without HX/XOD.

Table 2. A percentage of the anti active caspase3-positive HSG and HSG-AZA3 cells (mean ± S.D.)

<table>
<thead>
<tr>
<th>Group (cells)</th>
<th>control</th>
<th>treated with HX/XOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSG cells</td>
<td>0.254±0.027</td>
<td>0.574±0.074*</td>
</tr>
<tr>
<td>HSG-AZA3 cells</td>
<td>0.364±0.129</td>
<td>0.758±0.081*</td>
</tr>
</tbody>
</table>

Table 2 Summary of the anti active caspase-3-positive percentages on HSG and HSG-AZA3 cells. HSG and HSG-AZA3 cells treated with HX (0.1 mg/ml) and XOD (5munit/ml) for 10 minutes. After these cells were treated with HX/XOD, active caspase-3 was detected on these cells by immunocytochemistry using an antibody against active caspase-3. A significant increase in anti active caspase-3-positive cells that were treated with HX/XOD was recognized. *p < 0.05, compared with control in medium without HX/XOD.

Discussion

The functional impact of aging on adult salivary glands appears to be relatively modest, based on most criteria, and tends to be selective (15) while the submandibular gland shows an array of responses that accompany aging, which are also usually modest. In contrast, submandibular acinar-cell mucin shows a substantial decline in total amount produced per gland as humans age, and a number of factors that may influence either parenchymal loss or a change in its cellular composition have been proposed. Liu et al. (16) studied aging-related changes of apoptosis and found an increased rate of apoptosis for acinar cells, which they reported was one of the main reasons for the decline in the proportion of acinar cells in the submandibular gland.

Apoptosis in salivary glands has also been found with several pathological conditions. For example, the diminished salivary flow in Sjögren’s syndrome is the result of an increased frequency of apoptosis among ductal epithelial cells (17) while Fas is strongly expressed on ductal epithelial cells (18). Using a TUNEL method, Takahashi et al. (19) reported that submandibular gland duct ligation induced a marked depletion of myoepithelial cells by apoptosis, and the binding of the Fas ligand to the receptor was found to lead to receptor activation and the induction of intracellular signals that result in apoptosis. As for HSG cells, Morimoto et al. (9) reported that the expression of mRNAs and proteins of the Fas receptor and Fas ligand were expressed in those cells after treatment with okadaic acid while an antagonistic anti-Fas ligand monoclonal antibody prevented okadaic acid-induced apoptosis. These findings suggest that the Fas receptor-ligand system regulates apoptosis in HSG cells.

The mechanism on aging-related salivary secretion is still not entirely clear. Postmortem studies have demonstrated that, with aging, the parenchyma of the salivary glands is gradually replaced by fat, connective tissue, and oncocyes. Although apoptosis of salivary gland cells has been demonstrated in those pathological conditions, the role of apoptosis and its cellular mechanism in salivary glands with aging remains unknown, and the effect of the oxygen radical has not been well documented. It is very difficult to establish primary cultures of human salivary gland cells since the isolation of live tissue from healthy human subjects presents
ethical problems, and extended cultures of acinar and intercalated duct cells are known to be complicated. Thus, there may be further delay before studies of the cellular mechanism of apoptosis in human salivary glands caused by aging can be presented.

Shirasuna et al. (10) established an HSG clonal cell line from an irradiated human submandibular salivary gland that showed no neoplastic lesions, which resulted in stable growth and the formation of a duct-like structure with a mucinous eosinophilic substance. Furthermore, Sato et al. (13) treated HSG cells with 5-azacytidine and established an HSG-AZA3 cell line that exhibits phenotypes similar to those of acinar cells, such as microfibrils and myosin. These cells were employed in the present study to elucidate the effect of oxygen radicals on apoptosis in human salivary gland cells.

Oxidative stress resulting from the effects of oxygen radicals plays an important role in the pathogenesis of a variety of diseases, as well as important biological processes such as aging, inflammation, and injury response (20-22). The effects of oxygen radical metabolites, including superoxide anion, H$_2$O$_2$, and hydroxyl radicals, involve chemical alterations in proteins, lipids, carbohydrates, and nucleic acids (23-25). Further, low levels of oxygen radicals are produced as a normal part of cellular metabolism, and cells contain several enzymes whose function is to detoxify these radicals (26). Higher levels of oxygen radicals are generated upon activation of xanthine oxidase or during the respiratory burst of phagocytic cells, and these higher concentrations have been associated with tissue damage (27, 28). Faced with a decline in protective enzymes and as an adaptive capacity associated with aging, cells may sacrifice themselves by apoptosis, which results in the protection of surrounding healthy tissue from further damage.

To the best of our knowledge, results of the effect of oxygen radicals on the induction of apoptosis in human ductal and acinar cells have not been reported. For the present study, we used 2 cell lines, HSG and HSG-AZA3, as experimental ductal and acinar cells, respectively, to examine the effect of oxygen radicals on apoptosis.

Our results after treatment with HX/XOD clearly demonstrated induced apoptosis in both types of cells, as seen by a TUNEL method. The induced rates of apoptosis in both were similar and not at a high level. In an in vivo experiment of apoptosis associated with aging in mice, Liu using a TUNEL method found apoptotic cell populations in acinar cells: 0.0093% (3 months old) and 0.0189% (28 months old); intercalated-duct cells: 0.0320% (3 months old) and 0.0282% (28 months old); and glandular-duct cells: 0.0086% (3 months old) and 0.0075% (28 months old). The apoptotic cell population in all cells was 0.0124% (3 months old) and 0.0156% (28 months old). There was an increased rate of apoptosis for acinar cells that was moderately different between 3 and 28 months; however, no significant changes were seen in ductal cells (16).

Apoptosis is initiated either by ligand binding to cell surface receptors or by cell toxins via a pathway that targets mitochondria. In the receptor-mediated pathway, ligand binding to the tumor necrosis factor (TNF)/Fas family of receptors results in activation of the initiator caspase, caspase-8. Active caspase-8 then activates downstream “effector” caspases, which cleave cell proteins, and ultimately results in DNA fragmentation and cell death. Recently, Morimoto et al. (9) reported that treatment of HSG cells with the anti-Fas monoclonal antibody resulted in apoptosis, and Matsumura et al. (29) found that treatment with TNF-alpha also resulted in cell death. However, there are no reports of apoptosis in the mitochondria pathway caused by oxygen radicals in HSG cells. Various types of stress, including an oxygen-radical-instigated release of cytochrome c, activates caspase-9 from mitochondria, and then active caspase-9 activates caspase-3. Our TUNEL method results showed that the rate of apoptosis in both ductal and acinar cells was relatively modest. Therefore, we confirmed the rate by analyzing the expression of active caspase-3 in cultured HSG and HSG-AZA3 cells by immunocytochemistry using a monoclonal antibody against the active form of caspase-3. Active caspase-3 was observed expressed in cultured HSG and HSG-AZA3 cells treated with HX/XOD at greater levels than in the control, and the rates of TUNEL-positive cells and caspase-3 positive cells in both HSG and HSG-AZA3 cells were similar.

Our findings also suggest that a significant function of caspase-3 induced the apoptotic cell death of HSG and HSG-AZA3 cells and that caspase-3 is an important molecule regulating apoptosis in cultured HSG and HSG-AZA3 cells treated with HX/XOD. Activation of caspase-3 has been suggested to represent the point of no return in the execution
The relationships of HSG and HSG-AZA3 cells to salivary gland cells in normal and disease states is unknown; however, with only a few salivary gland cell lines presently available, the HSG cell line is by far the best characterized and has attributes similar to normal intercalated duct and acinar cells. We found that a challenge by oxygen radicals was able to induce apoptosis in HSG and HSG-AZA3 cells, suggesting that they may facilitate an understanding of the apoptosis mechanisms of signal transduction pathway and caspase activation cascade by oxygen radicals. Additional studies are now in progress in our laboratory.

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References


