Glyoxal-Induced Premature Senescence in Human Fibroblasts

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ABSTRACT: Mild stress-induced hormesis is an effective strategy to intervene in the aging process. Repeated exposure of human skin fibroblasts to 41°C heat shock for 1 h twice a week is an example of mild stress that has many hormetic effects, including improved resistance to other stressors. We are now developing an experimental model system of sugar-induced premature senescence, which can be useful to test the hormetic and antiaging effects of other stresses. Our present studies show that early-passage human skin fibroblasts treated with 1 mM glyoxal for 72 h undergo premature senescence in terms of enlarged cell size, inhibition of cell division, slowing down of cell growth, a decrease in the number of DNA synthesizing cells, and increased resistance to apoptosis.

KEYWORDS: sugar; senescence; aging; antiaging; human skin fibroblasts; hormesis; stress

INTRODUCTION

Oxidative reactions of glucose and many of its metabolites ultimately result in the formation of altered proteins and DNA damage products.[1,2] Aging and several age-related pathologies, such as Alzheimer´s disease and diabetes, are associated with the accumulation of altered proteins.[3] The aim of this study is to set up an experimental system in which the effects of glyoxal on cultured human fibroblasts resemble the phenotype of replicative senescence. The long-term aim is to use this experimental model to test the effects of mild stress (hormesis) and the effects of natural and synthetic molecules in the prevention and modulation of cellular aging.

EXPERIMENTAL METHODS

Cultures of human adult skin fibroblast cells, designated ASF-2, were established from the breast biopsy specimen of a consenting young healthy Danish

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woman.[4] ASF-2 cells were serially passaged at 1:2 or 1:4 split ratio at 37°C, 5% CO_2, and 95% humidity using culture medium (DMEM with 25 mM glucose, Cambex Bio Science, Vervierts, Belgium) with 10% fetal calf serum (Hyclone, Logan, Utah) and penicillin–streptomycin antibiotics (Cambex Bio Science). At 90% confluence the cell culture was split using the trypsin/EDTA method (BioWhittacker™, Cambex Bio Science). All experiments were performed with early-passage ASF-2 cells (population doubling levels between 7 and 25, which represent 15–50% replicative life span completed *in vitro*).

Cells were treated with 0.1–3.0 mM glyoxal 24 h after seeding. Glyoxal (40% aqueous solution, 8.72 M) was purchased from Sigma-Aldrich (Brondby, Denmark) and prepared in culture medium. Cells were analyzed for S-phase entry with an immunhistochemical 5-Bromo-2´-deoxy-uridine (BrdU) assay. Cells were seeded in slide flasks (growth area 10 cm^2) with input ranging from 5000 to 25,000 cells/cm^2. The BrdU labeling was initiated 12 h after seeding followed by 24 h of continuous labeling. The cells were fixed for 20 min at –20°C in ethanol, containing 16 mM glycine. After fixation, all incubation steps were performed for 30 min at 37°C without CO_2. Cell cycle progression of glyoxal-treated cells was analyzed with an enzyme-linked immunosorbent assay (ELISA) BrdU Assay Kit (Roche A/S Diagnostics, Hvidovre, Denmark). Apoptosis was evaluated with flow cytometry and samples were collected in duplicates. The culture medium was stored from all samples and centrifuged with the detached cells for 5 min at 2500 rpm. Binding of Annexin V and propidium iodide (PI) was performed with a kit (BD pharMingen, Franklin Lakes, NJ). Cell suspensions were loaded on the FACS Calibur cytometer (Becton Dickinson Biosciences, San Diego, CA). Cell Quest Pro was used for data acquisition and FlowJo for data analysis. Cell size and granularity were analyzed with flow cytometry. Cells were detached in 800 μL trypsin/EDTA solution and resuspended in 1 mL phosphate-buffered saline (PBS). Cell suspensions of samples in triplicates were loaded on the FACS Calibur cytometer (Becton Dickinson Biosciences). Acquisition and analysis of data were performed, as described above. The significance of changes in the experimental variables measured was assessed by Student's *t*-test.

RESULTS AND DISCUSSION

The present series of experiments were performed on early-passage ASF-2 cells, where cell growth had not been arrested as a consequence of *in vitro* aging. A BrdU immunohistochemical assay on untreated cells was used to evaluate cell cycle progression under normal conditions. Different cell densities were tested in order to see their effect on the number of cells entering S-phase. The proportion of cells able to enter S-phase at cell densities of 5000 and 25,000 cells/cm^2 was 90% and 60%, respectively. The results show that the majority of early-passage ASF-2 cells was able to enter S-phase even at high densities.

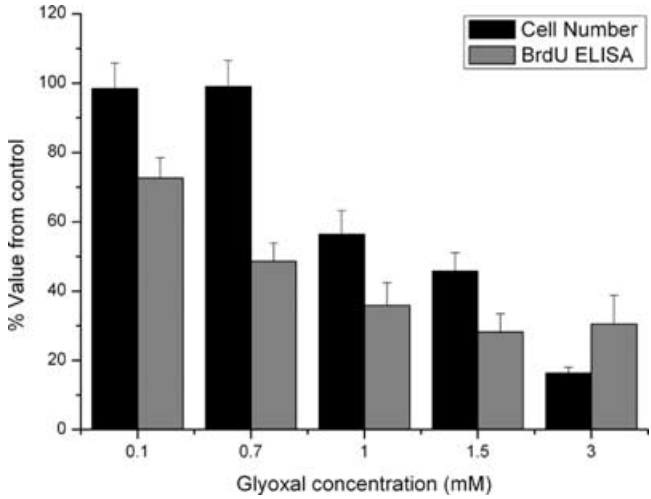

FIGURE 1. Effect of glyoxal on cell number and cell cycle progression of human skin fibroblasts (BrdU labeling).

FIGURE 1 shows the effect of glyoxal on cell number and BrdU incorporation 72 h after treatment. There was a dose-dependent decrease in cell number and BrdU signal after glyoxal treatment. At 1 mM concentration, the reduction in cell number and BrdU signal was 44% and 64%, respectively ($P < 0.05$).

The possibility that the reduction in cell number was due to enhanced apoptosis was investigated by flow cytometry and Annexin V and PI double staining after 1.0 mM glyoxal treatment. There was some induction of apoptosis within the first 24 h of glyoxal treatment (44% increase with respect to the control, $P < 0.05$), which may be an indication of selective death of most sensitive cells. However, after 48 and 72 h of glyoxal treatment, the extent of apoptosis was reduced by 37% and 41%, respectively, in glyoxal-treated cells compared with untreated cells ($P > 0.05$). Although this was not statistically significant, it may indicate a trend toward an increased resistance to apoptosis. No necrosis occurred during 72 h of treatment, as determined by the amount of Annexin V and PI double positive cells.

Changes in the cell morphology were observed in ASF-2 cells treated with various concentrations of glyoxal (FIG. 2). The most dramatic effects could be observed in cells treated with 1 mM glyoxal for 72 h. The cells were much larger, flattened, irregularly shaped, and had highly polymerized actin filaments. The appearance of glyoxal-treated ASF-2 cells was very similar to what is normally seen in late-passage senescent cells.[4]

Glyoxal-treated cells were further analyzed for cell size distribution and granularity. FIGURE 3 shows forward scatter (indicative of cell size) and side scatter (indicative of cell granularity) in histograms. Glyoxal treatment

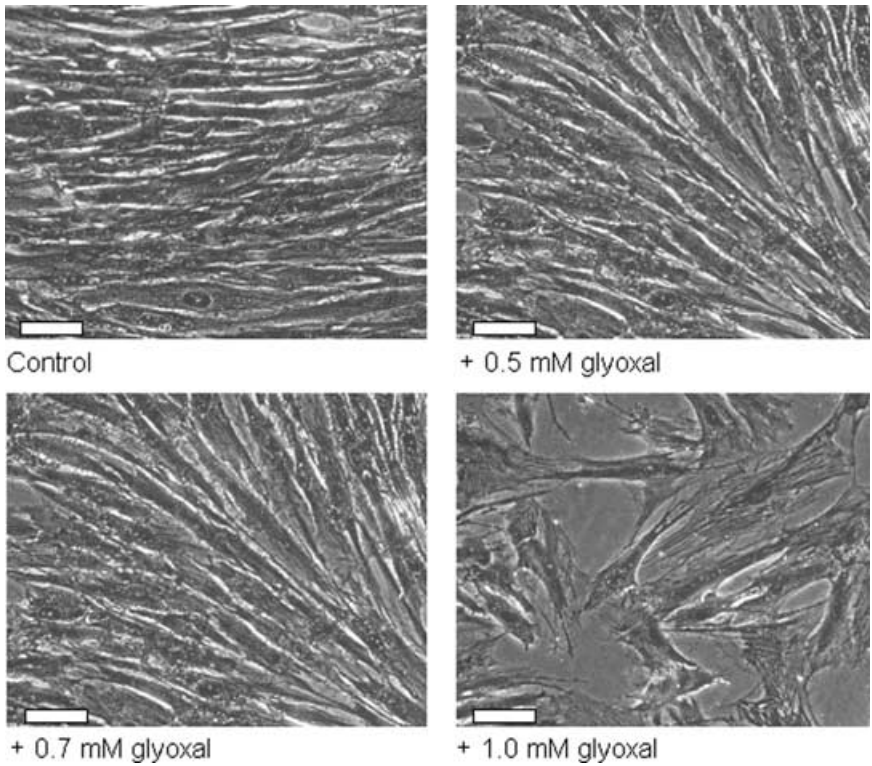

FIGURE 2. Effect of glyoxal treatment on the morphology of early-passage human skin fibroblasts (Bar: 100 μM).

increased the forward scatter of most cells, which was seen in a different distribution pattern with more cells toward the right end of the *x* axis (FIG. 3, panel A). The distribution of side scatter after glyoxal treatment showed more cells in the bins with higher values (FIG. 3, panel B), which could indicate an increase in the formation of residual bodies and protein aggregation.[3] The forward scatter median and side scatter median increased by 89% and 137%, respectively ($P < 0.05$).

Our results show that ASF-2 cells treated with glyoxal undergo premature senescence in terms of growth inhibition, cell cycle arrest, cell enlargement, and increased resistance to apoptosis. We are now investigating various other parameters of cellular senescence, such as levels of glycoxidatively damaged proteins, upregulation of senescence-related genes, and stress markers including heat shock proteins. Once the model of glyoxal-induced premature senescence is established, it can be useful for testing the aging modulating effects of natural and synthetic molecules and the hormetic effects of mild stress on human skin fibroblasts.[5]

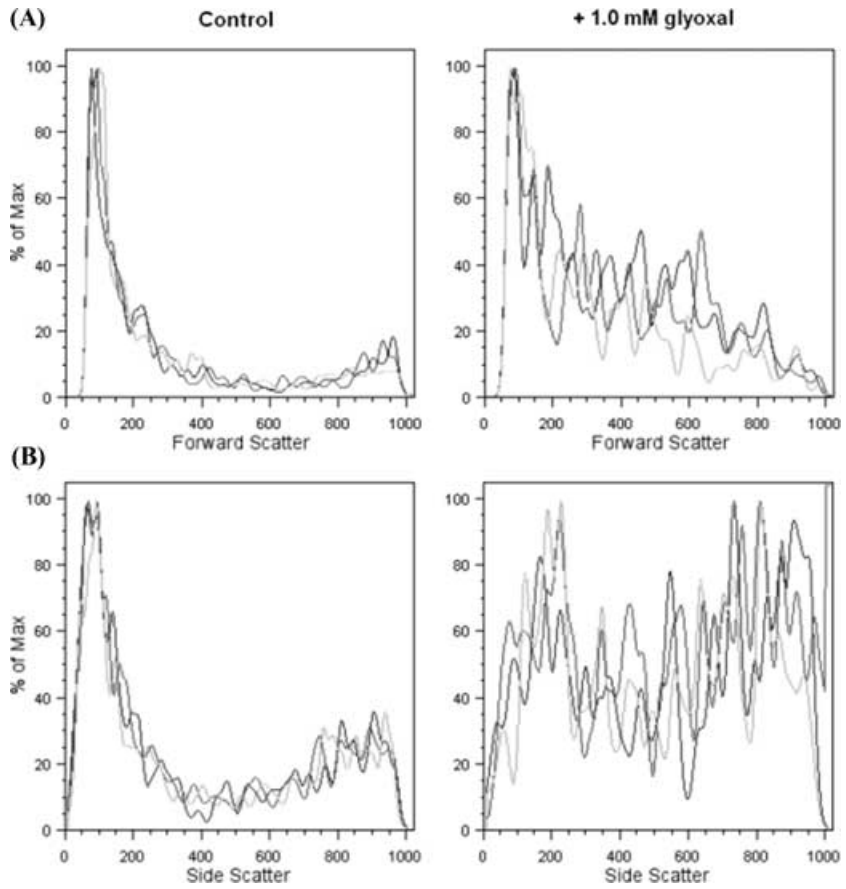

FIGURE 3. Effect of glyoxal on cell size (forward scatter, **A**) and cell granularity (side scatter, **B**) of early-passage human skin fibroblasts. Each bin on the *x* axis represents a group of cells giving rise to the same signal value.

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