Oxidative stress-induced apoptosis in dividing fibroblasts involves activation of p38 MAP kinase and over-expression of Bax: Resistance of quiescent cells to oxidative stress

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Oxidative stress has been postulated to be involved in aging and age-related degenerative diseases. Cell death as a result of oxidative stress plays an important role in the age-related diseases. Using human diploid fibroblasts (HDF) as model to study the mechanism of cell death induced by oxidative stress, a condition was standardized to induce apoptosis in the early passage sub-confluent HDFs by a brief exposure of cells to 250 \( \mu \text{M} \) hydrogen peroxide. It was observed that p38 MAP kinase (MAPK) was activated soon after the treatment followed by over-expression of Bax protein in cells undergoing apoptosis. An interesting finding of the present study is that the confluent, quiescent HDFs were resistant to cell death under identical condition of oxidative stress. The contact-inhibited quiescent HDFs exhibited increased glutathione level following H\(_2\)O\(_2\)-treatment, did not activate p38 MAP kinase, or over-express Bax, and were resistant to cell death. These findings indicated that there was a correlation between the cell cycle and sensitivity to oxidative stress. This is the first report to our knowledge that describes a relationship between the quiescence state and anti-oxidative defense. Furthermore, our results also suggest that the p38MAPK activation-Bax expression pathway might be involved in apoptosis induced by oxidative stress.

Keywords: apoptosis; cell death; oxidative stress; reactive oxygen species (ROS).

Introduction

Cell death is a physiological process essential for normal development and tissue homeostasis.\(^1\) Although the existence of cell death mechanisms is a protective measure for the organisms, which ensures the removal of unnecessary, damaged or potentially dangerous cells, any deregulation or inappropriate induction of this process leads to the loss of healthy cells that may result in disease. Particularly, cells dying in post-mitotic tissues such as brain and heart in adult organisms cannot be replaced by new ones resulting in functional compromise as occurs in Alzheimer’s disease (AD), Parkinson’s disease (PD) and stroke.\(^2\)\(^-\)\(^3\) Cell death induced by oxidative stress has been shown to be involved in the development of these pathologies.\(^4\)\(^-\)\(^5\)

Mammalian cells are under constant threat of oxidative damage due to production of reactive oxygen species from mitochondria by partial reduction of molecular oxygen. These cells also have a multitude of anti-oxidative defence mechanisms, including enzymes and antioxidants.\(^6\)\(^-\)\(^7\) Increase in oxidative stress has been shown to be involved in aging and in age-related diseases.\(^8\)\(^-\)\(^10\) However, the exact role of ROS in triggering different cellular signalling resulting in cellular damage and death is largely not understood. Induction of p53 and expression of Bax have been implicated in the signalling of cell death induced by DNA damage.\(^11\)

Mechanism of cell death induced by oxidative stress is still under intense investigation by many groups. Human diploid fibroblasts (HDF) have been used as a cellular model of replicative senescence.\(^12\)\(^-\)\(^13\) We have looked into the effect of direct oxidative stress on human fibroblasts. We have observed that sub-confluent dividing fibroblasts undergo apoptotic cell death following oxidative stress, and there was activation of p38 MAP kinase, and up-regulation of Bax expression during the early phase of apoptotic cell death. Interestingly, we found that confluent quiescent HDFs were resistant to the similar treatment. Our results indicated that confluent quiescent HDFs maintained or up-regulate the glutathione level; they neither activated p38 MAP kinase nor increased the expression of Bax in response to oxidative stress. We also observed activation of proteasome protease in cells...
undergoing apoptosis indicating the involvement of non-caspase proteases in this process.

Materials and methods

Cell culture

Human diploid fibroblasts (AG09309) were obtained from Coriell Institute for Medical research, New Jercy, (USA) and grown in Earle’s minimum essential medium supplemented with 15% (v/v) fetal bovine serum, L-glutamine, essential and non essential amino acids, vitamins and gentamycin (Gibco-BRL Life Technology), in 5% CO2 in a humid incubator at 37°C.

Oxidative stress

Cells were grown to desired confluence. The growth medium was replaced with fresh complete media containing 250 µM H2O2, and cells were incubated for 1 hour at 37°C. Then the media was replaced with fresh complete media without H2O2, and incubations were carried out for various time intervals as indicated in the figures.

Cellular staining and microscopy

Cells were grown and treated as described above and stained with Hoechst 33342 (10 µM) (0.75%, 37°C). Cells were photographed using a fluorescent microscope (Zeiss, Axiovert 200) and images were processed using Northern Eclipse 6.0 software.

Comet assay

The comet assay was performed using a slight modification of a previously described method.14 Briefly, 10,000 cells from control or treated samples (10 µl in volume) were mixed with 80 µl of warm low-melting point agarose (LMP) (0.75%, 37°C) in a microtube and spread on a glass slide pre-coated with 200 µl of 0.1% agarose in such a way that half of the gel was on the rough surface and other half on the smooth, transparent surface (agarose gel tend to slide away from smooth surface during processing). A cover slip was laid over the gel mixture and slides were kept at 4°C. After gelling at 4°C, the cover slip was peeled off. Slides were immersed immediately in a jar containing a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100 and 10% DMSO). Lysis was done at 4°C for 1 h in the dark. Slides were washed in a freshly prepared alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na2 EDTA, pH > 13). The DNA was electrophoresed at 300 mA (0.8 V/cm) and washed twice in a neutralizing buffer (0.4 M Tris, pH 7.5) and stained with Hocchst 33,342 (10 µM). Cells were photographed using a fluorescent microscope (Zeiss, Axiovert 200) and images were processed using Northern Eclipse 6.0 software.

Western blot analysis

Treated or untreated cultured cells were rinsed three times with PBS, scraped, and lysed in a lysis buffer consisting of 25 mM HEPES, 5 mM MgCl2, 1 M MgSO4, 1 mM EGTA and 1% Triton x-100. The buffer contained protease inhibitors; leupeptin, pepstatin (both at 1 µg/ml) and PMSF (0.5 mM). The cell suspensions were homogenized in a glass homogenizer and incubated on ice for 10 min. The lysates were pre-cleared by centrifugation, and equal amount of proteins per lane (15 µg) were resolved on a 10% SDS gel. Proteins were electro-transferred on to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk and probed with anti-Bax, anti-p38 MAP kinase (Sigma Mississauga, Ontario) or antiphospho-p38 MAP kinase (Cell Signaling, Beverly, MA). The blots were washed and exposed to HRP conjugated secondary antibodies (Sigma Chemical Company, Mississauga, Ontario) and then developed using ChemiGlow West kit (San Leonardo, CA) and visualized with Alpha Innotech Corporation Imaging System (San Leonardo, CA). The molecular masses of the proteins were estimated relative to Precision Protein Standards (Bio Rad Laboratories, Hercules, CA).

Proteasome and caspase activity

Total cell lysates were assayed for Proteosome and Caspase-3 activity based on fluorogenic substrate (a tetra peptide sequence corresponding to the substrate cleavage site), LLVY-AFC for proteasome and DEVD-AFC for caspase-3 from Enzyme System Products (Livermore, CA), using manufacturer’s protocol. The fluorescence (excitation 400 nm/emission 505 nm) was measured in a 96 well micro-titer plate using the Spectra Max Gemini XS from Molecular Devices, Sunnyvale, California. Protein concentration was estimated using the Bio Rad protein assay reagent (Mississauga, Ontario), and equal amounts of protein were taken for the Proteosome and Caspase3 activity. Microsoft Excel version 6.0 software was used for data representation and statistical analysis.

Glutathione measurement

Glutathione was measured using a procedure described by Kamencic et al.15 Treated cells were washed three times with PBS, lysed in 100 µM monochlorobimane (MCB).