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Hydrogen Peroxide in Relation to Proliferation and Apoptosis in BHK-21 Hamster Fibroblasts

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Addition of H_2O_2 at 100 μM, or 1 mM, to the culture medium of BHK-21 fibroblasts results in increased intracellular levels of H_2O_2. Whilst exposure of BHK-21 cells to lower levels of H_2O_2 (1 μM) actually stimulates proliferation, these higher oxidant concentrations not only depress proliferation rates but also lead to an increase in the appearance of apoptotic-like cells in the cultures. Other agents such as inhibitors of glutathione peroxidase and catalase, or mimics of superoxide dismutase, which also bring about elevated cellular levels of H_2O_2 in BHK-21 cells, similarly lead to decreased proliferation and an apparent increase in cells with apoptopic features. Thus intracellular conditions which are considered more prooxidant than normal, appear to favour apoptosis over proliferation in BHK-21 fibroblasts. Additionally these abnormal cellular conditions also appear to favour excessive DNA replication, in remaining non-apoptotic cells.

Key words: proliferation, apoptosis, H_2O_2, glutathione peroxidase, catalase, superoxide dismutase, DNA, BHK-21 fibroblasts

INTRODUCTION

The maintenance of homeostasis in normal mammalian tissues reflects a critical balance between cell proliferation and cell death.[1,2] In the latter case this mainly appears to be programmed by genetically controlled pathways leading to a morphologically distinct type of death known as apoptosis. Cells dying in this way show a variety of characteristics including blebbing of the plasma membrane, condensation of nuclei and chromatin as well as endonucleolytic cleavage of chromosomal DNA into nucleosomal length fragments.[1,2]

Some recent data implicate reactive oxygen species in cell death by apoptosis. In particular this view arises from work on a novel oncogene-derived protein Bcl-2 which functions as a repressor of apoptotic cell death in a genetic pathway of cellular suicide common to multicellular animals.[2,3] Bcl-2 has been localised to mitochondria and membranes of the endoplasmic reticulum,[4,5] sites where reactive oxygen species are generated intracellularly.

Although Bcl-2 does not appear to affect the rate of cellular generation of superoxide, gene transfection experiments in lymphocytes indicate that Bcl-2 will protect cells from the lethal effects of H_2O_2 and will suppress oxidative damage to

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vital cell structures such as lipid membranes.[2,6] Bcl-2 has also been shown to inhibit cell death that follows depletion of glutathione from neural cell lines.[7] In *E. coli* cells deficient in superoxide dismutase, transfection with mammalian *bcl-2* genes leads to a hundred-fold increase in resistance to H_2O_2 which is accompanied by a thirteen-fold increase in KatG activity.[8] It is suggested that Bcl-2 may be protective by virtue of inducing antioxidant enzyme activity in these bacterial cells. Another role for Bcl-2 has been suggested in as much as its homologue in *C. elegans*, Ced9, appears to repress the activity of interleukin-1β converting enzyme (ICE),[9] which is a cysteine protease with specificity for aspartic acid residues.[2] ICE-like molecules are believed to be important in a proteolytic cascade leading to cell death.[10] A suggestion has been that Bcl-2 influences the redox state of cysteine proteases such as ICEs and hence their activities.[2] A complicating factor however is that Bcl-2 appears to antagonise the cellular effects of another protein, Bax. Whilst Bcl-2 opposes cell death, Bax promotes death by as yet unknown pathways.[2]

In the past we have provided evidence that H_2O_2 and superoxide at low levels are important growth promoting signals.[11] In particular it was suggested[11] that this may constitute a novel redox system of control superimposed upon the established growth signal transduction pathways. Such a mechanism could involve the direct oxidation by H_2O_2, or superoxide, of growth signal transduction proteins such as growth factor receptors,[12] protein kinases,[13,14] protein phosphatases,[15] transcription factors,[16–20] transcription factor inhibitors.[18] Although the redox states of these signal transduction proteins could be modulated directly, they could also be modulated indirectly through changes in cellular levels of oxidised and reduced glutathione brought about by H_2O_2 or superoxide.[11]

Whilst superoxide and H_2O_2 can be generated normally in a variety of normal cell types, either constitutively or in response to various stimuli,[11] the intracellular levels of H_2O_2 appear to have an influence on the rates of BHK-21 (baby hamster kidney) fibroblast proliferation.[21] Treatment of BHK-21 cells with inhibitors of enzymes that metabolise H_2O_2 increases cellular levels of H_2O_2 and impairs cell proliferation.[21] Additionally exposure of cells to mimics of superoxide dismutase also increases cellular levels of H_2O_2 in these cells and reduces proliferation. Such observations have suggested an important and novel growth regulatory role for the antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutases in BHK-21 fibroblasts.[21]

In view of the suggested role for H_2O_2 and superoxide in the process of apoptosis, the effects of modulating intracellular levels of H_2O_2 in relation to apoptosis in BHK-21 cells has been explored. Whilst procedures that increase cellular levels of H_2O_2 above normal levels decrease BHK-21 cell proliferation, they can also increase the population of cells exhibiting apoptotic properties.

EXPERIMENTAL PROCEDURES

Cell Culture

Baby hamster kidney fibroblasts (BHK-21/C13) were grown at 37°C as monolayer cultures in 3.5 cm petri-dishes in 2 ml Eagle's minimal essential medium supplemented with 10% (v/v) calf serum (Gibco BRL, Paisley).[21,22] During growth a proportion of the cells detach from the monolayer. These 'detached' or 'non-adherent' cells can be harvested from the medium by centrifugation at 800 g for 5 mins and their numbers assessed separately using a haemocytometer. The proliferation of the live or 'adherent' cells remaining attached to the monolayer was assessed as previously described.[21,22]

Determination of Hydrogen Peroxide

H_2O_2 was determined in the culture media and within adherent BHK-21 cells using the methods previously used.[21] The medium was first removed

from the monolayer cultures. The monolayers were then washed two times with phosphate buffered saline (PBS). 2 ml 0.2 M perchloric acid were then added and the suspension containing precipitated cellular material was transferred to centrifuge tubes and centrifuged at 2500 g for 3 minutes at 4°C. The supernatant was removed and neutralised to pH 7.5 with 5 M KOH. This was then centrifuged 10 000 g for 2 minutes at 4°C. 200 µl of the supernatant was applied to a 1 ml column of anion exchange resin (AG 1-x2, Bio Rad) and the column washed with 800 µl distilled water and the eluate used for the determination of H_2O_2. Reaction mixtures contained 1 ml eluate, 400 µl 12.5 mM 3-dimethylaminobenzoic acid (DMAB) in 0.37 M phosphate buffer pH 6.5, 80 µl 0.01 M 3-methyl-2-benzothiazoline hydrazone (MBTH) and 20 µl horse radish peroxidase (17 units/ml). After 3 minutes at 20°C the absorbance of the solution was determined at 590 nm and compared with increases elicited by standard samples of hydrogen peroxide.

To determine the levels of H_2O_2 in the medium, the medium (2 ml) was removed and made 0.2 M with respect to perchloric acid. The precipitated proteins were removed by centrifugation at 2500 g for 3 minutes at 4°C. The supernatant was removed and neutralised to pH 7.5 with 5 M KOH. This was then centrifuged at 10 000 g for 2 minutes at 4°C and 1 ml samples used for assay of H_2O_2 after ion-exchange chromatography using DMAB, MBTH and horse radish peroxidase as above.

DNA Isolation

DNA was isolated from cells that became detached from monolayer cultures of BHK-21 cells and from live cells that remained attached to the culture dishes. In the case of the detaching cells these were collected from the medium by centrifugation as above. They were then re-suspended in 150 mM NaCl-10 mM tris-HCl pH 7.5 – 10 mM EDTA (NTE) at 10^6 cells per 6 ml. Cells remaining attached to the monolayer were first washed twice with phosphate buffered saline (PBS) then scraped off into NTE, again at 10^6 cells per 6 ml.

To both cell suspensions, sodium dodecyl sulphate was added to a final concentration of 0.5% (w/v). Ribonuclease solution (previously heated to 100°C for 15 min) was then added to a final concentration of 100 µg/ml and the solution incubated at 37°C for 30 min. Proteinase K was added to 100 µg/ml and incubation continued but at 40°C for 60 min. An equal volume of water saturated phenol was then added and the mixture shaken at 4°C for 20 min. The phases were separated by centrifugation at 2000 g for 10 min. The top layer was removed and extracted with chloroform: isoamyl alcohol (24:1). Again phases were separated by centrifugation at 2000 g for 10 min. The top layer was removed and mixed gently with 0.5 vol 7.5 M ammonium acetate. 2 vol ethanol was then added and mixed thoroughly. After a period of 18 hr at –20°C the DNA was collected by centrifugation at 2000 g for 30 min. The pellet was washed briefly with 2.5 M ammonium acetate – 66% (v/v) ethanol and then with 70% (v/v) ethanol. After air drying, the sticky DNA was dissolved in 10 m tris-HCl pH 7.5 – 10 mM EDTA (TE). The concentration of DNA was determined from its absorbance at 260 nM, an absorbance of 1.0 corresponding to a concentration of 50 µg/ml double standard DNA.

Electrophoretic Analysis of DNA

DNA samples from detached and adherent cells were analysed by electrophoresis in 1% agarose under neutral conditions. The DNA standards used for size comparisons were a Hae III digest of φX174 phage DNA and a Hind III digest of λ-phage DNA containing fragments of 1353, 1078, 872, 603, 310, 280 and 23 130, 9416, 6557, 4361, 2322, 2027 bp respectively.

Electron Microscopy

Detaching, or adherent cells, were collected by centrifugation and fixed with 2% gluteraldehyde

in 0.1 M cacodylate buffer pH 7.2 for 4 hr at 4°C. The cells were then washed twice in cacodylate buffer and then post fixed with 1% osmium tetroxide in distilled water. These fixed cells were then dehydrated in graded alcohols to propylene oxide and then held overnight in propylene oxide/Taab embedding resin (medium hardness), then pure resin for 3 days, changing twice per day. Polymerisation was at 60°C for 48 hr and blocks were cut on an LKB Ultratome using glass knives and the sections mounted onto copper grids. The sections were then stained with uranyl acetate (1 hr) and lead citrate (5 min), and viewed with an AEI6B microscope.

Assay of Lactic Dehydrogenase

As an indication of possible necrotic cell death, the media from various BHK-21 cell cultures was assayed for lactic dehydrogenase (LDH) activity. This was carried out using a diagnostic kit supplied by Sigma Chemical Co which was used according to their instructions. A low level of LDH activity was routinely determined (less than 2mU/ml) in the medium used for BHK-21 cell cultures but when cells were exposed to 1 mM and 10 mM H_2O_2, levels of LDH above these values were detected.

Reagents

Mercaptosuccinate, aminotriazole, 3-dimethylaminobenzoic acid, 3-methyl-2-benzothiozole hydrazone, horseradish peroxidase, α-tocopherol, butylated hydroxytoluene, ribonuclease, proteinase K and agarose were obtained from Sigma Chemical Co. Copper II (3,5-

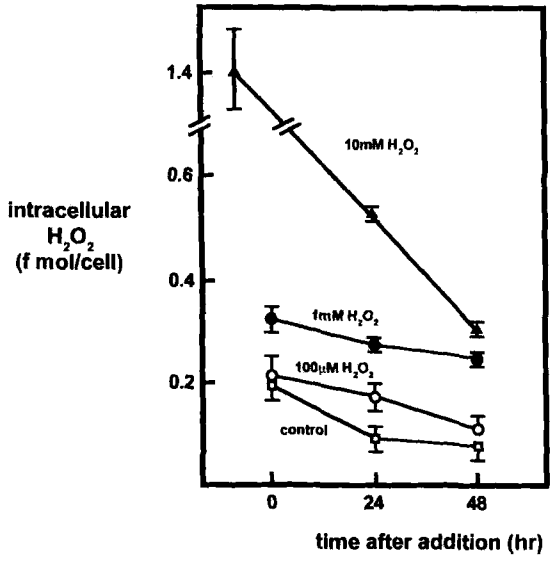

FIGURE 1 Intracellular levels of hydrogen peroxide in BHK-21 fibroblasts in relation to growth. Triplicate monolayer cultures (0.3×10^6 cells) were established in 3.5 cm petri-dishes in 2 ml growth medium. After growth for 24 hr at 37°C, H_2O_2 was added to the medium at 100 μM, 1 mM and 10 mM. Then and at times thereafter, intracellular H_2O_2 levels were determined as described in EXPERIMENTAL PROCEDURES in control cells (□), in cells exposed to 100 μM H_2O_2 (○), in cells exposed to 1 mM H_2O_2 (●), in cells exposed to 10 mM H_2O_2 (▲). Results are presented as the means of triplicate experiments ± s.d.(n = 3).

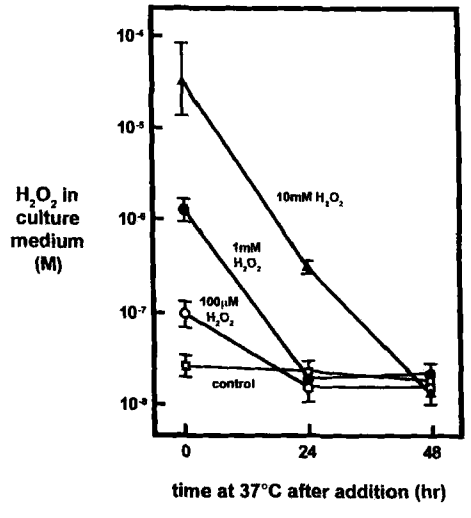

FIGURE 2 Concentrations of hydrogen peroxide in the culture medium following addition of exogenous hydrogen peroxide to cultures of BHK-21 cells. Triplicate monolayer cultures of BHK-21 cells were established (0.3×10^6 cells per 3.5 cm petri-dish in 2 ml growth medium). After 24 hr of growth at 37°C, H_2O_2 was added at 100 μM, 1 mM and 10 mM. Growth was allowed to continue and the medium was removed from the cultures at the times indicated for determination of H_2O_2 concentrations from control cells (□), from cells exposed to 100 μM H_2O_2 (○), from cells exposed to 1 mM H_2O_2 (●), from cells exposed to 10 mM H_2O_2 (▲). Results are presented as the means of triplicate experiments ± s.d.(n = 3).

diisopropylsalicylate)$_2$ was from Aldrich Chemical Co. Copper-D-isohistidine was a kind gift from Dr N Roberts, Roche Pharmaceuticals.

RESULTS

In order to assess intracellular levels of H_2O_2 in BHK-21 fibroblasts in relation to proliferation as well as apoptosis, an initial approach was to increase these intracellular levels by addition of increasing amounts of exogenous H_2O_2 to the culture medium. From Figure 1 it can be seen that the addition of H_2O_2 at 100 μM, 1 mM and 10 mM does in fact increase intracellular levels although not in direct proportion. In part this may be a reflection of the growth associated decline in cellular H_2O_2 levels previously observed in these cells.[21] Another factor likely to influence final cellular levels is the extremely rapid metabolism of the exogenously added H_2O_2 from the medium.[21] Figure 2 shows that even in the time taken to collect the culture medium for assay after H_2O_2 addition, very considerable reductions in the medium concentrations of H_2O_2 are evident.

Whilst the outcome of adding a single 'bolus' of exogenous H_2O_2 to the culture medium of BHK-21 fibroblasts is to elevate temporarily the relative levels of intracellular H_2O_2, Figure 3 shows that different effects of cell growth and death are produced. Even without addition of exogenous hydrogen peroxide (Figure 3a) to the growing cultures of BHK-21 cells, there is a low but progressively increasing detachment of a proportion of cells from the surface of the culture dish. Studies on cultured Rat-1 fibroblasts[23] had previously indicated dying cells to exhibit reduced adherence

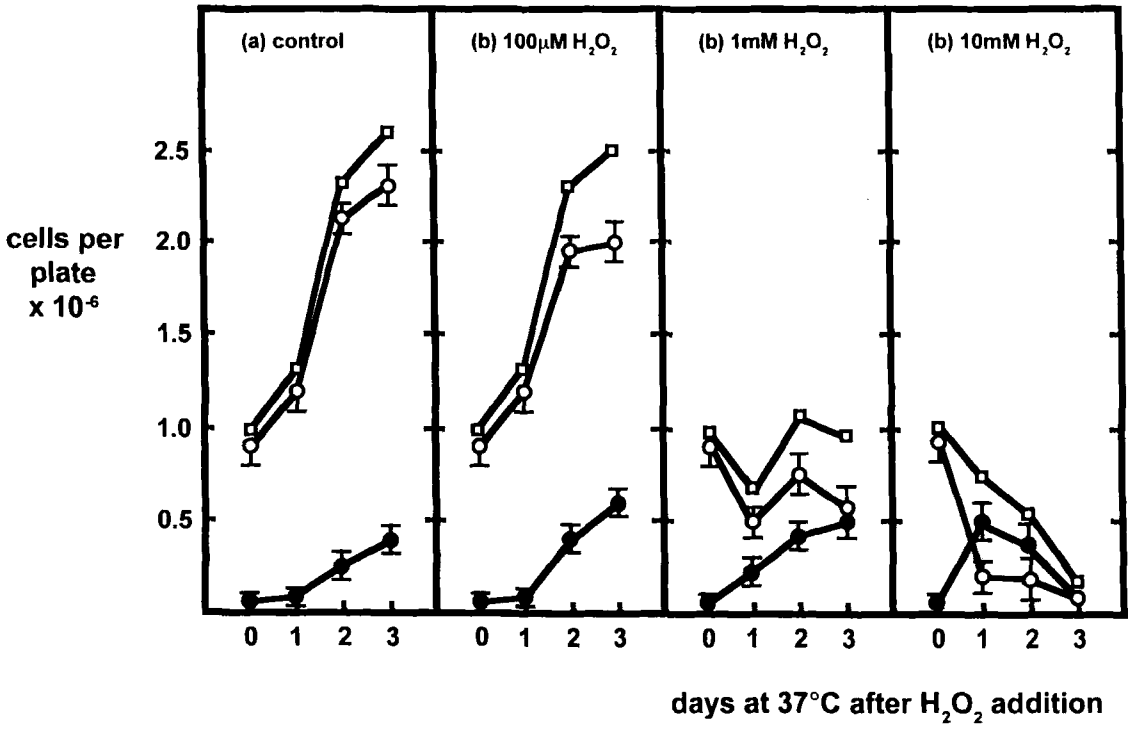

FIGURE 3 The effects of H_2O_2 on BHK-21 fibroblast growth. Triplicate 3.5 cm petri-dishes were seeded with 0.8×10^6 BHK-21 cells in 2 ml growth medium and allowed to grow for 24 hr at 37°C. At that time some cultures were exposed to H_2O_2 added to the medium at zero concentration (a), at 100 μM(b), at 1 mM (c), or at 10 mM (d). At various times thereafter the cells detaching from the monolayer (non-adherent cells) were collected from the medium and enumerated (●) as described in EXPERIMENTAL PROCEDURES. The number of adherent cells remaining with the monolayer were then assayed (○) as described in EXPERIMENTAL PROCEDURES. The total cells recovered (□), is the sum of the non-adherent and adherent cells determined. Results are presented as the means of triplicate experiments ± s.d.(n = 3).

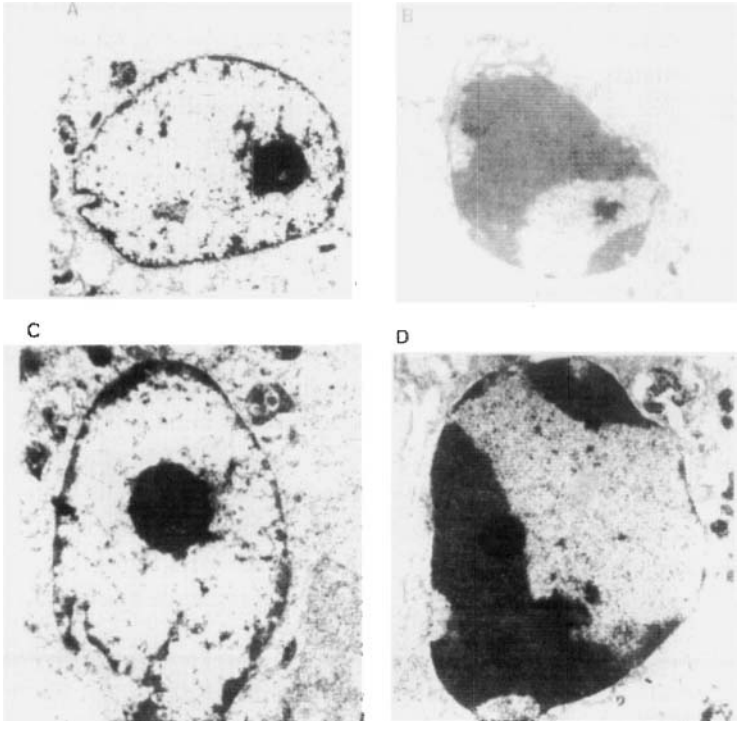

FIGURE 4 Transmission electron microscopy of adherent and non-adherent BHK-21 cells. A: adherent cells from untreated cultures (×9000) B: detached or non-adherent cells from untreated cultures (×8000) C: adherent cells from cultures treated with 100 μM H_2O_2 for 48 hr (×16 000), D: detached or non-adherent cells from cultures treated with 100 μM H_2O_2 for 48 hr (×17 000).

and when these were separately harvested and examined, they showed apoptotic properties such as nuclear condensation and fragmentation of chromosomal DNA into nucleosome ladders.[23] When the detached BHK-21 fibroblasts in this study were examined by electron microscopy they also displayed morphological signs of apoptosis in as much as significant condensation of chromatin adjacent to inner nuclear membranes was observed (Figure 4B). Additionally when the DNA of these cells was isolated and examined by agarose-gel electrophoresis there was also evidence of DNA ladders indicative of internucleosome cleavage (Figure 5B) which is a characteristic of apoptotic cells.[1,2] When H_2O_2 was added to the medium at 100 μM, not only was proliferation retarded as previously experienced[24] (Figure 3b), but also there was an even greater progressive increase in the level of cells with apoptotic properties. By 3 days (Figure 3b) the level of detached apoptotic-like cells (Figure 4D) had increased to around 26% of the total population. If H_2O_2 is added at 1 mM (Figure 3c), there was no net proliferation or extensive loss of BHK-21 fibroblasts, but a yet higher percentage of detached apoptotic-like cells were observed. There was also a small release of lactic dehydrogenase into the medium (an increase in medium levels of 5 mU/ml 24 hr after H_2O_2 addition) indicating some cell death may be due to necrosis in addition to possible apoptosis. When the exogenously added H_2O_2 was increased to 10 mM, there was an extensive net loss of cells from the culture. This is likely to be due to necrotic cell death as there was a considerable release of lactic dehydrogenase into the medium (an increase in medium levels of 35 mU/ml was detected 24 hr after H_2O_2 addition). For each concentration of H_2O_2, the DNA in

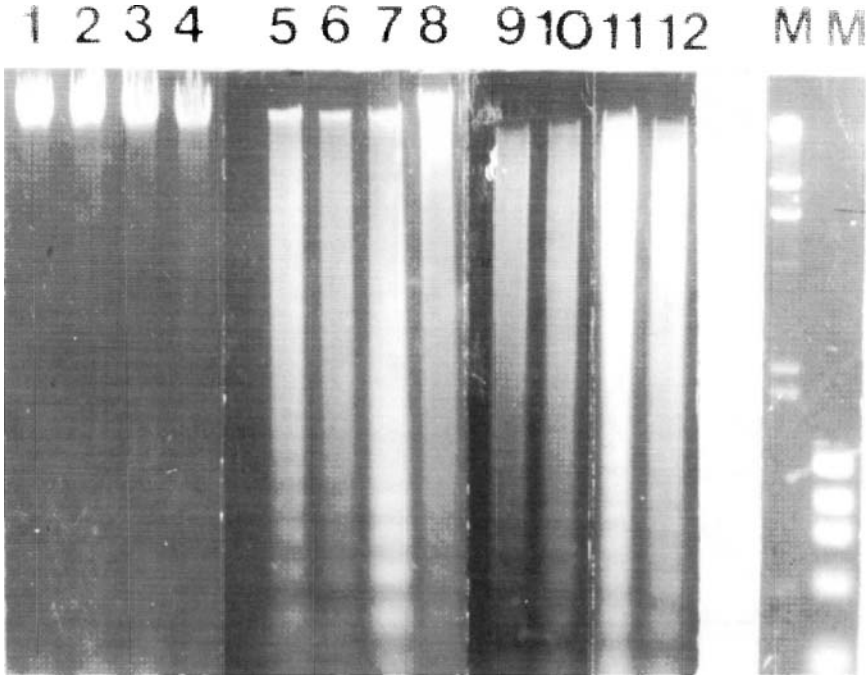

FIGURE 5 Neutral agarose gel electrophoresis of DNA isolated from adherent and detached BHK-21 cells. DNA from adherent cells remaining with monolayers after treatment of BHK-21 cell cultures for 48 hr with no added H_2O_2 (lane 1), with 100 μM H_2O_2 (lane 2), with 1 mM H_2O_2 (lane 3), with 10 mM H_2O_2 (lane 4), DNA from BHK-21 cells that have detached from the monolayer after treatment of BHK-21 cell cultures for 48 hr with no added H_2O_2 (lane 5), with 100 μM H_2O_2 (lane 6), with 1 mM H_2O_2 (lane 7), with 10 mM H_2O_2 (lane 8). DNA from BHK-21 cells that have become detached from the monolayer after treatment for 48 hr with 150 μM mercaptosuccinate (lane 10), with 150 μM aminotriazole (lane 11), with 10 μM CuDIPS (lane 12). Lanes M are DNA molecular weight markers of sizes (from top to bottom), 23 130, 9416, 6557, 4361, 2322 and 2027, and 1353, 1078, 872, 603, 310 and 280.

adherent cells remaining attached to the culture dishes after 48 hr was compared with the DNA from the detached, or non-adherent cells. From Figure 5 (lanes 1–4) it can be seen that in the case of cells remaining attached, surprisingly little extensive degradation of cellular DNA was evident from electrophoresis on neutral agarose gels. Moreover a striking feature of cells exposed to 100 mM and 1 mM H_2O_2 (Table 1) was a higher than normal content of cellular DNA. From Table 1 it can be seen that there was almost a four-fold increase in DNA content in the case of cells remaining attached to the monolayer after exposure to 1 mM H_2O_2. In contrast in the case of the DNA from the non-adherent cells that detach under the different conditions of H_2O_2 exposure, there was clear evidence of specific internucleosomal cleavage or laddering of DNA characteristic of apoptotic cells (Figure 5, lanes 5–8).

This initial approach suggested that intracellular levels of H_2O_2 are important. Moreover it

TABLE 1 DNA content of BHK-21 cells

Additions	Cellular DNA content pg/cell ± s.d. (n = 3)
none	6.60 ± 0.78
100 μM H_2O_2	9.50 ± 1.15
1 mM H_2O_2	26.90 ± 3.50

Triplicate 3.5 cm petri-dishes were seeded with BHK-21 cells in 2 ml growth medium and allowed to grown for 24 hrs at 37°C. At that time the indicated additions were made to the culture medium and growth allowed to continue for a further 48 hr at 37°C. DNA was then isolated from cells remaining attached to the monolayers and estimated as described in EXPERIMENTAL PROCEDURES.

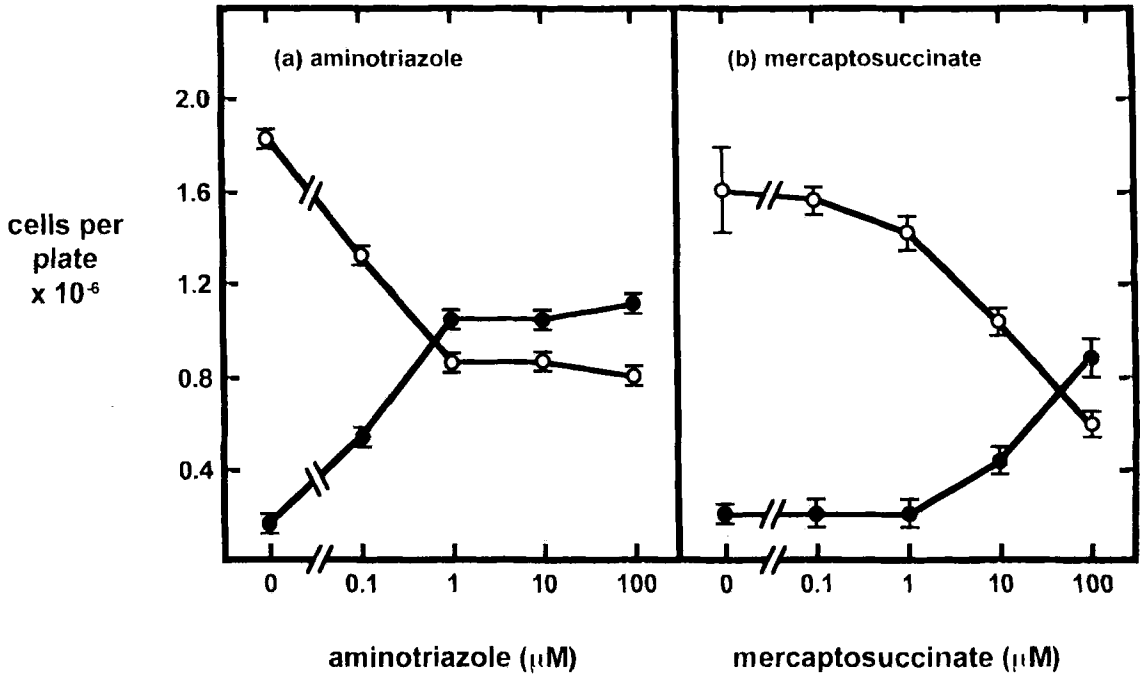

FIGURE 6 The effect of aminotriazole and mercaptosuccinate on BHK-21 cell growth. Triplicate 3.5 cm petri-dishes were seeded with 0.5×10^6 BHK-21 cells in 2 ml growth medium and the cultures were allowed to grow for 24 hr at 37°C. At that time cultures were exposed to various concentrations of aminotriazole (a), or mecaptosuccinate (b). After 3 days further growth at 37°C, cells detached from the monolayer (non-adherent) were assessed (●) as were adherent cells remaining attached to the monolayer (○) as described in EXPERIMENTAL PROCEDURES. Results are expressed as the means of triplicate experiments ± s.d.(n = 3).

is possible to modulate intracellular levels of H_2O_2, and apparently the subsequent rates of fibroblast proliferation or apoptosis (and also necrotic cell death) by alteration of the amount of exogenous H_2O_2 added to the culture medium. In view of this potential relevance of intracellular levels of H_2O_2, the effect of inhibitors of cellular enzymes involved in the metabolism of cellular H_2O_2 were examined in relation to fibroblast growth and possible apoptosis. Previous experience had shown such inhibitors to raise intracellular levels of H_2O_2 in BHK-21 cells.[21] Figure 6 shows that exposure of BHK-21 cells to either mercaptosuccinate or aminotriazole, inhibitors of glutathione peroxidase and catalase respectively,[21,22] both led to diminished cell proliferation as well as to increased levels of apoptotic-like cells detaching from the culture dishes. When BHK-21 cells were exposed to the low molecular weight lipophilic mimic of superoxide dismutase, copper II (3,5-diisopropylsalicylate)$_2$ (CuDIPS) the rate of cell proliferation progressively declined, but the rate at which apoptotic cells detached from the culture dish increased markedly day by day (Figure 7). Previous experience had shown that exposure of BHK-21 cells to CuDIPS also elevated intracellular levels of H_2O_2.[21] Figure 5 shows that when the DNA from cells progressively detaching from the culture dishes after exposure of the cultures to aminotriazole, mercaptosuccinate or CuDIPS was analysed by electrophoresis through agarose gels, the internucleosomal DNA cleavage

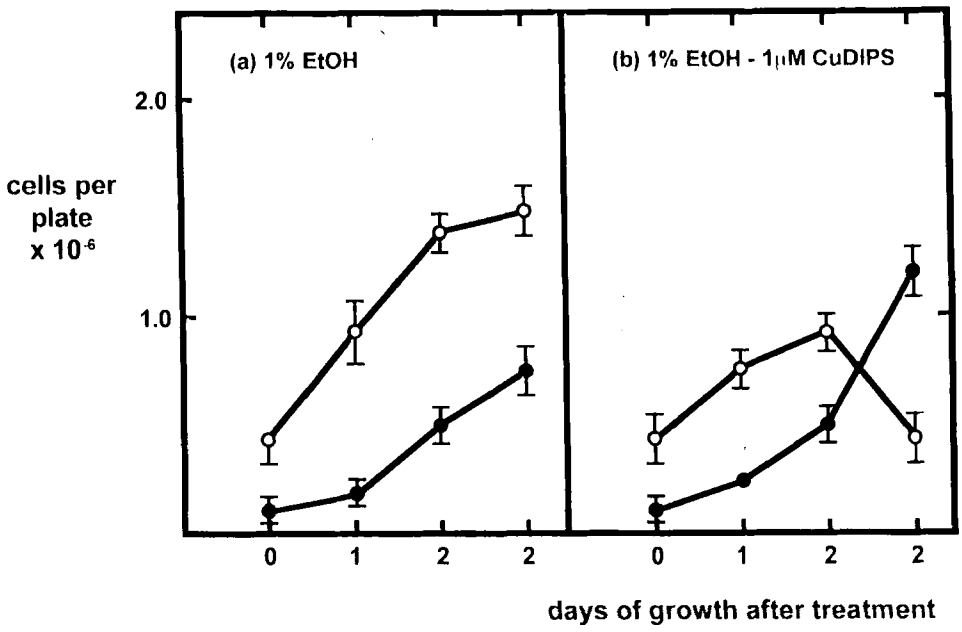

FIGURE 7 The effects of copper II (3,5 diisopropylsalicylate)$_2$ on BHK-21 cell growth. Triplicate 3.5 cm petri-dishes were seeded with BHK-21 cells in 2 ml of growth medium. Cultures were then either exposed to 1 μM copper II (3,5-diisopropylsalicylate)$_2$ in 1% ethanol (b), or to 1% ethanol alone and growth allowed to continue. The numbers of adherent (O) and non-adherent (●) cells were assessed at various times thereafter as described for Figure 6. Results are expressed as the means of triplicate experiments ± s.d.(n = 3).

patterns characteristic of apoptotic cells was again observed (Figure 5, lanes 10–12). Similar results (not shown) were obtained with another mimic of superoxide dismutase, copper-D-isohistidine.[23,24] In the case of the cells remaining attached to the culture dishes under these conditions no extensive DNA degradation was evident from electrophoresis on neutral aragose gels (not shown).

DISCUSSION

Apoptosis has been studied following serum depletion of rodent fibroblasts (Rat-1) expressing *c-myc* constitutively.[25] In that particular study the apoptopic cells were specifically harvested by virtue of their reduced adherence to the culture surface. Such detaching cells which increased after serum removal displayed apoptic features like nuclear condensation and internucleosomal fragmentation of chromosomal DNA. In the case of mouse embryonic CH3/10T½ cells, again serum depletion brought about an increase in apoptotic-like cells which were characterised by their reduced adherence to the surface of the culture dishes, condensation of nuclear chromatin and internucleosomal DNA fragmentation.[26] From our initial studies with hamster fibroblasts (BHK-21) a similar situation seems to prevail.[27] Although we found that a significant number of BHK cells detached from the monolayer with increasing time of growth, this can be significantly increased by serum depletion.[27] Fibroblasts that detach from the culture dish during culture show signs of apoptosis in as much as chromatin condensation is observable and internucleosomal cleavage of chromosomal DNA is apparent. In contrast the BHK fibroblasts remaining attached to the culture dish after the various treatments do

not show these characteristics, their DNA showing little sign of extensive fragmentation.

Whilst fibroblasts cultured in the absence of added H_2O_2 show a small accumulation of cells exhibiting apoptotic properties, the proportion of such cells can nevertheless be increased if the cultures are exposed to a single 'bolus' of 100 μM H_2O_2 when cell proliferation is reduced but not prevented. At 1 mM H_2O_2, growth is essentially prevented but apoptosis occurs at a rate similar to that in cell cultures exposed to 100 μM H_2O_2. In contrast exposure of cultures to 10 mM H_2O_2 whilst initially giving rise to some apoptotic cells, results mainly in a dramatic reduction in the total number of cells. As judged by the conspicuous release of lactic dehydrogenase into the culture medium, this overall loss of cells is likely to be due to necrotic cell death. On this basis there may also be a small level of necrotic cell death in cell cultures exposed to 1 mM H_2O_2. However no lactic dehydrogenase release was detected in cells exposed to 100 μM H_2O_2.

These results with H_2O_2 added at concentrations of 100 μM to 10 mM are in contrast with the observed effects of exogenous H_2O_2 added at 1 μM. In previous reports[21,22] we showed this low concentration to be growth ***stimulatory*** (10 μM H_2O_2 was neither growth stimulatory or significantly growth inhibitory). Whilst the addition of H_2O_2 at 100 μM to 10 mM raised the intracellular levels of H_2O_2, the addition of H_2O_2 at 1 μM actually had no significant effect on intracellular levels of H_2O_2 in BHK-21 cells. Rather it brought about a modest decline in cellular levels of reduced glutathione(GSH).[21]

In view of the observation that increasing cellular levels of H_2O_2, for example by exposure to 100 μM H_2O_2, decreased proliferation rates and increased apoptosis in BHK cells, the effect of inhibitors of catalase and glutathione peroxidase are significant. Both of these inhibitors will increase levels of H_2O_2 in BHK cells[1] and both will decrease rates of proliferation and increase the appearance of apoptotic-like cells in the culture.

Another type of agent capable of increasing cellular levels of H_2O_2 in BHK cells is low molecular weight superoxide dismutase mimics e.g. CuDIPS and Cu-D-isohistidine. Again these will decrease proliferation rates and progressively increase the rate of apparent apoptosis. Thus the antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutases take on a novel role as potential cell regulators of growth and apoptosis. The regulatory role of Mn-superoxide dismutase in relation to cellular behaviour has previously been indicated from work with melanoma cells transfected with a gene for Mn-superoxide dismutase.[28] Such cells exhibited a reduced malignant phenotype as do sarcoma virus-infected mammalian cells with increased superoxide dismutase activity.[29] Moreover certain cells treated with organometallic superoxide dismutase mimics are also reported to display a reduced tumour phenotype.[30] Thus the ability of superoxide dismutase mimics, or inhibitors of catalase and glutathione peroxidase, to induce apoptosis may have important therapeutic potential in treatment of diseases characterised by increased cell survival.[31] Indeed CuDIPS has already been shown to be useful in inhibiting skin tumour development in mice.[32]

The observation that either exposure of BHK fibroblasts to 100 μM levels of H_2O_2, or to agents that bring about increased cellular levels of cellular H_2O_2, also leads to progressively increased rates of apparent apoptosis, certainly supports the view that oxidative stress may be central to apoptotic mechanisms. Whether these mechanisms involve peroxidation of vital cell structures such as membranes cannot be decided from our experiments. However apoptosis induced in thymocytes by exposure to oxidant stress in the shape of a H_2O_2/ferrous sulphate mixture can be repressed by addition of Trolox, a water-soluble analogue of vitamin E.[33] In contrast we found that when α-tocopherol itself, or butylated hydroxytoluene, was added at 10 μM to BHK cell cultures exposed to 100 μM H_2O_2 no reduction in the extent of apoptosis was observed (results not shown). It may be that the water-soluble Trolox reduced the induction of apoptosis in the stressed

thymocytes by affecting extracellular events unrelated to the inhibition of lipid peroxidation. Alternatively Bcl-2 may simply be a more effective intracellular antioxidant in this particular context. Another possibility is that Bcl-2 may have properties that specifically regulate the redox state of key proteins such as ICEs which may play a role in proteolytic processes now believed to be associated with the apoptosis pathway.[2]

In general it could be that apoptosis is the outcome of signal transduction pathways involving the participation of a spectrum of signal transduction proteins analogous to growth signal transduction proteins. Indeed signal transduction proteins such as Myc and Fos have been implicated both in the regulation of proliferation and apoptosis.[25,34–38] Either reactive oxygen species such as H_2O_2 may directly 'trigger' the activity of proteins of apoptotic pathways[34] or by increasing cellular oxidative stress, such as exemplified by increased cellular levels of H_2O_2, may generally bring about redox conditions favouring the functioning of signal transduction proteins in apoptotic pathways rather than in pathways promoting growth. Thus we find that as oxidative stress increases, apoptosis is favoured over growth, although it should be emphasised that severe oxidative stress can be destructive and likely to lead to necrotic cell death (Figure 3d).

In these studies the oxidative stress was imposed by exposure of BHK-21 fibroblasts to increasing levels of exogenous H_2O_2. H_2O_2 as previously reported occurs normally even in unexposed BHK cells and its intracellular concentration is related to growth.[22] An alternative approach has been to impose an oxidative stress using redox cycling quinones, which would generate superoxide and H_2O_2 by redox cycling reactions catalysed by enzymic systems within the exposed cells. Dypbukt *et al.*[39] have shown that increasing concentrations of 2,3-dimethoxy-1, 4-naphthoquinone (DMNQ) stimulated growth, or apoptosis and necrotic death, in pancreatic RINm5F cells depending on the dose and extent of exposure. For instance at 10 μM, proliferation was enhanced, ornithine decarboxylase elevated and polyamine biosynthesis increased. However at 30 μM, apoptotic cell death was evident with depressed ornithine decarboxylase and depleted levels of polyamines. Increasing the DMNQ levels to 100 μM resulted in necrotic cell death.

Our data from H_2O_2 exposed BHK-21 cells is broadly very similar to those of Dypbukt *et al.*[39] from DMNQ exposed RINm5F cells. However, the range of H_2O_2 concentrations (from 1 μM to 10 mM) required to cover the contrasting effect of growth stimulation and cell death is broader than that observed for DMNQ (from 10 μM to 100 μM). This difference may result from the high level of single bolus H_2O_2 metabolism encountered in BHK-21 cells and their culture medium, compared with the likely ***continuous*** intracellular generation of superoxide and H_2O_2 in the case of DMNQ treated cells.

From our studies and those of Dypbukt *et al.*[39] it would appear that if conditions are excessively 'oxidative', apoptosis, or even necrosis, appear to be favoured over proliferation. On the other hand whether oxygen derived species are specifically obligatory for apoptosis signalling has recently been called into question. For example established inducers of apoptosis in lymphocytes can bring about similar levels of apoptosis at widely differing oxygen tensions,[40] suggesting that oxygen derived species may not always be required for apoptopic signalling. Moreover in lymphocytes and fibroblasts, Bcl-2 appears to provide protection from apoptosis in very low oxygen.[41,42] In addition to this dilemma, it may also be significant even in non-apoptotic BHK-21 fibroblasts (i.e. those still adhering to culture dishes) that conditions of 'oxidative' stress, can lead to increases in DNA content of three to four-fold higher than normal levels. Despite the increased DNA content, the DNA isolated from the adherent cells does not appear to be extensively fragmented as judged by crude electrophoretic analysis. Whilst oxidative stress could in some way cause aberrant DNA replication in growth arrested cells possibly at the G_2/M check point no plausible explanation

can be offered at present. However in this context, Handeli and Weintraub[42] have reported a temperature sensitive mutant (ts41) in Chinese hamster cells which leads to successive phases of DNA replication in the absence of intervening G_2 and G_1 phases of the cell division cycle. Normally cells complete only one round of DNA replication before entering the M-phase. The ts41 mutant at non-permissive temperatures exhibits a time-dependent lethality and accumulates excess DNA. Thus it is possible that a protein product of a similar gene in BHK-21 cells is somehow oxidatively modified to prevent stringent control of DNA replication and permit accumulation of excess cellular DNA.

In general therefore it seems that progress towards higher cellular concentrations of H_2O_2, whilst initially favouring proliferative responses in BHK-21 fibroblasts, subsequently results in increased apoptotic and necrotic cell death if the oxidative stress is severe.

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