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ENDOGENOUSLY GENERATED ACTIVE OXYGEN SPECIES AND CELLULAR GLUTATHIONE LEVELS IN RELATION TO BHK-21 CELL PROLIFERATION

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In BHK-21 cells (baby hamster kidney fibroblasts) cellularly generated active oxygen species such as hydrogen peroxide and superoxide appear to be important growth regulatory signals as judged from the growth inhibitory effects of catalase, superoxide dismutase and superoxide dismutase mimics. These active oxygen species may contribute a novel redox system of regulatory control superimposed upon established growth signal pathways. This may be achieved by direct oxidative modification of cell regulatory proteins such as transcription factors or protein kinases or indirectly through, for example alterations in levels of glutathione (GSH). This latter possibility is suggested from observations that catalase, or superoxide dismutase treatment of BHK-21 cells brings about increased cellular levels of GSH. However during the normal growth phase cellular levels of GSH actually decline although this effect can be partly reversed by N-acetylcysteine and by mercaptosuccinate which also impair proliferation of these cells.

KEY WORDS: Cell proliferation, glutathione, catalase, hydrogen peroxide, superoxide, BHK-21 cells.

INTRODUCTION

In a previous paper[1] we provided evidence that hydrogen peroxide and superoxide at low levels are important growth modulatory signals. It was suggested that they may constitute a novel redox system of control superimposed upon the established cell growth signal transduction pathways. For example the proliferation of hamster BHK-21[2,3] and HeLa cells[1] can be stimulated by the exogenous addition of low levels of active oxygen species such as hydrogen peroxide or superoxide. In addition in the case of HeLa cells evidence has been presented to suggest that endogenously generated active oxygen species has a similar positive effect on proliferation. For example exposure of HeLa cells,[1] BHK-21[4,5] or rat 208F[4] cells, to the lipophilic superoxide dismutase mimic (copper II(3,5-diisopropylsalicylate)$_2$ at micromolar concentrations markedly reduces proliferation. In the case of HeLa cells the mimic also causes the reduction of cellular levels of superoxide.[1] Another feature of these experiments was the progressive accumulation of cells stainable with trypan blue, and detaching from the culture dish surface following exposure of the cultures to these mimics.[4]

At a mechanistic level present views of cell communication are dominated by a simple paradigm that signalling is achieved by molecules that bind non-covalently to specific receptors through complementarity of shape. It is possible that there is

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an additional means of cellular signalling in which the signal molecule affects its target molecule covalently on the basis of its redox potential. For example the activity of a number of transcription factors such as AP-1 (Fos/Jun heterodimer),[6] NF-kappa B,[7-9] Rel,[11] Myb[10] and Ets[12] can be modified by redox changes. Certain protein kinases including protein kinase C can also be modified by oxidation-reduction.[13-15]

Here we further explore the possible importance of endogenously generated superoxide and hydrogen peroxide as growth modulatory signals in baby hamster kidney fibroblasts (BHK-21/C13) and their interrelationship with cellular glutathione (GSH) levels during cell growth. Exposure of BHK-21/C13 cells to catalase, SOD or SOD mimics all depress proliferation and decrease cell viability.[4] Catalase and SOD treatment also brings about elevated cellular levels of GSH. However throughout the growth phase of BHK-21/C13 cells cellular levels of GSH actually decrease although this decline can be partly reversed by addition of N-acetylcysteine or mercaptosuccinate which also impair the proliferation of these particular cells. It appears that there may be a special relationship between proliferation, cell viability and cellular GSH levels.

EXPERIMENTAL PROCEDURES

Cell Culture

Baby hamster kidney fibroblasts (BHK-21/C13) and HeLa cells were grown 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) and proliferation determined as previously described.[1,12]

Glutathione Determination

The medium was first removed from the cultured BHK-21 or HeLa monolayers and the cells washed two times with PBS (phosphate buffered saline). The remaining monolayer was scraped off into 2 ml fresh PBS and the cells collected by centrifugation. The pellet of cells was then lysed with 0.5 ml 5% metaphosphoric acid and the lysate centrifuged at 3000 g for 8 min at 4°C to remove precipitated proteins. 0.1 ml samples of protein-free lysate were then used for determination of reduced glutathione (GSH) using the colorimetric assay kit specific for GSH (the GSH-400 method) supplied by Bioxytech S A, France and following the makers instructions. In the context of this study it is particularly relevant that when these instructions are followed there is no interference from N-acetylcysteine or mercaptosuccinate at the concentrations used in this work.

For determination of GSH levels in the media, samples were first treated with an equal volume of 10% metaphosphoric acid. After centrifugation at 3000 g for 8 min to remove insoluble material, 0.1 ml samples were taken to determine media levels of reduced glutathione by the specific procedure detailed above.

Reagents

SDS, superoxide dismutase (bovine erythrocytes, 5100 U/mg protein), catalase (bovine liver, 3800 U/mg protein) GSH, N-acetylcysteine, and mercaptosuccinate were from Sigma Chemical Co.

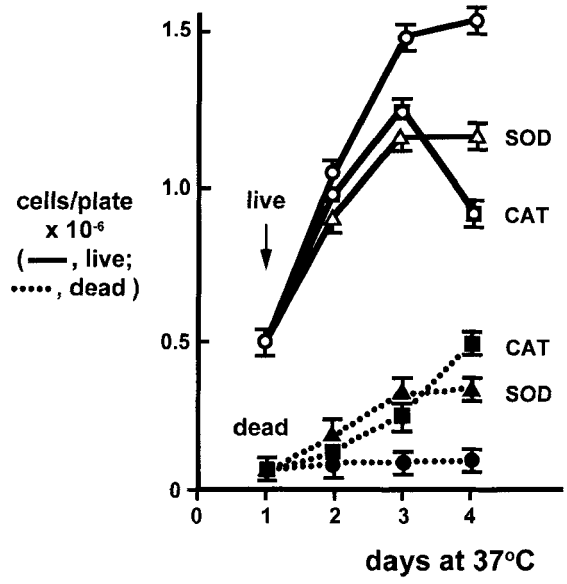

FIGURE 1 The effects of Cu,Zn-superoxide dismutase and catalase on the growth of BHK-21/C13 cells. Cell monolayers were initiated (0.4×10^6 cells per 3.5 cm petri dish in 2 ml Eagle's MEM supplemented with 10% calf serum). After growth for 24 hr the medium was removed and replaced with fresh medium containing 100 μg/ml Cu,Zn-superoxide (SOD) or catalase (CAT). After various periods of further growth at 37°C, cell numbers were assessed as described in EXPERIMENTAL PROCEDURES. Live cells (—) were taken as those remaining attached to the monolayer on the petri dish as distinct from those that became detached and stained with trypan blue (. . .). Results are expressed as the means of triplicate experiments ± s.d. Control cells (○, •); SOD treated (△,▲); CAT treated (□,■).

RESULTS

As was found for HeLa cells,[1] the addition of exogenous Cu, Zn-SOD, at high levels, to the culture medium of BHK-21 cells (Figures 1 and 2) markedly depressed proliferation. Whilst this enzyme may remain outside the cultured cells there are a number of reports to indicate its endocytosis by a variety of cells.[16,19] In this regard it is notable that relative to the concentrations of the mimic that were effective,[4] large doses of enzyme were required to elicit comparable growth inhibitory effects. Catalase addition, also at high levels, was similarly growth inhibitory towards BHK-21/C13 cells (Figures 1 and 2). Again a notable feature (Figure 1) was the progressive accumulation of cells staining with trypan blue and becoming detached from the surface of the culture dish.

In the case of HeLa cells, it was suggested that active oxygen species like hydrogen peroxide (or superoxide) might act as growth modulatory signals.[1] This might be achieved through the augmentation of normal growth signal transduction pathways possibly through the adjustment of the redox state of key proteins such as transcription factors or protein kinases.[1] Cellularly generated H_2O_2 can of course be metabolised through glutathione peroxidase[20] and as such potentially depress cellular levels of GSH. When BHK-21/C13 cells are exposed to high levels of catalase, not only does growth decline, but intracellular levels of GSH are increased

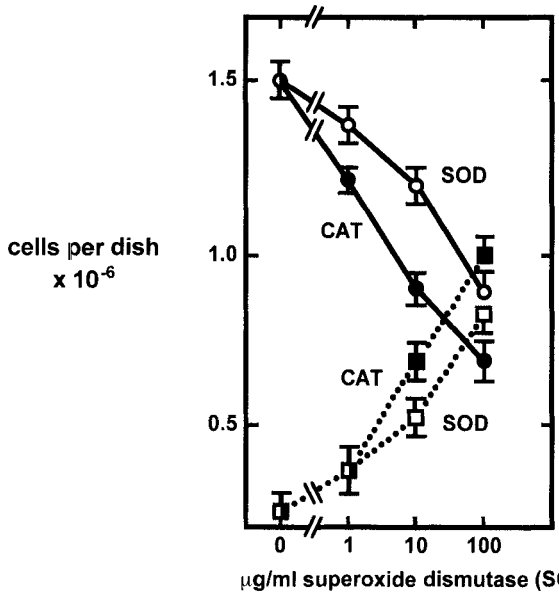

FIGURE 2 The effects of Cu,Zn-superoxide dismutase and catalase on the growth yield of BHK-21/C13. Cell monolayer cultures (triplicate) were initiated (0.5×10^6 cells per 3.5 cm petri-dish in 2 ml Eagle's MEM supplemented with 10% calf serum). After growth for 24 hr the medium was removed and replaced with fresh medium containing various levels of Cu,Zn-superoxide dismutase (SOD) or catalase (CAT). After 3 days of further growth at 37°C, cell numbers were assessed as described in EXPERIMENTAL PROCEDURES. Live cells (—) were taken as those remaining attached to the monolayer on the petri-dish as distinct from those that become detached and stained with trypan blue (. . .). Results are expressed as the means of triplicate experiments ± s.d.

compared with those in untreated cells (Table 1). This is also the case in cells exposed to Cu, Zn-SOD (Table 1). As shown in Figure 1, growth of cells in the presence of catalase also led to a significant proportion of cells becoming detached from the monolayers. These stain with trypan blue and show some of the characteristics of apoptosis. In particular they manifest considerable condensation of chromatin around the nuclear membrane.

In view of this apparent connection between H_2O_2, proliferation and cellular GSH levels, an examination of the GSH levels during growth of BHK-21/C13 cells was carried out. The cells were seeded into culture dishes in normal medium. Thereafter proliferation ensues, and when intracellular GSH levels were determined, there appears to be a notable decline during this period of growth which lasts about 3 days under the conditions used (Figure 3). Addition of N-acetylcysteine to the growing cells during this period diminished the rate of GSH decline and also reduced cell proliferation (Figure 3). A reduction in growth rate and increase in detaching trypan blue staining cells can also be brought about by exposure of BHK cells to mercaptosuccinate,[21] an inhibitor of glutathione peroxidase (Figure 4). Besides reducing proliferation, exposure to mercaptosuccinate also resulted in increased levels of intracellular GSH (Figure 5). Under the experimental conditions of Figure 5, exposure of cells to mercaptosuccinate for 24 hr led to a 39% reduction in cellular glutathione peroxidase activity.

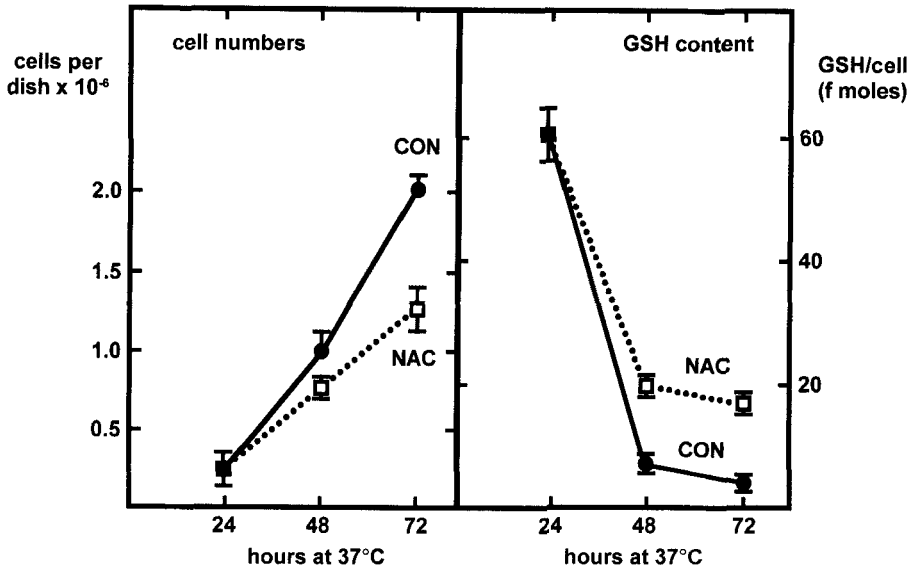

FIGURE 3 Effect of N-acetylcysteine on growth and GSH levels in BHK-21/C13 cells. Triplicate monolayers (0.12×10^6 cells per dish) were established and allowed to grow for 24 hr. As indicated N-acetylcysteine (NAC) was added at 10 mM. Growth was continued at 37°C and live cell numbers and their GSH levels were determined at various times thereafter as described in EXPERIMENTAL PROCEDURES. Control (CON) cells, (•—•) NAC treated cells (□. . .□). Results are expressed as means of triplicate determinations ± s.d.

TABLE 1
Effects of catalase and superoxide dismutase on glutathione (GSH) levels in BHK-21/C13 cells

Expt	Additions	Cells/dish $\times 10^{-6}$	GSH/cell (f moles)
1	none	2.08 ± 0.31	5.6 ± 0.6
	catalase (100 μg/ml)	1.39 ± 0.27	9.4 ± 0.2
2	none	2.34 ± 0.09	7.1 ± 1.1
	Cu,Zn-superoxide dismutase (100 *mg*/ml)	1.85 ± 0.02	9.5 ± 0.9

Triplicate monolayers were established with 0.15×10^6 cells per 3.5 cm dish. After 24 hr of growth, catalase or Cu,Zn-superoxide dismutase added as indicated. After a further 48 hr of growth, the cells remaining attached to the monolayer were collected, washed and used for GSH determination as described in EXPERIMENTAL PROCEDURES.

Whilst the experiments with mercaptosuccinate suggest that glutathione peroxidase may have a role in the processes modulating the growth associated decline in cellular levels of GSH, it also appears that a proportion of cellular GSH may be "exported" to the growth medium (Figure 6). On the other hand withdrawal of serum reduces cell proliferation and this is accompanied by a diminished decline in cellular levels of GSH and an apparent lack of GSH "export" (Figure 6).

To investigate more closely the possible relationship between hydrogen peroxide, cell proliferation and cell GSH levels, low levels of hydrogen peroxide (1 μM) were added exogenously to BHK cell cultures. Whilst proliferation increased, there was

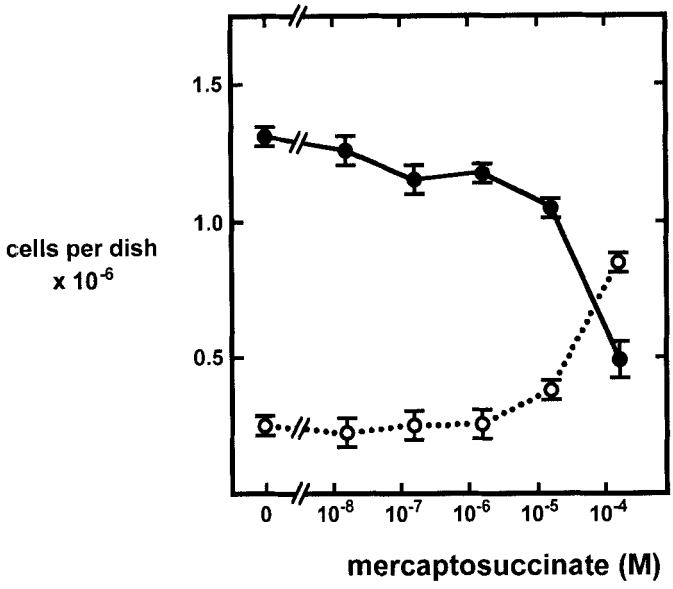

FIGURE 4 Effect of mercaptosuccinate on the growth and viability of BHK-21/C13 cells. Triplicate monolayers were established as in Figure 2. After growth for 24 hr the medium was removed and replaced with fresh medium containing various levels of mercaptosuccinate. After 3 days of further growth at 37°C cell numbers were assessed as described in EXPERIMENTAL PROCEDURES. Live cells (—) were taken as those remaining attached to the monolayer as distinct from those that became detached and stained with trypan blue (. . .). Results are expressed as the means of triplicate experiments ± s.d.

a modest initial drop in cellular GSH levels but thereafter the decline in cellular GSH was similar to that encountered in untreated cells (Figure 7). In the case of growth inhibitory levels of hydrogen peroxide (e.g. 100 μM) the initial drop in intracellular GSH was even greater (Figure 7). Treatment of BHK cells with L-buthionine-SR-sulphoximine which severely depletes cells of GSH by inhibiting γ-glutamyl cysteine synthetase, also reduced proliferation and increased the numbers of detaching tryplan blue staining cells (Figure 8).

In order to ascertain whether the growth related decline in cellular GSH levels was peculiar to BHK-21 cells, similar studies were carried out on HeLa cells (Figure 9). During the growth phase of HeLa cells intracellular GSH levels also declined, but importantly when the growth rate declines, intracellular levels of GSH begin to increase, again underlining the possibility of some special relationship between proliferation, viability and critical intracellular GSH levels.

DISCUSSION

As was the case for HeLa cells, the proliferation of BHK-21/C13 cells is adversely affected by SOD, SOD mimics as well as by catalase when these are added exogenously to the culture medium.[4] Whilst these approaches imply that endogenously generated superoxide, or hydrogen peroxide, are important growth modulatory signals, the question of mechanism remains to be addressed. We have

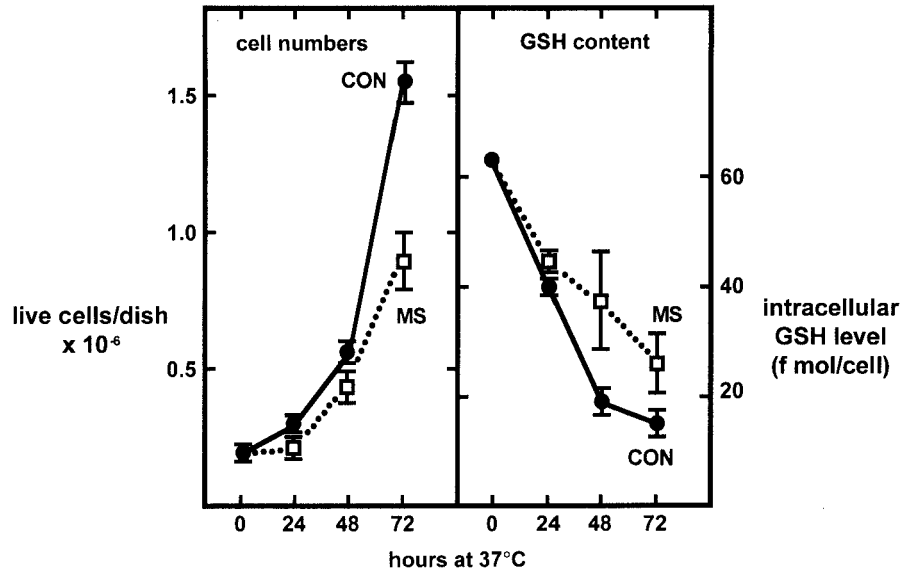

FIGURE 5 Effect of mercaptosuccinate on growth and GSH levels in BHK-21/C13 cells. Triplicate monolayers were established in 2 ml Eagle's medium supplemented with 10% calf serum. As indicated mercaptosuccinate (MS) was added at 150 μM. Growth was continued and live cell numbers and their GSH levels were determined at various times thereafter as described in EXPERIMENTAL PROCEDURES. Control (CON) cells (•—•); MS treated cells (□. . .□). Results are expressed as means of triplicate determination ▪ s.d.

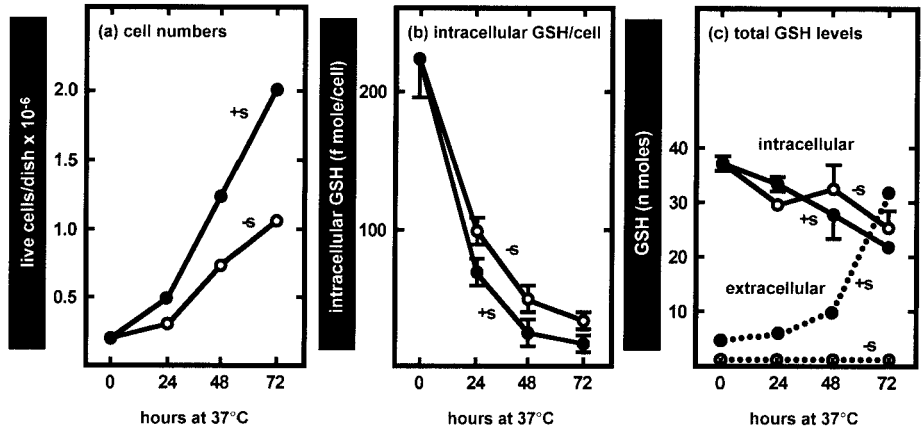

FIGURE 6 Effect of serum withdrawal on glutathione levels in BHK-21/C13 cells. Triplicate petri-dishes (3.5 cm) were seeded with 0.17×10^6 cells in 2 ml Eagle's MEM medium, with or without 10% v/v calf serum, and allowed to grow for 72 hr. At the times indicated live cell numbers (i.e. remaining with the monolayer) were enumerated (a), intracellular GSH levels/cell assessed (b) as well as *total* intracellular and extracellular GSH levels (c). The extracellular levels (. . . .) are presented as the increase in medium GSH levels *over these detected in the media before addition to the cells*. Cells with serum supplementation (+ S, ●); cells without serum supplementation (-S, O). Results are expressed as means of triplicate experiments ± s.d.

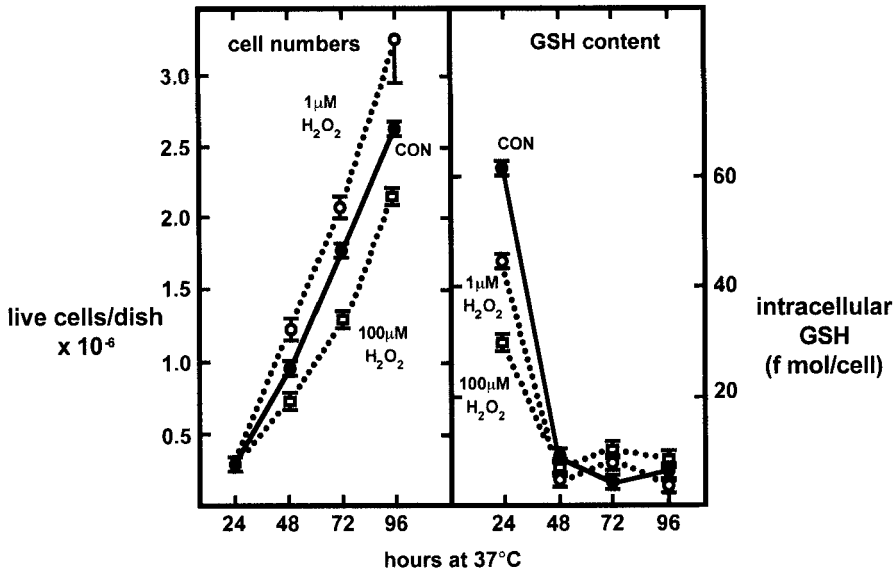

FIGURE 7 The effect of exogenous hydrogen peroxide on growth and cellular levels in BHK-21/C13 cells. Triplicate monolayer cultures of BHK-21/C13 cells (0.27×10^6 cells/3.5 cm petri-dish) were established in 2 ml Eagle's MEM medium supplemented with 10% (w/w) calf serum. To some cultures hydrogen peroxide at 1 μM or 100 μM was added after 24 hr and growth was followed at 37°C. At the times indicated determinations were made of live cells in the monolayers (i.e. that do not detach) and GSH content per cell. Results are presented as means of triplicate experiments ± s.d. Control cells (CON) —; hydrogen peroxide treated cells . . .

suggested[1] that these species may be involved in the adjustment of the redox status of key regulatory proteins of growth signal transduction pathways such as protein kinases and transcription factors. As such this was proposed as a novel redox growth regulatory paradigm superimposed upon established cellular signal transduction pathways. It is possible that adjustment of the redox state of proteins involved in these pathways is a prerequisite for their optimal functioning.

An important question that stems from this hypothesis is whether hydrogen peroxide, or superoxide, directly affects the redox state of signal transduction proteins or whether this is achieved indirectly for example by modulation of cellular GSH levels. In the case of NF-Kappa B, experiments suggest that *in vivo*, hydrogen peroxide can activate forms of the factor cytoplasmically sequestered with the inhibitor I-kappa B.[8,22] This activation can be inhibited by N-acetylcysteine and cysteine.[8] In the nucleus the released factor binds to DNA as a hetrodimer of structurally related DNA-binding subunits, but paradoxically such binding is augmented by thiols and inhibited by oxidation[6,7,22] probably of the conserved Cys62.[23]

Hydrogen peroxide on the other hand is a powerful inducer of *c-fos* and *c-jun* gene transcription,[24-26] *in vivo*, but again paradoxically the *in vitro* DNA-binding by Fos in combination with factors such as Jun is sensitive to oxidation-reduction. Reduction of a conserved cysteine residue located in the DNA-binding domain,[6] either by reducing agents or by a nuclear redox protein, Ref-1, is required for

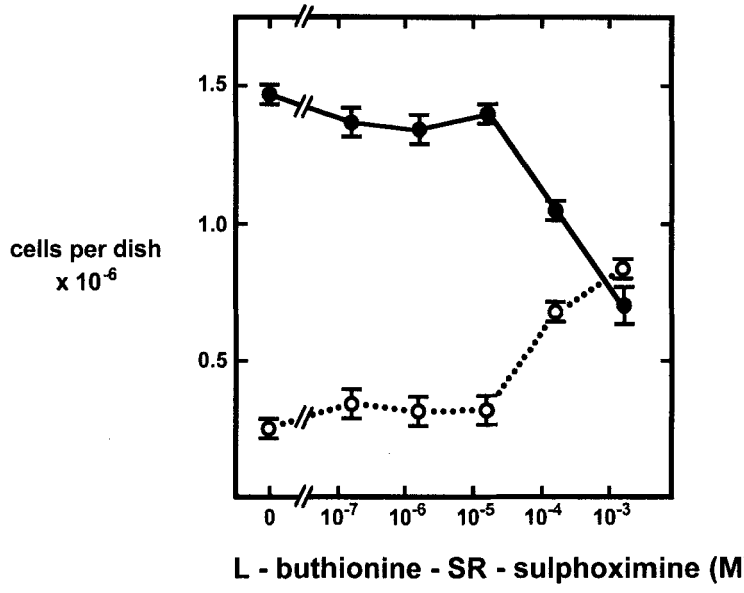

FIGURE 8 The effect of buthionin sulphoximine on the growth and viability of BHK-21/C13 cells. Triplicate monolayers were established as in Figure 2. After growth for 24 hr the medium was removed and replaced with fresh medium containing various levels of L-buthionin-SR-sulphoximine. After 3 days of further growth at 37°C cell numbers were assessed as described in EXPERIMENTAL PROCEDURES. Live cells (—) were taken as those remaining on the monolayer as distinct from those that became detached and stained with trypan blue (. . .). Results are expressed as the means of triplicate experiments ± s.d.

optimal DNA-binding activity.[27] Ref-1 stimulates DNA binding of Fos-Jun heterodimers as well as other transcription factors including NF-kappa B.[27] It is thus possible that continuing changes in cellular redox potential may act to finely regulate NF-Kappa B or Fos-Jun driven responses initiated *directly* or *indirectly* by reactive oxygen species.

The possible involvement of GSH was suggested from the observation that when cells were treated with catalase, or SOD, not only did proliferation decline but GSH levels were elevated. A connection between cellular levels of GSH and BHK cell proliferation is further supported by the observation that during the growth phase of the culture intracellular GSH levels notably decline. However in the case of BHK-21 cells exposed to N-acetylcysteine or mercaptosuccinate, the decline in cellular levels of GSH was diminished but so also was proliferation. N-acetylcysteine is reported to bring about elevated GSH levels in cultured cells, such as Chinese hamster ovary cells (CHO), by promoting cystine uptake and utilisation for GSH,[28] or possibly by directly serving as a precursor for the production of cysteine.[29] Whilst N-acetylcysteine can raise cellular levels of GSH, it has to be conceded that this may not be the only means whereby it acts to inhibit proliferation. It could also act by directly scavenging hydrogen peroxide (or superoxide). Mercaptosuccinate on the other hand is known to be an effective inhibitor of glutathione peroxidase.[21] Thus its adverse effects on cell proliferation and concomitant ability to reduce the proliferation associated decline in cell GSH, are supportive of a hypothesis whereby

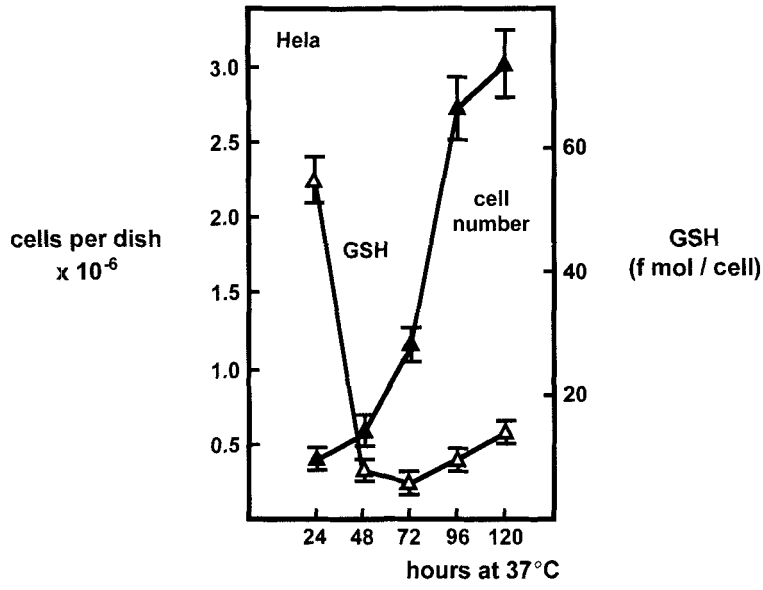

FIGURE 9 The effect of growth on intracellular GSH levels in HeLa cells. Triplicate monolayer cultures of HeLa cells (0.2 × 10^6 cells/3.5 cm petri-dish) were established in 2 ml Eagle's medium supplemented with 10% calf serum. At the times indicated the number of cells in monolayer were assessed together with levels of cellular GSH as described in EXPERIMENTAL PROCEDURES. Results are expressed as the means of triplicate experiments ± s.d. Cell numbers in monolayers, ▲; GSH content (f moles/cell) △.

growth modulatory effects of hydrogen peroxide may be at least partly linked with GSH levels.

Another feature of the growth phase was the *apparent* export of GSH to the growth medium. Export of GSH from cells has been reported as an important determinant of intracellular levels (see ref 30) and has previously been observed in cultured cells.[31,32] However, any accumulation of GSH in the growth medium is nevertheless a function both of cellular levels of γ-glutamyl transpeptidase[32] and the rate of GSH oxidation in the medium.[31]

The decline of GSH levels associated with the growth phase of BHK-21 cells is at first sight surprising as a previous report in the literature had suggested that an elevation of intracellular GSH was associated with the mitogenic stimulation of quiescent 3T3 fibroblasts.[33] However a subsequent study[34] showed that increased levels of GSH in quiescent serum-stimulated rat fibroblasts (NRK-49F) are associated, not with a response to growth factors, but with nutrient repletion. In conditions where this problem was eliminated, it was in fact observed that stimulation of quiescent NRK-49F cells with epidermal growth factor as mitogen, actually led to a significant decline in cellular GSH levels.[34]

In lymphocytes there are also reports of declining GSH levels following mitotic stimulation.[35,36] On the other hand separate reports suggest increased levels after proliferative stimuli.[36,37] These differences may be important and reflect cell type as opposite effects were observed when thymocytes were compared with splenocytes.[37] In recent years it has become clear that the various functions of cells

of the immune system are differentially sensitive to active oxygen species and cellular levels of GSH. Superoxide and hydrogen peroxide at physiologically relevant concentrations augment the production of IL-2 whereas IL-2 production is inhibited by GSH.[38] Other functions such as IL-2 dependent proliferation, development of $CD8^+$-T cell blasts and cytotoxic T-lymphocyte activity require high GSH levels.[38]

In the case of BHK-21 cells it is suggested that low concentrations of hydrogen peroxide, or superoxide, added exogenously or generated endogenously, act as positive signals for proliferation. These appear to exert their effects at least partly through a modest reduction of the intracellular level of GSH, which as pointed out above nevertheless declines normally throughout the growth phase. This may involve the participation of glutathione peroxidase although the export of GSH must not be discounted. In contrast agents such as catalase, superoxide dismutase, N-acetylcysteine and mercaptosuccinate, or procedures such as serum withdrawal, which all result in higher than normal levels of cellular GSH, all favour reduced BHK-21 cell proliferation, and progressive loss of cell viability with some characteristics of apoptosis. Nevertheless a critical feature to emerge from these studies was that exposure to moderately high levels of hydrogen peroxide (eg 100 μM) which can lower cellular GSH levels to a greater extent than 1 μM hydrogen peroxide (Figure 7) rather than stimulating cell proliferation, instead favoured reduced cell proliferation and progressive loss of cell viability. Buthionine sulphoximine, an established inhibitor of cellular GSH synthesis,[30] also has a similar effect (Figure 8). It is possible then that specific intracellular levels of GSH are critical in determining whether proliferation is enhanced or diminished, although the higher levels of hydrogen peroxide may also be significant in as much as hydrogen peroxide itself may directly and deleteriously affect growth regulatory molecules.

In this latter context it is always possible that the reduced cell proliferation observed in the presence of mercaptosuccinate (Figure 4) could also be partly explained by accumulation of higher than normal levels of hydrogen peroxide resulting from inhibition of glutathione peroxidase. In this context it may be significant that treatment of BHK-21 cells with the catalase inhibitor, aminotriazole, whilst modestly growth stimulating at low concentrations (1–10 μM) is nevertheless significantly growth inhibitory at higher concentrations (10–100 μM) (unpublished observations).

Whether our general findings extend to other non-inflammatory cell types however remains to be tested. However initial experiments with HeLa cells are encouraging (Figure 9). During the growth phase of HeLa cells intracellular GSH levels also decline, but particularly when the growth rate declines, intracellular levels of GSH begin to increase, again underlining the possibility of some special relationship between proliferation, viability and intracellular GSH levels in these cells. Finally in terms of possible apoptosis, recent observations indicate an important role for the product of the *bcl-2* gene in suppressing the process in certain cell types.[39,40] Although Bcl-2 may not interfere with superoxide generation, it is suggested that it may act to prevent peroxidation of cell lipids. Previous studies of ours involving BHK-21 cells indicated that levels of cellular lipid peroxidation were higher in cells with reduced rates of proliferation.[41] Moreover, withdrawal of serum from the growth medium increases cellular lipid peroxidation both in BHK-21 cells (unpublished observations) and in HeLa cells.[42] As shown in Figure 6, serum withdrawal not only reduces the rate of proliferation of BHK-21 cell but also

increases in cellular levels of GSH. It will thus be of importance to ascertain how the expression of *bcl-2* is influenced by cellular levels of GSH.

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