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FREE RADICALS AND THE REGULATION OF MAMMALIAN CELL PROLIFERATION

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The question of whether free radicals or free radical-related species play a role in the modulation of mammalian cell proliferation is examined. Although a positive role for free radicals as specific components of mitogenic pathways is not apparent it is clear that certain free radical-derived species can have a significant modulatory influence on components of major growth signal transduction mechanisms. Free radical-derived species are also involved in the production of prostaglandins which themselves can modulate cell growth. Free radicals themselves appear to have a down regulatory effect on cell proliferation inasmuch as protection from oxidative stress enhances cell proliferation. On the other hand, in certain cases low levels of active oxygen species can enhance cell proliferation.

KEY WORDS: Proliferation, hydrogen peroxide, prostaglandins, fatty acids, superoxide radicals.

1. INTRODUCTION

There is a growing body of evidence to suggest that free radicals or free radical-related species may be important in processes that modulate mammalian cell proliferation. Such evidence has recently been derived from a variety of systems which include studies on cell antioxidant levels in relation to DNA synthesis, the effects of antioxidants on rates of cell proliferation, levels of cellular lipid peroxidation in relation to cell proliferation and growth promoting agents, the effects of polyunsaturated fatty acids on cell growth, lipid peroxide breakdown products and their effect on growth promoting signal transduction mechanisms etc. On the other hand it has to be conceded that the processes that regulate cell proliferation are already known to be complex. For this reason some introduction to our present understanding of proliferative control mechanisms is necessary in order to assess any additional mechanisms involving free radicals or related species.

2. DIVISION AND PROLIFERATION OF MAMMALIAN CELLS

(a) The cell cycle

Before cell division can take place a cell must double its mass and duplicate all its components. Most of the processes involved in this preparation go on continuously during the period between cell divisions known as interphase.[1] Within interphase there is a specific period during which DNA is replicated. This is referred to as the S-phase of the cell cycle and the period between mitotic division and the S-phase is known as the G1 phase. Cells can become arrested in the G1 phase in the absence of specific growth factors or by adding inhibitors of protein synthesis. There appears to be a

restriction point (R) late in G1 and cells arrested at this point can remain viable for considerable periods.

At present it is not known what determines whether a mammalian cell will go beyond R in G1 and start a new cycle. One suggestion has been that cells have to accumulate a threshold amount of some unstable "trigger" protein to pass through R.[2,3] For example, conditions that stimulate rates of overall protein synthesis would permit the amount of trigger protein to rise above threshold levels whereas in conditions of growth factor deprivation where protein synthesis is reduced the concentration of trigger protein will be correspondingly reduced. As will be discussed in section 5, conditions of oxidative stress bring about altered rates of protein synthesis which may be relevant to cell cycle control.

(b) Requirements for cell growth

From cell culture studies,[4] it is clear that cells have to be maintained at pH 7.0–7.3 and the media must provide essential inorganic ions, correct osmolarity as well as a source of energy such as glucose. It is well known that most cell cultures grow better at lower oxygen tensions than atmospheric.[5] However, for proliferation other components are required. These include most of the common amino acids, eight B- group vitamins and around 10% serum. Although bovine serum is most commonly used its overall contribution is not fully understood. It is clear that it includes components such as hormones (eg. insulin, which stimulates glucose and amino acid uptake) polypeptide growth factors which specifically stimulate cell proliferation (eg. EGF, epidermal growth factor) and essential proteins (eg. transferrin, which binds iron and makes it available to the cell). Serum is also believed to supply trace elements, for example selenium, copper and zinc, fatty acids important for cell growth (see Section 3), as well as components which may serve an antioxidant function, including ascorbate and α-tocopherol. Omission of serum from the growth medium, besides depressing cell growth, results in increased cellular oxidative stress (see Section 5a)

(c) Gene expression and growth control

The cells of an organism divide at very different rates, the major differences being in the time they spend in G1. The rate of cell division, however, is controlled by mechanisms that allow cells to divide if and when new cells are required, uncontrolled cell division being a feature of tumour growth. At the genetic level it is now becoming clear that specific genes known as "proto-oncogenes" (eg. c-fos, c-myc, c-ras) exert a controlling influence on the molecular events that enable cells to proliferate.[6] One of the earliest events following a proliferative stimulus is the expression of the c-fos gene.[7,8] This precedes the expression of c-myc and c-ras. Whilst the products of c-fos and c-myc[9] exert their effects in the cell nucleus probably at the level of transcriptional control,[10] the c-ras product may function in a manner analogous to that of a G-protein in the regulation of adenylate cyclase.[11] As discussed in Section 5, a potentially important inducer of protooncogene transcription is oxidant stress.

(d) Signal transduction mechanisms and second messengers

Certain polypeptide growth factors can dramatically increase the formation of diacylglycerol (DG) and inositol triphosphate (IP_3) in cells by virtue of activating the

enzyme phospholipase C.[12] IP_3 causes the release of calcium ions from the endoplasmic reticulum and these ions together with DG bring about the activation of protein kinase C.[13] This important kinase can phosphorylate various cellular proteins which are believed to be involved in proliferative control. Besides IP_3 and DG another second messenger of possible significance for cell proliferation is cAMP.[14] Bursts of elevated cAMP production in response to various mitogens have been observed prior to S-phase in a number of cell types and it appears that such transient elevations are coupled to DNA synthesis.[15] On the other hand continuous exposure of cells to high levels of cAMP appears to inhibit the overall mitogenic process.[16] These effects of cAMP may be due to its well-established ability to regulate the activity of various protein kinases. cAMP can also regulate the transcription of genes such as c-fos[17] by stimulating a system involving proteins that bind to DNA regions upstream of the genes to be activated.[18] Such regions are referred to as cAMP regulatory elements. The intracellular level of cAMP is under the influence of a variety of regulators including hormones and growth regulating substances. These regulators act by binding to specific cell surface receptors which communicate a stimulatory signal to adenylate cyclase through a group of proteins referred to as G-proteins[19] (see Section 2c). Particularly important stimulatory ligands in the context of this review include prostaglandins.[16] Adenylate cyclase can also be stimulated by oxidants such as hydrogen peroxide (see Section 5b).

Although it has been suggested that cGMP might also be involved in growth regulation in opposition to cAMP,[16] there are still few data to indicate a clear role.

3. POLYUNSATURATED FATTY ACIDS AND CELL PROLIFERATION

Unsaturated fatty acids promote the growth of many cell lines when added to cultures at low concentrations but exhibit inhibitory effects at high concentrations. The growth of normal cells can be stimulated at concentrations of polyunsaturated fatty acids up to 30 μM but their potential ability to enhance growth of tumour cells operates within lower limits (1–20 μM).[20] In excess of these levels proliferation is inhibited.

Polyunsaturated fatty acids, such as C20:4, 5,8,11,14-eicosatetraenoic acid, C20:3 8,11,14 eicosatrienoic acid and C20:5 5,8,11,14,17-eicosapentaenoic acid, can be oxidized intracellularly by a variety of free radical reactions *enzymically* to prostaglandins or leukotrienes and *nonenzymically* to a variety of carbonyl compounds. In the prostanoid pathway, via cyclooxygenase, cyclic endoperoxides are intermediates; hydroperoxytetraenoic acids (HPETE) are intermediates in the lipoxygenase pathway (Figure 1). Whereas cyclooxygenases are present in most mammalian tissues, different lipoxygenases are of different importance depending on the cell or tissue studied. It is possible that it is the HPETE levels within the cell which control the prostaglandin pathway through the activation or inactivation of cycloxygenase; higher levels of HPETE increase the conversion of polyunsaturated fatty acids into prostaglandins.[21,22] The nonenzymic mechanism involves the formation of acyclic fatty acyl hydroperoxides through a radical-mediated peroxidative pathway.

Prostaglandins can modulate the rate of cell proliferation and differentiation, although prostaglandin synthesis is not a pre-requisite for cell proliferation. Possible means whereby cell proliferation may be modulated by polyunsaturated fatty acids include:

i) the prostaglandin and leukotriene products of polyunsaturated fatty acid metabolism;
ii) the endoperoxide, hydroperoxy and free radical intermediates formed during eicosanoid synthesis;
iii) the generation of reactive oxygen species during non-enzymic peroxidation;
iv) the elevation of cyclic nucleotide levels through the activation of enzymes by certain of the metabolites mentioned above.

Differential effects on cell growth and survival displayed by individual prostaglandins are shown in Table 1. PGE_2 displays concentration-dependent effects. A stimulation is observed at physiological levels with a transition to a cytostatic response at the upper end of the range.[23-25] Stimulation of prostacyclin (PGI_2) synthesis leads to elevated cAMP levels. The effects of prostaglandins may relate to the phase of the cell cycle at which the prostaglandin is added: a cursory increase in cAMP levels at the end of the G1 phase can stimulate the initiation of DNA synthesis, but a prolonged increase is inhibitory to cell proliferation.[23]

It has been proposed that the stimulatory action of PGF_{2a} on normal cell growth[26,27] may be related to increased phosphatidyl inositol metabolism[28] which may mediate the stimulation of DNA synthesis. PGD_2 itself does not inhibit cell growth but its metabolites are cytotoxic. For example, high concentrations of the deoxy-derivatives of PGD_2 degradation inhibit cell division by blocking the progression from G1 to the S phase of the cell cycle through a cAMP-independent mechanism, possibly mediated by free radicals.[29,30] The cyclopentenone prostaglandins PGA_2 and Δ^{12}-PGJ_2 are, however, potent inhibitors of cell growth by causing a block in the G1 phase of the cell cycle.[31] They appear to be actively transported into cells by a specific carrier and accumulate in cell nuclei binding to nuclear proteins.

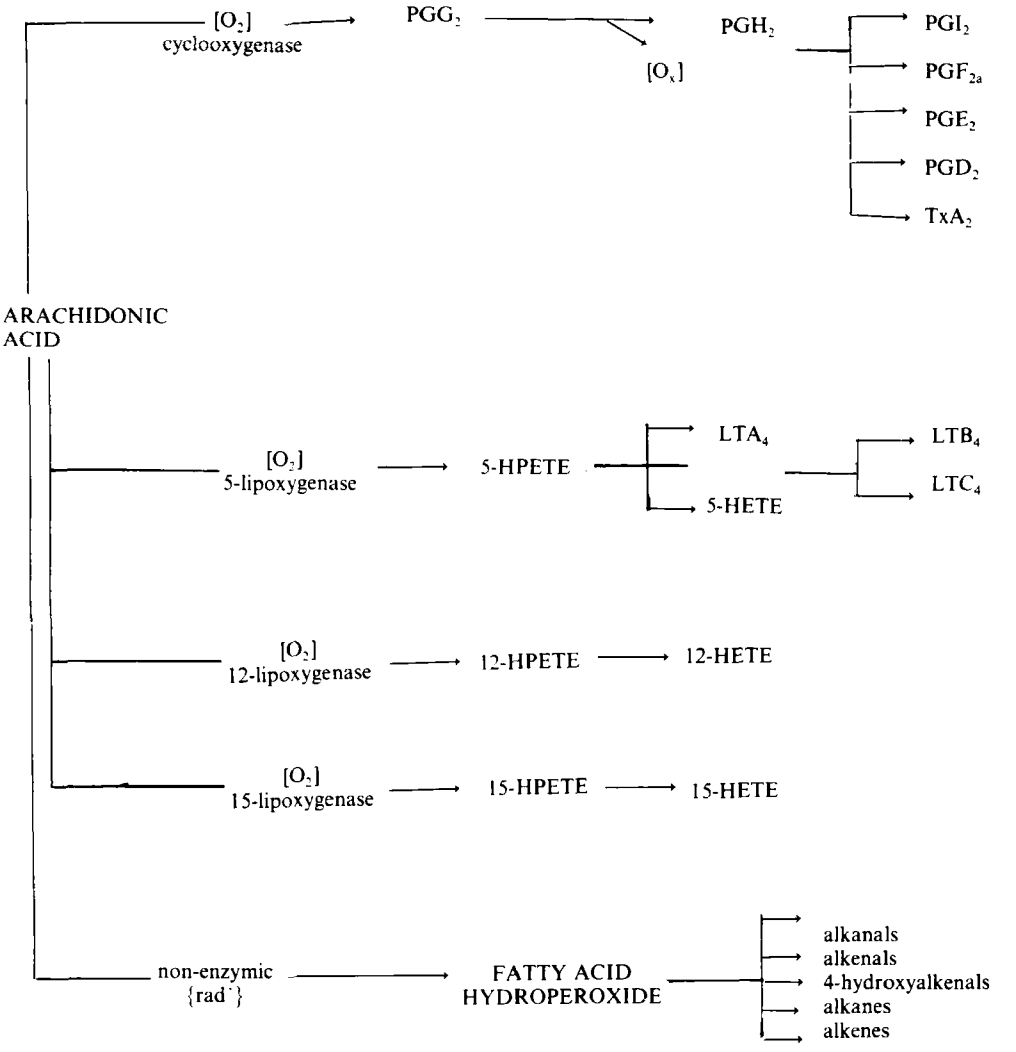
Experiments to investigate the involvement of polyunsaturated fatty acid oxidation in the modulation of cell proliferation have shown that prevention of prostaglandin synthesis does not suppress these inhibitory effects by polyunsaturated fatty acids but several antioxidants do (Section 5).[26,32-35] Such observations suggest that the inhibitory effects may relate to nonenzymic fatty acid peroxidation processes rather than to effects on enzymic prostanoid metabolism. This is consistent with the observation that all of the polyunsaturated fatty acids that inhibit cell proliferation generate lipid peroxides when added to cells in culture. Cornwell has summed up the situation in what he describes as the prostaglandin paradox:[20] prostaglandin synthesis increases as polyunsaturated fatty acid concentration increases, and cell proliferation continues until the capacity of the prostaglandin hydroperoxidase enzyme for prostaglandin synthesis is exceeded.

On a cautionary note it should be pointed out that commercial samples of polyunsaturated fatty acids may contain variable amounts of autoxidised products[36] which may complicate the interpretation of experiments such as those described above. For example, autoxidised arachidonic acid has a potent prostaglandin-like activity.[37] It is thus essential to establish the extent of oxidation of applied fatty acid preparations.

4. EFFECTS OF FATTY ACYL HYDROPEROXIDES AND THEIR METABOLITES

Cells undergo lipid peroxidation on exposure to polyunsaturated fatty acids and

FIGURE 1 Products of enzymic and nonenzymic oxidation of arachidonic acid



generate increased amounts of precursors of aldehydic secondary metabolites as well as eicosanoid intermediates and products. The growth and proliferation of cells in culture are affected by lipid peroxides and their metabolites whether generated endogenously or added exogenously.[32,38-40]

However, it has long been known that rapidly proliferating cells in regenerating tissue are resistant to lipid peroxidation.[41] Chain-breaking antioxidants such as α-tocopherol and butylated hydroxytoluene (BHT), promote cell proliferation[26,42] suggesting that cell growth and proliferation are blocked by the formation of lipid peroxides, their breakdown products and the overproduction of free radicals (see also Section 5a).

Antioxidants have been reported to alleviate the polyunsaturated fatty acid-

TABLE 1
Effects of prostaglandins on cell growth and survival

Prostaglandin	Response
PGE_2	Concentration-dependent effects on cell growth and proliferation[23-25]
PGE_1	Inhibition[26]
PGF_{2a}	promotes growth of normal cells, no effect on tumour cells in vitro[26,27]
PGI_2	increase in cAMP levels[23]
PGD_2	no direct inhibition of cell growth, cytotoxic response from metabolites[29,30]
PGA_2	block in cell cycle progression in G1[31]
Δ^{12}-PGJ_2	block in cell cycle progression in G1[31]

induced inhibition of cell growth. This again may be interpreted as a mechanism acting through the inhibition of the formation of peroxy radicals, hydroperoxides, HPETEs and aldehydic decomposition products of lipid hydroperoxides.[38] HPETEs, HETEs (hydroxytetraenoic acids) and leukotrienes have deleterious effects on cells, one hypothesis proposed being the possibility of damage to DNA polymerases[43] induced by radical intermediates or the formation of breakdown products of hydroperoxides.

Direct addition of various aldehydic metabolites of lipid and fatty acyl hydroperoxides have been shown to restrict cell proliferation although the mechanisms are not understood. Among the nonenzymic peroxidative breakdown products of unsaturated fatty acids, the 4-hydroxy-2,3-transenal series appears to display important biological actions.[44] 4-hydroxy nonenal (HNE), the most abundant of these products, exhibits cellular toxicity at higher concentrations but appears to have a possible regulatory function at lower concentrations: concentrations of HNE of 1 μM have been found in normal intact hepatocytes.[45] The activities of adenylate cyclase,[46] guanylate cyclase and phospholipase C are stimulated by HNE concentrations lower than 1 μM.

Dianzani *et al.*[46] have suggested that aldehydes, and therefore peroxidation processes, might modulate cellular functions through the regulatory G-proteins (see Section 2d). It has also been demonstrated that HNE at 1 μM concentrations blocks the expression of the c-myc proto-oncogene in a human erythroleukaemic cultivated line (K-562),[47] although some other genes in the same cell are activated suggesting that HNE also may affect differentiation pathways.

These observations raise mechanistic questions. 4-Hydroxynonenal is considered to act on proteins either by forming Schiff bases with amino groups or by interacting with free thiol sidechains. The mechanism of the HNE-induced stimulation of adenylate cyclase may involve blockage of either an amino or a free thiol group. The latter may be important in the inhibition of the regulatory function of the G-proteins.

5. DIRECT EFFECTS OF EXOGENOUS ANTIOXIDANTS AND OXIDANTS ON CELL PROLIFERATION

As already mentioned (Sections 3 and 4) there have been a number of observations that suggest an apparent inverse relationship between levels of cellular lipid peroxidation and rates of cell proliferation.[38] In regenerating liver the periodic bursts of DNA synthesis are linked to corresponding depressions in the rate of lipid peroxidation[41]. Studies in regenerating liver also indicate that the levels of α-tocopherol rise in relation to periods of DNA synthesis.[41] In order to explore further the relationship between oxidative stress, lipid peroxidation and cell proliferation, several investigators have examined the effects of exogenously added antioxidants and oxidants on mammalian cell proliferation.

(a) Antioxidants

Experiments with HeLa cells[49] show that when deprived of serum, not only does the proliferative rate decrease but there is a concomitant rise in the level of cellular lipid peroxidation. These effects can nevertheless be abated by the inclusion of mannitol (50mM) in the medium lacking serum.[9] However, mannitol addition between 1 μM and 100 μM will also enhance the growth of BHK-21 cells (hamster fibroblasts) even in the presence of 10% calf serum with optimal effects at 10 μM. The effects of mannitol, for example, are likely to be at the cell surface where the operation of receptors in mediating growth responses by intracellular transduction mechanisms may be affected. Similar experiments with BHK-cells were carried out with α-tocopherol or BHT addition instead of mannitol.[42] Whilst both were also able to enhance growth in medium containing serum the latter was more effective. Both α-tocopherol and BHT also promote the proliferation of cultured aortic smooth muscle cells.[26]

In view of the known antioxidants present in serum (e.g. albumin, ascorbate), it is perhaps not suprising that mammalian cells cultured aerobically in the absence of serum accumulate increased levels of lipid peroxides.[49] Our observations that mannitol can not only counter this effect[49] but also improve the growth of serum-deprived cells is consistent with the notion that oxidative stress, lipid peroxidation and cell proliferation may be interconnected. In situations where the growth medium is supplemented with serum it is normally only to the extent to 10% (v/v). Under such conditions it may well be that such cells are still not adequately protected. Indeed low but significant "steady-state" levels of lipid peroxidation have been reported in cultured mammalian cells.[49,50] Thus the stimulatory effects of mannitol, tocopherol or BHT may be due to their ability to provide extra protection even in 10% serum supplemented cells.

The above points raise the question of what particular types of growth regulatory mechanisms are impaired in conditions of oxidative stress. A wide variety of cellular signal transduction systems operating at the level of cell membranes might be primary targets (see Section 5d). Prostaglandin synthesis may be another pathway affected (see also Section 5c).

Despite these reported stimulatory effects of antioxidants on mammalian cells it should be emphasised that the cells involved are of epithelial or fibroblastic origin. Studies on lymphoid cells provide contrary evidence. Antioxidant addition to "activated" T-lymphocytes appears to impair proliferation. The antioxidants studied include iron chelators as well as the lipid-soluble free radical scavenger butylated

hydroxyanisole. Such observations have led to the suggestion that free radicals may be involved in processes occurring in the early stages of lymphocyte activation.[51,52] Whilst there may be distinct differences between different cell types no suggestions regarding mechanisms for free radical mediated processes in "activated" lymphocytes have yet been proposed.

(b) Hydrogen peroxide and superoxide radicals

A considerable proportion of mammalian cells and tissues are normally exposed from time to time to oxidants. For example considerable levels of oxidants are produced by cells involved in the inflammatory response. Cultured human fibroblasts have been reported to release low levels of superoxide radicals, similar to those of unstimulated endothelial and phagocytic cells.[53] Such observations raise the question of whether oxidant species such as hydrogen peroxide and superoxide radicals when generated intracellularly or released extracellularly might play a role in the modulation of cell proliferation. This may be of significance in wound healing and tissue regeneration.

Leukocytic oxidants, generated during inflammatory reactions, attack various targets in cells (endothelial cells, fibroblasts, lymphocytes, macrophages) including DNA.[54] Rapid damage is elicited by leukocytic oxidants resulting in single strand breaks of DNA and base hydroxylation. Hydrogen peroxide is capable of penetrating cells and DNA damage has been observed[54–56] proportional to the concentration of external hydrogen peroxide. While hydrogen peroxide at concentrations lower than 40 μM induced single strand breaks in DNA, hypochlorous acid and chloramines failed to damage DNA at a ten-fold higher concentration. Cochrane *et al.*[54] have implicated hydroxyl radical rather than hydrogen peroxide itself from studies involving hydroxyl radical scavengers.

Recent experiments of ours[42] have shown that low concentrations (10nM) of extracellular hydrogen peroxide will stimulate the proliferation of both polyoma virus transformed and non-transformed hamster fibroblasts (BHK-21 cells) (Figure 2). In fact, hydrogen peroxide at 10nM was capable of stimulating hamster fibroblast growth even in the absence of serum.[42] Our observation that hydroxyl radical scavengers improve the growth promoting properties of hydrogen peroxide[42] argues in favour of a direct role for hydrogen peroxide rather than any hydroxyl radicals derived from it.

Despite these stimulatory effects of hydrogen peroxide at low concentrations, it has to be conceded that high concentrations can severely impede growth of the tumor-virus transformed PyY cells (see Figure 2). Other workers have also reported differential sensitivity of tumor cells to exogenous hydrogen peroxide and have suggested that this may be due to deficiencies in glutathione-dependent hydrogen peroxide metabolism[57]. For example, GSSG is well-known to inhibit protein synthesis.[58,59] These deleterious effects at high concentrations ($> 10\,\mu$M) on the transformed cells can nevertheless be minimised by inclusion of hydroxyl radical scavengers in the growth medium implicating hydroxyl radicals as the actual growth inhibitors.[42]

Since hydrogen peroxide can be derived from superoxide radicals by dismutation, the effect of addition of exogenous superoxide radicals has also been investigated. When BHK-21 cells were exposed to low levels of exogenous superoxide radicals by adding mixtures of xanthine and xanthine oxidase to the growth medium quite different effects were observed on the polyoma-virus transformed cells (BHK-21/PyY) compared with the corresponding non-transformed cells (BHK-21/C13) (see Figure

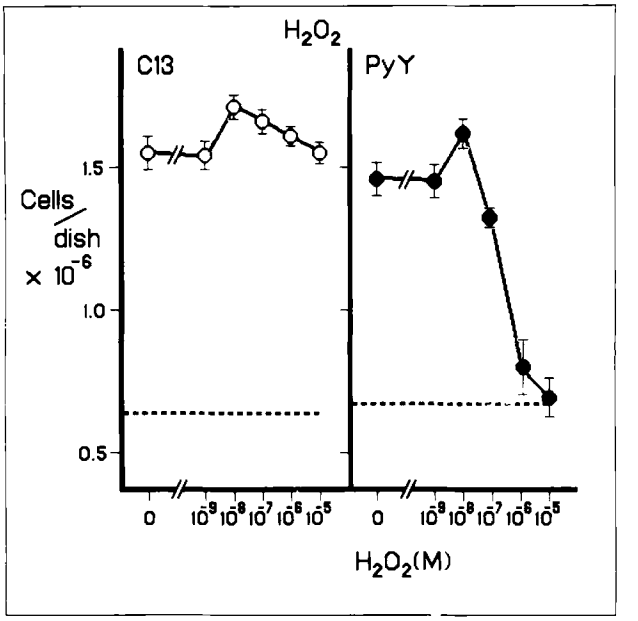


FIGURE 2 The effects of added hydrogen peroxide on BHK-21 cell growth. The triplicate monolayer cultures (0.5 × 10^6 cell/dish) were allowed to grow for 24 hr at 37°C in DMEM supplemented with 10% calf serum. The dotted lines indicate the number of cells per dish after that period. The medium in each dish was then removed and the monolayers washed three times with serum-free medium and replaced with DMEM and calf serum along with varying amounts of hydrogen peroxide. After 3 days at 37°C the cells were removed from each dish and counted. Results are expressed as the mean of triplicate determinations ± s.d. (O BHK-21/C13 cells; ● BHK-21/PyY cells).

3). The *inhibitory* effect of superoxide radicals on the non-transformed hamster fibroblasts was nevertheless converted into a stimulatory effect if superoxide dismutase was added to the medium in addition to the superoxide-generating system (Table 2). However, if catalase is added along with the superoxide dismutase the stimulatory effect is lost and is thus likely to be due to the formation of exogenous hydrogen peroxide from the added superoxide radicals. In the case of the polyoma-virus transformed fibroblasts it appears that the exogenous superoxide radicals are *stimulatory* rather than inhibitory. Moreover, after addition of exogenous superoxide dismutase the stimulatory effect is retained although the combination of superoxide dismutase and catalase reduces the stimulation (Table 2) again suggesting a central role for hydrogen peroxide in the stimulation of fibroblast proliferation.

Exogenous superoxide radicals and/or hydrogen peroxide have also been observed to promote the proliferation of certain strains of mouse epidermal cells,[60] mouse embryo fibroblasts[61] although it is not clear whether hydrogen peroxide or superoxide is primarily responsible. Nevertheless[62] the addition of such exogenous oxidants can induce the expression of some protooncogenes known to be involved in the processes of cell growth initiation eg. c-fos and c-myc (see Section 2c). The mechanisms responsible for the activation of these growth related genes are not clear but might involve the post-translational modification of proteins specifically regulating the

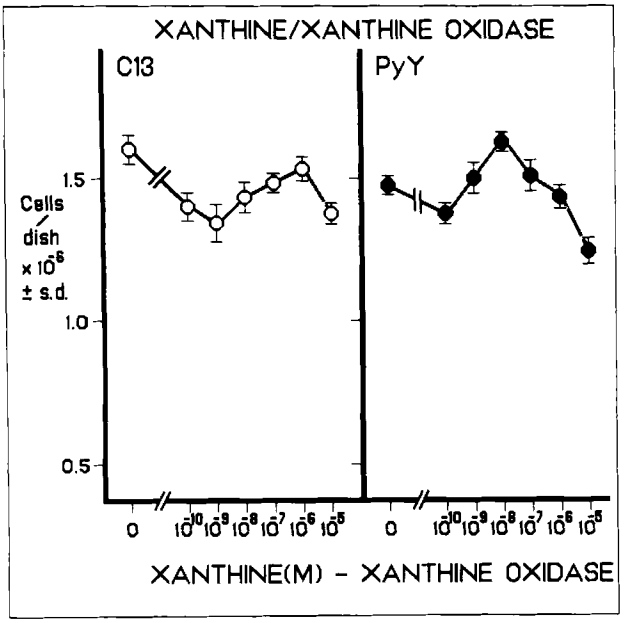


FIGURE 3 The effects of added superoxide radicals on BHK-12 cell growth. Experimental procedures were as described for figure 2 except that varying dilutions of a xanthine-xanthine oxidase mixture were added after the initial 24 hr growth period i.e. where xanthine was at 10^{-5} M, xanthine oxidase was added at final concentration of 3 μg ml^{-1}; where xanthine was at 10^{-6} M, xanthine oxidase was added at 0.3 μg ml^{-1} etc etc. (O BHK-21/C13 cells; o BHK-21/PyY cells).

TABLE 2
Superoxide dismutase and catalase addition and the effect of superoxide radicals on BHK-21 cell growth

Conditions	Cells/dish × 10^{-6} BHK-21/C13	BHK-21/PyY
no additions	1.55 ± 0.03	1.45 ± 0.02
xanthine/xanthine oxidase	1.39 ± 0.03	1.62 ± 0.02
xanthine/xanthine oxidase plus superoxide dismutase (100 μg/ml)	1.64 ± 0.03	1.67 ± 0.02
xanthine/xanthine oxidase plus superoxide dismutase (100 μg/ml) plus catalase (100 μg 1ml)	1.38 ± 0.07	1.55 ± 0.01

Triplicate monolayer of BHK-21 cells (0.5 × 10^6 cells per dish) were grown for 24 hr in DMEM supplemented with 10% calf serum.

The medium was removed, the monolayers washed three times with serum-free medium and the medium replaced as indicated above. Xanthine was added at 10 nM and xanthine oxidase at 3pg. ml^{-1}. After 3 days the cells in each dish were removed and counted in a haemocytometer. The results are expressed as the mean of triplicate experiments ± s.d.

transcription of these protooncogenes. A suggestion is that such a modification could involve phosphorylation or ADP-ribosylation.[62]

(c) Oxidants and the modulation of prostaglandin and lipoxygenase pathways

In addition to the effects discussed above exogenous oxidants will stimulate the metabolism of arachidonic acid in ways that are likely to affect cell proliferation. For example, Cerutti[63] has observed a strong stimulation of the synthesis of the prostaglandins F_{2a} and E_2, but not of lipoxygenase products, in lung epithelial cells exposed to exogenous xanthine/xanthine oxidase mixtures. In phorbol ester-stimulated mouse skin these particular prostaglandins appear necessary for the hypoproliferation subsequently observed.[64,65] Superoxide and hydrogen peroxide have also been reported to stimulate prostaglandin synthesis in a number of other cell types (see 63). Such observations indicate regulatory control possibly at the level of cyclooxygenase which may become activated in cells exposed to exogenous oxidants (see 63).

(d) Oxidants and signal transduction mechanisms

The observations that low levels of exogenous oxidants can stimulate mammalian cell growth even in the absence of serum clearly raise questions with regard to possible mechanisms. In this connection it may be relevant that although superoxide will not readily penetrate cells, hydrogen peroxide will do so. Hydrogen peroxide has recently been reported to have effects that mimic those of insulin,[66] an important component of the serum normally added to the medium of proliferating cells (see Section 2b). For instance, in rat adipocytes addition of hydrogen peroxide leads to an increase in glucose uptake.[67] These manifestations however do not appear to be a direct effect of hydrogen peroxide on the insulin receptor itself but require the participation of non-plasma membrane cellular constituents. The ability of hydrogen peroxide to oxidise critical protein thiol groups may be important in eliciting these regulatory effects.

Hydrogen peroxide has also recently been shown to stimulate adenylate cyclase activity in rat testis Leydig cells (S Sandu, B A Cooke and C Rice-Evans, unpublished observations). In addition experiments with cell-free protein synthesizing systems from HeLa cells show how low levels of hydrogen peroxide to be stimulatory in vitro, (R H Burdon and V Gill, unpublished observations). As mentioned in section 2, whilst cAMP may play a role as a second messenger in mechanisms regulating cell proliferation in response to hormones or prostaglandins, the rates of overall cellular protein synthesis are also likely to be relevant to the levels of possible "trigger" proteins required to proceed past R, the restriction point in G1 of the cell cycle (see Section 2a).

6. CONCLUDING REMARKS

It might be argued that at this early stage in the development of our knowledge regarding free radicals in relation to cell proliferation any concluding remarks may be premature. Indeed in the face of large bodies of evidence for the positive role of the mitogenic pathways involving cAMP or phosphatidyl inositol-Ca^{2+}-diaglycerol systems a similar positive role for free radicals is not discernible. Despite lack of

evidence for a regulatory pathway specifically involving free radicals it is clear that certain free radical-derived species such as 4-hydroxynonenal can have a significant modulatory influence on the activity of vital components of transduction systems such as adenylate cyclase. Another potentially modulatory role for free radical-related species relates to the production of prostaglandins and leukotrienes which themselves modulate cell proliferation. On balance whilst it is unlikely that such effects have sufficient specifity for a truly regulatory role they are nevertheless likely to have considerable significance in specific disease states where radical formation is a notable outcome of the cellular pathology.

On the whole free radicals *per se* appear to have a "down regulatory" effect on cell proliferation, in as much as protection from, or alleviation of, oxidative stress has an enhancing effect on cell proliferation. This can be achieved through exogenous or endogenous antioxidants or through activities of cellular enzymes such as superoxide dismutase and those involved for instance in glutathione-dependent hydrogen peroxide metabolism. A reasonable view might be that protection of components of positive signal transduction pathways from free radical induced damage is likely to have generally beneficial effects on proliferation, rather than being specifically mitogenic. Indeed future chemotherapy might be aimed at exploiting differences between cells in their ability to detoxify free radicals of one sort or another. Certainly the genetic consequences of defects in the genes for these critical enzymes may be of considerable clinical significance.

Although free radicals appear to have a general negative effect on cell proliferation, the positive effects of low levels of hydrogen peroxide itself on fibroblastic or epithelial cells could be significant in tissue repair or wound healing in pathological situations. Clearly the effects of hydrogen peroxide require careful investigation especially if the reported insulinomimetic effects are taken as an indicator of possible mechanisms. Can hydrogen peroxide activate major mitogenic pathways by stimulating integral components such as receptors, G-proteins or adenylate cyclase etc? Whilst such effects may overlap, other targets for hydrogen peroxide may exist at the level of specific gene activation. Hydrogen peroxide is already known to activate the transcription of a group of stress proteins (known as heat shock proteins) but it is not known whether this involves upstream genetic regulatory sequences similar to those required for the activation of the proto-oncogenes c-fos and c-myc during cell cycle progression.

Despite the present limitations to our knowledge, it is clear that free radicals and related species are important considerations in mammalian cell physiology especially in relation to responses elicited in pathological situations. An integrated view of the full significance of free radicals in the context of cell proliferation control will emerge only following detailed study at both the chemical and molecular biological levels.

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