Effects of hydrogen peroxide on the metabolism of human rheumatoid and osteoarthritic synovial fibroblasts in vitro

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Abstract

The effects of hydrogen peroxide (H_2O_2) on the metabolism of cultured human synovial fibroblasts derived from joints of four patients with rheumatoid arthritis and three with osteoarthritis have been investigated. The exposure of rheumatoid cell cultures to this oxygen derived species at sublethal concentrations (1–100 $\mu\text{mol/l}$) induced a dose related inhibition of both hyaluronic acid (HA) and DNA synthesis. In contrast, in osteoarthritic cell lines a biphasic response was shown. At low concentrations of H_2O_2 (<10 $\mu\text{mol/l}$) a stimulatory effect on HA synthesis was noted, whereas in the presence of higher concentrations (>10 $\mu\text{mol/l}$) a significant inhibition of synthesis occurred. These deleterious effects of H_2O_2 were partially reduced by the addition of catalase to the culture media. The finding that both HA and DNA synthesis were inhibited at concentrations of H_2O_2 less than those which caused loss of cell integrity (>200 $\mu\text{mol/l}$) suggests oxidation of intracellular components, such as glyceraldehyde-3-phosphate dehydrogenase, and subsequent depletion of ATP concentrations.

In inflammatory arthritides such as rheumatoid arthritis infiltration of large numbers of polymorphonuclear leucocytes and macrophages into the joint space occurs.¹ When activated, these phagocytic cells are known to undergo a respiratory burst which is accompanied by the generation of highly reactive oxygen species, superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2).² The release by these cells of O_2^- and H_2O_2 can also result in the formation of other metabolites such as hydroxyl radical ($OH^\bullet$), singlet oxygen, and hypochlorous acid.³ The liberation of these oxygen derived reactive species upon stimulation of phagocytes in the synovial cavity by immune complexes, particulate matter—for example, urate or pyrophosphate crystals, cartilage debris—and other inflammatory agents is considered to be responsible for the decrease of synovial fluid viscosity owing to depolymerisation of hyaluronic acid (HA).^{4–10}

Additionally, free radicals within inflamed joints may be generated from other sources, including the prostaglandin cascade and the autoxidation of small molecules such as flavoproteins, hydroquinones, pyrogallols, etc.¹¹ Even though it has been realised that the production of oxygen radicals during inflammation can be limited by oxygen concentration¹² and that inflammatory joints generally have

lower oxygen tensions than normal joints,¹³ there is considerable indirect and direct evidence showing that oxidant scavenging activity in rheumatoid synovial fluid is low and is not sufficient to control damage in the joint mediated by reactive oxygen species.^{14–17}

Furthermore, in vitro studies have shown the ability of the reactive oxygen metabolites (O_2^- and $OH^\bullet$ radicals) generated by different radical producing systems to degrade a number of extracellular matrix components, including collagen¹⁸ and proteoglycans.^{18–22} More recently, H_2O_2 has been shown to depolymerise the proteoglycan aggregates of human articular cartilage in vitro.²³ It has been suggested that this aggregate disruption may be attributed to degradation of HA and chemical modification of link proteins which are necessary for stabilisation of proteoglycan aggregates.²⁴

In addition to its ability to mediate fragmentation of proteoglycans, H_2O_2 has been shown to inhibit in a concentration dependent manner in vitro proteoglycan and HA biosynthesis by bovine and murine articular cartilage chondrocytes.^{25–27} Exposure of endothelial cells,²⁸ skin fibroblasts,³⁰ epithelial cells,³² hepatocytes,³³ and tumour cells³⁴ to H_2O_2 generating systems has also been reported to cause permanent cellular injuries. It has been proposed that H_2O_2 penetrates the cell membrane, inducing degradation of DNA³⁵ and suppression of ATP production.³⁷

In inflamed synovial joints fibroblasts of the synovial lining (type B cell) are exposed to H_2O_2 liberated from stimulated phagocytes,³⁹ but the effects that this oxidant might have on the metabolism of these cells had not been reported. We therefore undertook this study to consider that question.

The results obtained indicate that the metabolism of synovial fibroblasts derived from both rheumatoid and osteoarthritic knee joints can be modified by H_2O_2 ; the effects produced depending on the concentration of H_2O_2 to which these cells are exposed.

Materials and methods

MATERIALS

Dulbecco's modified Eagle's medium and trypsin (from porcine pancreas (EC 3.4.21.4)) were products of Gibco Laboratories, Grand Island, NY, USA. Collagenase type I (from *Clostridium histolyticum* (EC 3.4.24.3)), diethylenetriaminepenta-acetic acid, and catalase (from bovine liver (EC 1.11.1.6)) were purchased from Sigma Chemical Co, St Louis, MO, USA. Fetal calf serum was obtained from

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Flow Laboratories (Australia) Pty Ltd, Seven Hills, NSW, Australia. Gentamicin was from David Bull Laboratories Pty Ltd, Mulgrave, Victoria, Australia. The [^3H]acetic acid, sodium salt (specific activity 74–185 GBq/mmol), [methyl- ^3H]thymidine (specific activity 185 GBq/mmol) were purchased from Amersham Australia Pty Ltd, North Ryde, NSW, Australia. Hyaluronidase (from *Streptomyces hyalurolyticus* (EC 4.2.99.1)) and peroxidase (from horseradish roots (EC 1.11.1.7)) were supplied from Miles Laboratories Australia Pty Ltd, Epping, NSW, Australia. Superoxide dismutase (Palosein (EC 1.15.1.1)) was purchased from Intervet (Australia) Pty Ltd, Artarmon, NSW, Australia. Diphenylamine was manufactured by Ajax Chemicals, Auburn, NSW, Australia. Hydrogen peroxide (pure medicinal quality) was from Pacific Mfg Co Pty Ltd, Balmain, NSW, Australia and phenol red from Alpha Chemicals (Australia) Pty Ltd, Dee Why, NSW, Australia. Sepharose CL-2B together with all chromatography instruments were supplied by Pharmacia (Australia) Pty Ltd, North Ryde, NSW, Australia. All other reagents were of analytical grade and double distilled water was used in all buffer and media preparations.

SOURCES OF SYNOVIAL TISSUE

Synovial tissue from osteoarthritic and rheumatoid arthritic knee joints was provided by Dr David Sonnabend and Dr Nigel Hope, from patients undergoing total joint replacement surgery at the Royal North Shore Hospital of Sydney or the North Sydney Community Hospital. The classification of the synovium as being derived from osteoarthritic or rheumatoid joints was determined by the attending surgeons using both clinical and pathological methods of assessment. Altogether seven cell lines (four rheumatoid, three osteoarthritic) were used in this study.

SYNOVIAL FIBROBLAST CULTURE METHOD

Synovial fibroblasts were prepared from the tissues described above by the Smith and Ghosh modification⁴⁰ of the Dayer *et al* procedure.⁴¹ Briefly, explants of the synovial membrane were washed three times and finely diced in sterile Dulbecco's calcium and magnesium free phosphate buffered saline (CMF-PBS). Tissues were then placed in Dulbecco's modified Eagle's medium containing 23 mM sodium bicarbonate, 40 $\mu\text{g}/\text{ml}$ gentamicin, and 4 mg/ml collagenase for three to four hours at 37°C with occasional mixing. After the incubation, an equal volume of 0.05% (w/v) trypsin and 0.02% (w/v) ethylenediaminetetra-acetic acid (EDTA) in CMF-PBS was added. Incubation was continued for one hour under the same conditions as above. The suspension was then centrifuged for five minutes at 400 g. The supernatant was aspirated and the cell pellet washed three times in Dulbecco's modified Eagle's medium containing 23 mM sodium bicarbonate, 40 $\mu\text{g}/\text{ml}$ gentamicin, and 10% (v/v) heat inactivated fetal calf serum, and then plated in 75 cm² culture

flasks (Lux; Miles Laboratories). The fibroblasts were grown and maintained in a monolayer culture in the latter previously described medium, at 37°C in a humidified atmosphere of 95% (v/v) air and 5% (v/v) carbon dioxide. The medium was changed every two to three days and the cells were subcultured on reaching confluency.

HYALURONIC ACID SYNTHESIS

Cells, passaged five to eight times, were seeded into 2 cm² wells (24 wells/plate) (Linbro; Flow Laboratories) at a density of 30 000 cells/well. Once they reached 70% confluency (three to four days later depending on the cell line, at which the rate of HA synthesis was found to be greatest⁴²) the medium was replaced with 0.4 ml of medium containing 925 kBq/ml [^3H]acetate in the presence and absence of test substances. The cells were further incubated under standard conditions for 24 hours. The medium was then removed from each well and the cell layer was washed twice with 0.3 ml of CMF-PBS. The washings were combined with the media and assayed for radiolabelled HA by the method described by Smith and Ghosh.⁴⁰ In this assay the media and washings from each culture well were dialysed against 0.1 M sodium acetate pH 5.6 at 4°C over two days with four changes. After dialysis each sample was made up to 2 ml and then 2 $\times$ 0.8 ml aliquots were taken. To one set of these aliquots was added five turbidity reducing units (TRU) (one TRU = the amount of enzyme which causes a 50% decrease in optical density at 660 nm in 30 minutes at 60°C) of *Streptomyces hyaluronidase*. Aliquots, both with and without enzyme were incubated at 60°C for three hours. After incubation the samples were dialysed as previously. The redialysed sample volumes were then made up to 2 ml, and a 0.5 ml aliquot was counted for radioactivity.

The amount of newly synthesised HA was calculated as disintegrations per minute (dpm) of the blank sample minus the dpm of the hyaluronidase treated sample. This value was corrected for cell number or DNA content to eliminate a potential source of variation between the wells. Results were expressed as a percentage in HA production when compared with the control (no test substances) cultures.

The measurement of DNA in samples was determined by trypsinising cells with 0.1% (w/v) trypsin and 0.02% (w/v) EDTA in CMF-PBS, then solubilising the DNA in 0.5 M perchloric acid, followed by measuring DNA concentration with the diphenylamine reagent as described by Burton.⁴³

DNA SYNTHESIS

Cells were plated in 0.38 cm² wells (96 wells/plate) (Linbro; Flow Laboratories) at a density of 15 000 cells/well. After 24 hours' incubation in 0.2 ml Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum the culture medium was changed and cells were further incubated under the same culture conditions but in media containing 185 kBq/ml [^3H]-

thymidine in the presence and absence of test substances for 24 hours. The media were then discarded and the cell layers washed twice with CMF-PBS, and released with 0.2% (w/v) trypsin, 0.1% (w/v) EDTA in CMF-PBS. The cells were lysed with distilled water and the lysates filtered through glass fibre filters (Titertek; Flow Laboratories). The filters were then air dried, and the radioactivity was determined by scintillation spectrophotometry.

Results were expressed as a percentage in [3H]thymidine uptake when compared with the control (no test substances) samples.

GEL EXCLUSION CHROMATOGRAPHY

Tissue culture media (1 ml) directly obtained from the [3H]HA synthesis experiments were chromatographed on a Sepharose CL-2B column (1.5×90 cm) equilibrated with 0.5 M sodium acetate pH 6.8 at 4°C, eluting at 12 ml/h. The column was calibrated with highly purified HA of molecular weight 3.8×10^6 (Healon; Pharmacia Leo Therapeutics AB, Uppsala, Sweden) and [3H]acetate to mark the void volume and total volume respectively. Fractions of 2 ml were collected and monitored for radioactivity.

CELL INTEGRITY

Cell membrane integrity of the treated fibroblasts was measured by the trypan blue exclusion assay. An equal volume of 0.1% (w/v) trypan blue in CMF-PBS was added to a suitable cell suspension and the number of intact and damaged—that is, stained—cells was counted with a haemocytometer. The number of intact cells was expressed as a percentage of the total number of cells.

DETERMINATION OF HYDROGEN PEROXIDE (H_2O_2)

The concentration of stock H_2O_2 solutions was determined by 0.1 M potassium permanganate titration,⁴⁴ and the concentration of H_2O_2 remaining in the culture medium at various times during incubation with synovial cells was determined by the method used on the peroxidase dependent oxidation of phenol red by H_2O_2 ,⁴⁵ described by Pick.⁴⁶ The assay was performed in a microtitre plate with a final concentration of phenol red of 0.56 mmol/l and of peroxidase 20 units/ml in each well. (One unit = the amount of enzyme which catalyses the conversion of 1 μ mol peroxide per minute at 25°C.) Absorbances were read at 620 nm instead of 600 nm as originally described.

STATISTICAL METHODS

The data generated on HA and DNA synthesis in the various cell lines, for each treatment group (three and six replicates in each group on HA and DNA synthesis respectively), were analysed by an unpaired Student's *t* test. The null hypothesis was that test substances exerted no effects on HA or DNA synthesis with the control group for comparison. An effect was considered to occur when the difference

between the two groups corresponded to $p \leq 0.05$.

Results

EFFECTS OF HYDROGEN PEROXIDE (H_2O_2) ON HA SYNTHESIS

Figure 1 shows the effects of different concentrations of H_2O_2 on the incorporation of [3H]acetate into newly synthesised HA after incubation with the human synovial fibroblast cultures for 24 hours. Fibroblasts derived from osteoarthritic joints showed a biphasic response (fig 1A). Hydrogen peroxide concentrations of 10 μ mol/l or less produced stimulation of HA synthesis in these cells, the maximum being 123% above the control value at 2 μ mol/l. The increased biosynthesis of radiolabelled HA at 5 μ mol/l H_2O_2 could also be shown by Sepharose CL-2B chromatography of the media from the osteoarthritic cells. A higher proportion of high molecular weight 3H labelled HA (confirmed by its susceptibility to *Streptomyces* hyaluronidase digestion) eluted in the void volume fraction than in media from the control cultures (fig 2). At concentrations above 10 μ mol/l, however, H_2O_2 inhibited HA synthesis in the osteoarthritic cell line in a concentration dependent manner (fig 1A). Sepharose CL-2B chromatography of the media from the 20 μ mol/l cultures confirmed the low incorporation of radioactivity into the high molecular weight HA fraction (fig 2).

In contrast with the osteoarthritic cell lines, no stimulation of HA synthesis by H_2O_2 was

Figure 1 Effects of various concentrations of hydrogen peroxide on the synthesis of [3H]hyaluronic acid by synovial fibroblasts derived from (A) osteoarthritic and (B) rheumatoid joints. Each column represents the mean (SEM) of three determinations. Columns marked with asterisks were significantly different from controls: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 2 Sepharose CL-2B chromatograms of culture media obtained after 24 hours' incubation of synovial fibroblasts obtained from osteoarthritic joints with [^3H]acetate alone (—+—) and in the presence of H_2O_2 at concentrations of 20 $\mu\text{mol/l}$ (—■—), 5 $\mu\text{mol/l}$ (—●—), and 1 $\mu\text{mol/l}$ before (—○—) and after (---) incubation with 5 TRU *Streptomyces hyaluronidase* at 60°C for three hours.

found in fibroblasts derived from rheumatoid arthritic joints over the entire concentration range tested (1–200 $\mu\text{mol/l}$) (fig 1B). Decreased synthesis occurred in the presence of H_2O_2 at concentrations as low as 1 $\mu\text{mol/l}$, with maximum inhibition being found at 100 $\mu\text{mol/l}$ (fig 1B).

The inhibitory effect of H_2O_2 on HA synthesis in the rheumatoid synoviocyte lines was, however, reduced by inclusion of 250 units catalase in the cultures (fig 3). One unit of catalase corresponds to the amount of enzyme which decomposes 1.0 μmol of H_2O_2 per minute at pH 7.0. This activity was confirmed before use. Significantly, the addition of 250 units of superoxide dismutase (one unit=the

Figure 3 Effects of 250 units of superoxide dismutase (—●—), 250 units of catalase (---■---), or 5 mM diethylenetriaminepenta-acetic acid (...▲...) on the H_2O_2 inhibition of hyaluronic acid synthesis in cultured rheumatoid arthritic synovial fibroblasts compared with controls (—○—). Each point represents the mean (SEM) of three determinations. Catalase showed significant stimulation of hyaluronic acid synthesis ($***p \leq 0.001$) relative to the respective controls containing H_2O_2 alone at concentrations of 100 and 200 $\mu\text{mol/l}$, whereas diethylenetriaminepenta-acetic acid exacerbated the inhibitory effect of H_2O_2 .

amount of enzyme that would cause a 50% inhibition in the rate of reduction of cytochrome *c* at 25°C, pH 7.8) had no effect on the action of this oxidant on HA biosynthesis. The activity of the superoxide dismutase preparation was shown by the ferricytochrome *c* reduction method.⁴⁷ In contrast, 5 mM diethylenetriaminepenta-acetic acid seemed to potentiate the H_2O_2 inhibition of HA synthesis by these cells (fig 3).

EFFECT OF H_2O_2 ON DNA SYNTHESIS

Figure 4 shows the effect of various concentrations of H_2O_2 on DNA synthesis by synovial fibroblast lines derived from rheumatoid and osteoarthritic joints. When [^3H]thymidine incorporation was used as an index of DNA synthesis it was found that addition of H_2O_2 at concentrations of 2 $\mu\text{mol/l}$ and above to the rheumatoid synoviocytes depressed the synthesis of this macromolecule (fig 4B). At concentrations of H_2O_2 100 $\mu\text{mol/l}$ and above the synthesis of DNA in this cell line was almost completely inhibited. In the osteoarthritic cells, however, H_2O_2 inhibition of DNA synthesis was only evident at higher concentrations (10 $\mu\text{mol/l}$ and above), 70 and 80% inhibition occurring at 100 and 200 $\mu\text{mol/l}$ of H_2O_2 respectively (fig 4A).

No effect on DNA synthesis was observed when 250 units of superoxide dismutase were added to the rheumatoid synoviocyte cultures

Figure 4 Effect of various concentrations of hydrogen peroxide on [^3H]thymidine incorporation by (A) osteoarthritic and (B) rheumatoid arthritic fibroblast lines. Each column represents the mean (SD) of six determinations. Columns marked with asterisks were significantly different from controls: * $p \leq 0.05$; ** $p < 0.01$; *** $p \leq 0.001$.

Figure 5 Effects of 250 units of superoxide dismutase (—●—) or 250 units of catalase (—■—) on the H_2O_2 inhibition of DNA synthesis in a rheumatoid arthritic cell line compared with controls (—○—). Superoxide dismutase had no effect on inhibition, but catalase showed significant stimulation at 50 and 100 $\mu\text{mol/l}$ H_2O_2 compared with their corresponding controls: *** $p \leq 0.001$; * $p \leq 0.05$. Each point represents the mean (SD) of six determinations.

together with H_2O_2 (fig 5). On the other hand, 250 units of catalase afforded some protection, but only in the presence of 50 $\mu\text{mol/l}$ H_2O_2 . Diethylenetriaminepenta-acetic acid at a concentration of 1 mmol/l exacerbated the inhibitory effect of H_2O_2 on DNA synthesis at all concentrations (fig 6). At 5 mmol/l it caused complete inhibition of DNA synthesis even in the absence of H_2O_2 (data not shown).

EFFECT OF H_2O_2 ON THE INTEGRITY OF HUMAN SYNOVIAL FIBROBLASTS

To determine if the inhibition of HA and DNA synthesis produced by H_2O_2 in these cell lines was solely due to loss of cell integrity the trypan blue exclusion assay was used. The percentage of intact cells remaining after exposure to various concentrations of H_2O_2 was determined in the absence and presence of superoxide dismutase, catalase, and diethylenetriaminepenta-acetic acid. In the presence of H_2O_2 alone, up to a concentration of 20 $\mu\text{mol/l}$, the

Figure 6 Effects of no diethylenetriaminepenta-acetic acid (—○—), and 1 mmol/l (---▲---) and 5 mmol/l (...▲...) concentrations on the H_2O_2 inhibition of DNA synthesis in fibroblasts from rheumatoid arthritic joints. All cultures containing diethylenetriaminepenta-acetic acid show significant inhibition compared with controls that contained H_2O_2 alone, except at 50 $\mu\text{mol/l}$ H_2O_2 (NS), which was not significant ($p > 0.05$). * $p < 0.05$.

morphology of the rheumatoid synovial fibroblasts was not affected and their integrity remained at control level (fig 7). Above this concentration, however, cell viability declined significantly, complete cell staining occurring at 200 $\mu\text{mol/l}$. When 5 mM diethylenetriaminepenta-acetic acid was added to the medium at H_2O_2 concentrations as low as 1 $\mu\text{mol/l}$ there seemed to be an increased toxicity compared with cultures exposed H_2O_2 alone. The addition of 250 units of superoxide dismutase to the media failed to provide protection against loss of cell integrity produced by H_2O_2 at all concentrations tested. On the other hand, addition of the same amount of catalase clearly prevented cell damage induced by H_2O_2 up to the maximum concentration used (fig 7).

STABILITY OF H_2O_2 IN CELL CULTURES

Figure 8 shows the change in H_2O_2 concentration of the incubation media over a two hour period after the addition of freshly prepared 100 $\mu\text{mol/l}$ H_2O_2 to cultures in the absence and presence of osteoarthritic synovial fibroblasts.

Figure 7 Effects of 250 units superoxide dismutase (—●—), 250 units catalase (—■—), and 5 mM diethylenetriaminepenta-acetic acid (...▲...) on the H_2O_2 induced loss of cell integrity assessed by trypan blue exclusion compared with controls (—○—). Data are expressed as percentage of intact cells compared with the total cell number.

Figure 8 Kinetics of H_2O_2 elimination from the synovial cell culture media. After the addition of 100 μM H_2O_2 the concentrations of H_2O_2 in the media in the presence (■) and absence (□) of osteoarthritic synovial cells were determined at intervals. The ordinate depicts the mean of duplicate values (SD). No error bars are large enough in relation to the size of the symbol used in the diagram to be illustrated. The insert shows a linear semilogarithmic plot of the data.

The concentration of H_2O_2 declined exponentially as a function of incubation time. The rate of change of H_2O_2 concentration in the media alone and in the presence of cells was determined by calculating the half lives ($t_{1/2}$). In media alone $t_{1/2}$ was 45 minutes (see inset of fig 8), whereas in the presence of cells a more rapid decrease was observed with a half life of only 30 minutes. From these differences it was estimated that about 33 $\mu\text{mol/l}$ of H_2O_2 was removed by the osteoarthritic synovial fibroblasts from a solution containing initially 100 $\mu\text{mol/l}$ H_2O_2 over a 24 hour culture period.

Discussion

In these studies we showed that exposure of human synovial fibroblasts derived from both rheumatoid arthritic and osteoarthritic joints to H_2O_2 causes alterations in their biosynthetic behaviour. This is in agreement with the observed effects of this oxygen derived species on other tissues and cells.^{25-27 48 49} The addition of both the O_2^- scavenger—superoxide dismutase—and diethylenetriaminepenta-acetic acid, which is a specific chelator of iron that inhibits Fe^{2+} dependent conversion of H_2O_2 into the highly reactive $\text{OH}^\bullet$ radical,⁵⁰ failed to reverse the inhibitory effects of H_2O_2 in the cellular system studied. This indicates that the effects noted were mediated by H_2O_2 . If diethylenetriaminepenta-acetic acid penetrated the cell plasmalemma, however, it might mobilise intracellular non-protein bound divalent iron, thereby preventing Fe^{2+} dependent formation of hydroxyl radical in situ as has been noted with desferrioxamine.⁵¹ Further support for the role of H_2O_2 as a mediator was provided by the observation that catalase generally afforded some protection to fibroblast biosynthesis of HA and DNA and consistently prevented the loss of cell integrity as shown by the trypan blue exclusion assay (fig 7). It is noteworthy that diethylenetriaminepenta-acetic acid at the concentration used in our studies (5 mmol/l), which is one tenth the concentration used by Bates *et al*^{25 26} in their cartilage studies, was found not only to be ineffective in abrogating the effects of H_2O_2 but exacerbated the action of this metabolite on synovial cells. Diethylenetriaminepenta-acetic acid when used alone significantly inhibited both DNA and HA synthesis, whereas superoxide dismutase or catalase had no effect or only slightly increased the synthesis of these macromolecules. The reason for these selective effects are presently unclear.

The mechanism(s) by which sublethal concentrations of H_2O_2 (1–100 $\mu\text{mol/l}$) inhibit HA synthesis in synovial cells is presently unknown. It is clear from the data obtained (fig 7), however, that neither cell viability nor alteration in the permeability of the cell membrane, as assessed by trypan blue exclusion assay, were compromised by the oxidative potential generated in vitro by concentrations of 20 $\mu\text{mol/l}$ H_2O_2 or less. This suggests that the effects produced at low concentrations of H_2O_2 are mediated by damage to intracellular components rather than through non-specific cell toxicity.

Baker and colleagues, using cartilage explant cultures, have suggested that H_2O_2 acts by oxidising the thiol (-SH) residue at the active site of glyceraldehyde-3-phosphate dehydrogenase.³⁸ Inactivation of glyceraldehyde-3-phosphate dehydrogenase leads to a reduction in the rate of glycolytic ATP synthesis and subsequent depletion of intracellular ATP concentrations, resulting in a suppression of DNA, protein, proteoglycan, and HA synthesis. It is reasonable to assume that a similar oxidative 'lesion' is produced by H_2O_2 within synovial fibroblasts. As this H_2O_2 induced damage occurs intracellularly it might explain why catalase was not very effective in modulating H_2O_2 activity at low concentrations. Catalase is a large protein (mol wt 232 000) and this may restrict its entry into the cells and thereby reduce its ability to interact with intracellular H_2O_2 .

The mechanism(s) by which H_2O_2 at low concentrations stimulated HA synthesis in the osteoarthritic cell line (fig 1A) is still obscure. Although it is reported that thymidine incorporation by normal human dermal fibroblasts is stimulated by H_2O_2 at concentrations of 1 $\mu\text{mol/l}$,⁴⁹ this did not occur in any of our osteoarthritic or rheumatoid cell lines. As the rheumatoid synovial fibroblasts (and possibly some osteoarthritic lines) were derived from joints which had been previously exposed to oxygen derived reactive species and H_2O_2 it is possible that the intracellular activity of catalase and glutathione peroxidase, both of which serve to protect the cell,³ was exhausted. In the normal dermal fibroblast cell lines, however, sufficient amounts of these protective enzymes were present to inactivate the intracellular H_2O_2 at least at low concentrations. Possibly, cell mitosis is stimulated by a feedback mechanism which is activated when intracellular catalase or glutathione peroxidase activities, or both, are reduced by interaction with H_2O_2 . Should the intracellular concentrations of H_2O_2 exceed the antioxidant concentrations present, however, oxidation of sulphhydryl dependent enzymes essential for respiration—for example, glyceraldehyde-3-phosphate dehydrogenase—will occur and inhibition of metabolic activities will result. In the absence of experimental data this explanation must remain highly speculative.

Although anaerobic metabolism is well documented in rheumatoid synovial tissues,^{52 53} it is unlikely that these conditions would significantly alter the outcome of the present investigations as it has been shown that under aerobic and anaerobic conditions the Embden-Meyerhof glycolysis pathway remains the major energy source for these tissues.⁵²

Our finding that 200 $\mu\text{mol/l}$ of H_2O_2 was lethal to synovial cells was consistent with data reported for other cell types.^{29 33} It is noteworthy that this order of concentration of H_2O_2 can be generated in vitro in only 10 minutes by 10^6 activated human neutrophils.⁵⁴ In the inflamed joint 10^9 neutrophils may accumulate in the synovial cavity over a 24 hour period.¹¹ When stimulated, these cells might generate sufficient H_2O_2 to cause lethal damage to synovial fibroblasts in vivo. With fewer neutrophils, or reduced stimulation, non-lethal con-

centrations of H₂O₂ could be readily achieved within synovial joints causing intracellular damage—for example, ATP depletion or DNA breakage.⁵⁵

The clearance rate of 100 µmol/l H₂O₂ from the culture media in the presence of either rheumatoid (data not shown) or osteoarthritic synovial cells (the calculated half life (t_{1/2}) being 33 and 30 minutes, respectively) is in agreement with studies conducted previously using cultures of articular cartilage.^{27–56} The finding that H₂O₂ was depleted from the media more quickly in the presence of cells than in media alone (fig 8) strongly suggests that H₂O₂ was taken up and used by these cells. It is noteworthy, however, that the effects produced by H₂O₂ persisted beyond its elimination from the media (≤2 hours) because 24 hours after incubation with synovial cells (when most of the extracellular H₂O₂ had been lost) the suppressive effects on cell metabolism were still evident. These observations are in agreement with those of Bates *et al*, who found prolonged inhibition of proteoglycan synthesis (five days) after two hours' exposure of bovine articular cartilage to this oxidant *in vitro*.²⁵ In this connection Baker and coworkers have suggested that H₂O₂ mediated inhibition of cellular biosynthesis (HA, proteoglycan, DNA, and protein) arises from the disruption of a basal metabolic process which is required for each of these synthetic pathways to operate.³⁸ Accordingly, it would not be surprising if some time elapses before the effects can be observed and for cells to completely regain their ability to synthesise these macromolecules.

Notwithstanding the obvious limitations associated with extrapolation of *in vitro* findings to the human clinical situation we suggest that H₂O₂ generated by activated inflammatory cells or from other sources within the inflamed joint may modify synovial fibroblast metabolism, particularly the biosynthesis of HA. As HA represents an essential component of synovial fluid and is responsible for its unique rheological^{57–58} and lubricating properties,^{59–60} a decline in its synthesis must be considered to be contributory to the progression of the arthritic process.

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