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# Hydroxyl Radical Formation in Chondrocytes and Cartilage as Detected by Electron Paramagnetic Resonance Spectroscopy Using Spin Trapping Reagents

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Chondrocytes have been shown to produce superoxide and hydrogen peroxide, suggesting possible formation of hydroxyl radical in these cells. In this study, we used electron spin resonance/spin trapping technique to detect hydroxyl radicals in chondrocytes. We found that hydroxyl radicals could be detected as α-hydroxyethyl spin trapped adduct of 4-pyridyl 1-oxide N-tert-butylnitrone (4-POBN) in chondrocytes stimulated with phorbol 12-myristate 13-acetate in the presence of ferrous ion. The formation of hydroxyl radical appears to be mediated by the transition metal-catalyzed Haber-Weiss reaction since no hydroxyl radical was detected in the absence of exogenous iron. The hydroxyl radical formation was inhibited by catalase but not by superoxide dismutase, suggesting that the hydrogen peroxide is the precursor. Cytokines, IL-1 and TNF enhanced the hydroxyl radical formation in phorbol 12-myristate 13-acetate treated chondrocytes. Interestingly, hydroxyl radical could be detected in unstimulated fresh human and rabbit cartilage tissue pieces in the presence of iron. These results suggest that the formation of hydroxyl radical in cartilage could play a role in cartilage matrix degradation.

*Keywords:* Chondrocytes, cartilage, oxygen intermediates, hydroxyl radicals, electron spin resonance, arthritis

## INTRODUCTION

The pathogenesis of arthritis, which involves the aging process in osteoarthritis and inflammation in rheumatoid arthritis, results in a selective and irreversible degradation of bone and cartilage matrix.[1] The underlying mechanism of cartilage matrix degradation in arthritis is poorly understood but the matrix metalloproteinases and the reactive oxygen intermediates are implicated as the main causative factors.[2,3] Studies have shown that matrix metalloproteinases degrade collagen and proteoglycan matrix components of cartilage.[4] In contrast, Halliwell and others have

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suggested that reactive oxygen intermediates (ROI) derived from the phagocytic cells that infiltrate diseased joints may contribute significantly to matrix degradation process in arthritis.[3,5,6] Chondrocytes are the only cell types in cartilage and the possible contribution of chondrocyte-derived ROI in degradation of cartilage matrix has not received adequate attention.[7] We have previously reported that chondrocytes do secrete large quantities of hydrogen peroxide.[8] Other reports have since confirmed that chondrocytes indeed produce superoxide anion and hydrogen peroxide.[9,10] Since chondrocytes are the primary source of pathology in osteoarthritis, we speculate that free radicals inside the cartilage tissue could eventually lead to matrix degradation.

The production of reactive oxygen species in chondrocytes exhibits NADPH-oxidase dependent-burst-like process.[8,11,12,13] Using the electron paramagnetic resonance/spin trapping system, we have detected hydroxyl radical in phorbol ester-stimulated human and rabbit articular chondrocytes. Moreover, we detected hydroxyl radical in intact cartilage tissue. The significance of these findings is discussed.

## MATERIALS AND METHODS

### Reagents

Diethylenetriaminepentaacetic acid (DTPA), phorbol 12-myristate 13-acetate (PMA), superoxide dismutase, catalase, 4-pyridyl 1-oxide-*N*-tert-butylnitrone (4-POBN) were purchased from Sigma Chem. Co. (St. Louis, MO). Pure ethyl alcohol (U. S. P.) was from Quantum Chemical Corporation, Tuscola, IL. Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), phenol-free Hank's balanced salt solution (HBSS), L glutamine, gentamicin, HEPES buffer, penicillin, and streptomycin were purchased from GIBCO (Grand Island, NY). Recombinant human tumor necrosis factor-α (rhTNF-α) was a gift from Dr. M. Shephard, Genentech, Inc. (San Francisco, CA). Recombinant human interleukin-1α (rhIL-1α) was a gift from Drs. M. Stern and P. Lomedico, Hoffman-LaRoche (Nutley, NJ).

### Isolation of Rabbit Articular Chondrocytes

NZW rabbits of either sex (6 to 8 pounds) were killed by i.v. injection of Beuthanasia-D special (Schering Corp., Kenilworth, NJ). The chondrocytes were prepared as previously described.[8] The chondrocytes comprised a homogeneous population of cells, showing more than 98% viability as measured by trypan blue exclusion. Primary chondrocytes were resuspended in phenol-free Hank's balanced salt solution at $1–5\times10^6$ cell per ml.

### Culturing of Chondrocytes

The articular chondrocytes were suspended in DMEM containing 10% FBS and cultured at high cell density ($1\times10^6$/ml). The plates were incubated at 37°C with 5% $CO_2$/95% air humidified incubator. The media was changed every 5 to 7 days.

### Cytokine Treatment of Chondrocytes

Primary confluent articular chondrocytes in petri dishes (60 mm) were cultured in 10% FBS-DMEM in the presence or absence of cytokines for 24 hours. The cells were exposed to 100 U/ml of rhIl-1α and 100 ng/ml of rhTNF-α. Cells were washed, treated with PMA (100 ng/ml) for 45 minutes before electron paramagnetic resonance (EPR) spectroscopic measurement.

### Articular Cartilage

Rabbit articular cartilage was collected from ends of long bones and cut aseptically into small 2 mm×2 mm pieces. Fresh human articular cartilage tissue was obtained from patients with osteoarthritis undergoing arthroplasty and cut

into small pieces and used. Fresh human and rabbit cartilage tissue pieces were used for EPR spectroscopic measurement.

### Human Chondrocytes and Cartilage Samples

Cartilage samples from patients undergoing arthroplasty were collected and immediately transported to the laboratory. The human studies have been approved by Institutional Review Board. Small pieces of cartilage tissue were digested overnight by enzyme solution (8) and single cell suspensions were obtained. Chondrocytes were propagated at high cell density ($1\times10^6$/ml) conditions in 10% FBS-DMEM and split 1:3 after achieving confluence.

### Synovial Fluids

Joint fluids were collected from patients undergoing diagnostic or therapeutic arthrocentesis. The joint fluid was centrifuged to remove cells and cellular debris. The supernatant was stored in aliquots at −20°C. The samples were thawed and tested for supporting the formation of hydroxyl radical by PMA-stimulated rabbit articular chondrocytes. Four samples were from patients with rheumatoid arthritis, two samples were from osteoarthritic patients and one sample each was obtained from crystal-, calcium pyrophosphate- or urate-induced acute arthritis.

### EPR/Spin Trapping

Spin trapping reaction mixtures consisted of cells ($1–5\times10^6$/ml in HBSS), 4-POBN (10 mM), ethanol (170 mM), DTPA (0.1 mM) with or without PMA (100 ng/ml) and with or without ferrous sulfate (0.1 mM) and sufficient HBSS in a final volume of 0.2–0.5 ml (14). A 50–100 ul of the reaction mixture was transferred to quartz capillary tube. The tube was placed in the cavity of Varian E-12 EPR spectrometer (Varian Associates, Palo Alto, CA). The spectrum was recorded at 25°C. Instrument settings are given in the legends of each figure.

## RESULTS

### Hydroxyl Radical Formation by Chondrocytes as Detected by 4-POBN/ethanol

Figure 1 shows that an EPR signal appeared in PMA-treated chondrocytes, but not in the unstimulated cells, in presence of exogenous ferrous sulfate (0.1 mM) (spectra C vs B). The triplet nitroxide signal gives hyperfine splitting constants ($A_N$=15.6 G, $A_H$=2.4 G) identical to that reported for α-hydroxyethyl spin adduct of 4-POBN, 4-POBN-CH($CH_3$)OH[14]. Control experiments also showed that the formation of EPR signals depends on the presence of exogenously-added ethanol (data not shown) similar to that reported for neutrophils and monocytes.[14] Taken together, the results strongly suggest that hydroxyl radical was generated in the stimulated chondrocytes in the presence of ferrous ion, most likely via the Haber-Weiss type reaction (Spectra A vs B). The formation of α-hydroxyethyl spin adduct of 4-POBN by PMA-stimulated chondrocytes could be abolished in the presence of catalase (Spectra E) but not by superoxide dismutase (Spectra D), indicating that indeed hydrogen peroxide, not superoxide, is the immediate precursor of hydroxyl radical. This notion is consistent with findings that ferrous ion is required for generation of the EPR signals.

The signal intensity shown in Figure 1 reached its maximal value in chondrocytes 45–60 minutes after PMA stimulation, indicating a respiratory burst-like characteristic. The signal persisted up to 2 hours after chondrocyte stimulation (data not shown).

### Hydroxyl Radical Formation by Human Osteoarthritic Chondrocytes

In contrast to normal rabbit chondrocytes we found that human osteoarthritic chondrocytes could generate significant amounts of α-hydroxyethyl adduct of 4-POBN without prior PMA stimulation (Figure 2). Addition of PMA (100 ng/ml),

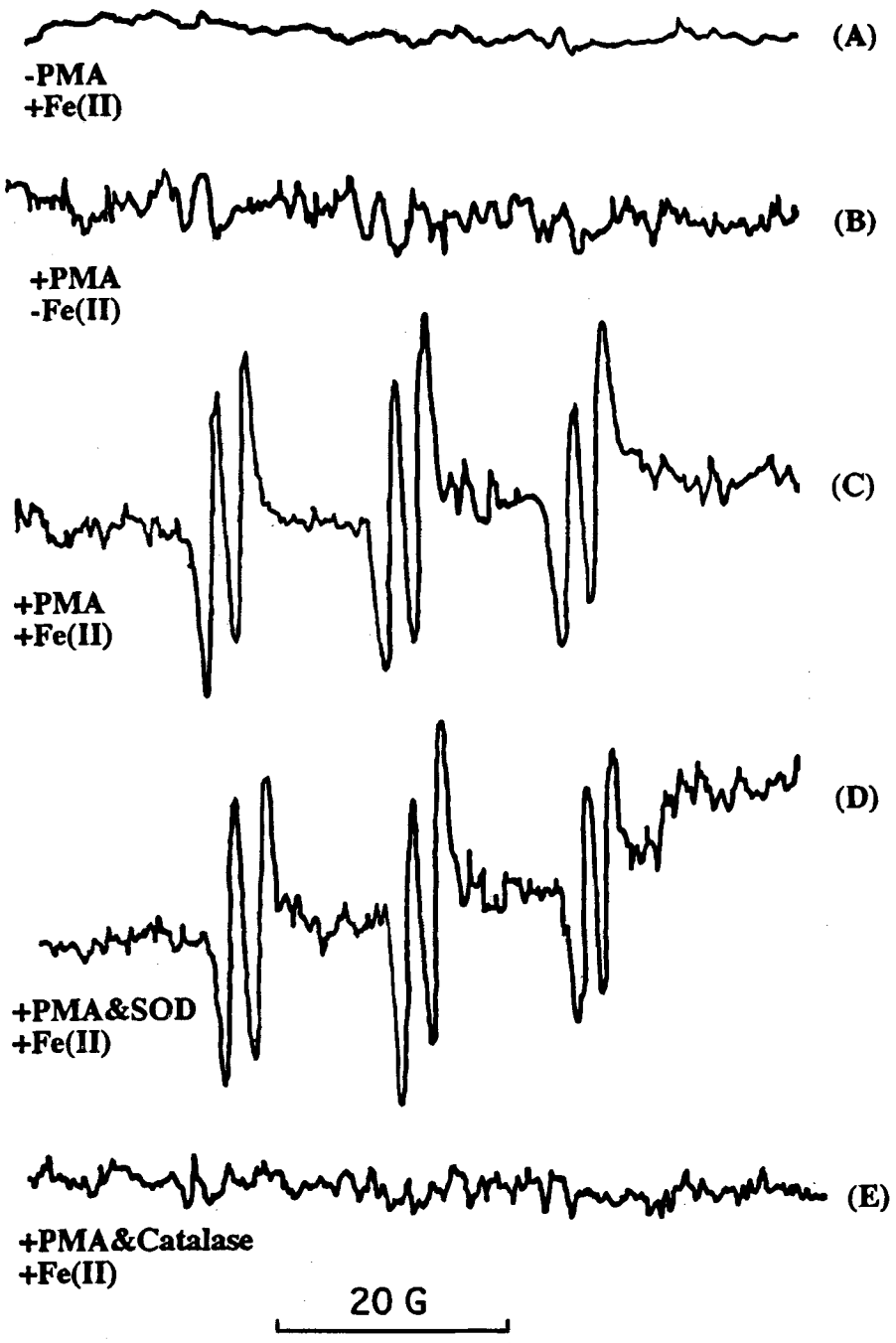



FIGURE 1 Hydroxyl radical formation by chondrocytes and effect of antioxidant enzymes. EPR spectrum of articular chondrocytes ($2\times10^6$) in spin trapping reaction mixture of 4-POBN (10 mM), DTPA (0.1 mM) and ethanol (170 mM) in HBSS in presence or absence of exogenous ferrous sulfate (0.1 mM) 1.5 hours after addition of PMA (100 ng/ml). A, unstimulated chondrocytes in the presence of iron. B, PMA-(100 ng/ml) stimulated chondrocytes in the absence of iron. C, PMA-stimulated chondrocytes in the presence of iron. D, same as in C in the presence of superoxide dismutase (SOD) (15 U/ml). E, same as in C in the presence of catalase (300 U/ml). Instrument settings were: modulation amplitude, 1.0 G; time constant, 0.1 sec; scan rate 50 G/min; receiver gain, $1.25\times10^4$; power, 10 mW. Experiments of unstimulated and stimulated chondrocytes repeated five time and with antioxidants done three times. A representative experiment with antioxidants.

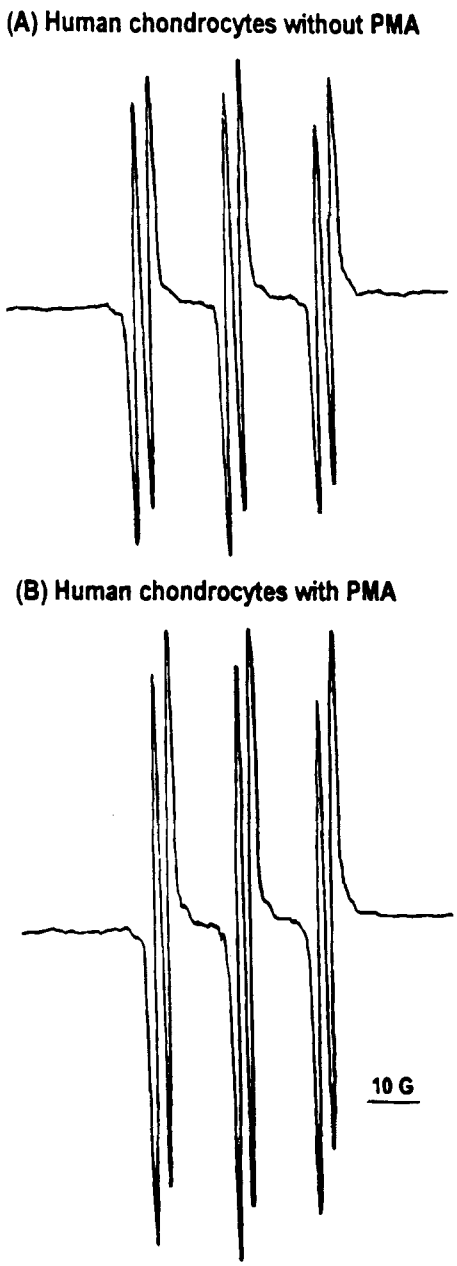



FIGURE 2 Hydroxyl radical formation by human osteoarthitic chondrocytes. EPR spectrum of primary human osteoarthitic chondrocytes ($1\times10^6$/ml) in spin trapping reaction mixture of 4-POBN (10 mM), DTPA (0.1 mM) and ethanol (170 mM) in HBSS in the presence of exogenous ferrous sulfate (0.1 mM) with (B) or without (A) PMA-(100 ng/ml) stimulation. Instrument settings were: modulation amplitude, 1.0 G; time constant, 0.03 sec; scan rate 50 G/min; receiver gain, $4\times10^3$; power, 10 mW. A representative experiment repeated twice.

however, led to a slight increase in EPR signals (Figure 2). This finding provides indirect evidence that there is an altered regulation of oxidative response in osteoarthritic chondrocytes as compared to the normal.

### Effect of Cytokines on the Hydroxyl Radical Formation by Chondrocytes

Cytokines such as IL-1 and TNF-α have been found to be important physiological agents that trigger and prime phagocytes for increased oxidative function.[8,11,12] We therefore examined the effect of pretreatment of IL-1 and TNF on hydroxyl radical formation in chondrocytes. Figure 3 shows that IL-1 or TNF treatment enhanced the generation of hydroxyl radical in PMA-treated chondrocytes by about three-fold as estimated from the EPR signal intensity. The results support the notion that cytokines IL-1 and TNF could enhance the oxidative response of chondrocytes to PMA.

### Effect of Synovial Fluid on Hydroxyl Formation by Chondrocytes

Articular chondrocytes derive nutrients from synovial fluid.[15] Rheumatoid synovial fluid, derived from immune inflammatory joints, contains low levels of iron[16] whereas osteoarthritic or crystal-induced arthritic synovial fluids representing either noninflammatory or acute inflammatory synovial fluid, respectively, have been reported to contain normal levels of iron.[16] We found that synovial fluid alone could not promote hydroxyl radical formation in PMA-treated chondrocytes in the absence of exogenous ferrous ion (data not shown). This may be either because the presence of DTPA (0.1 mM), a chelating agent, in the spin trapping mixture would make transition metal ions unavailable, or the iron present in the synovial fluid was unavailable for the Haber-Weiss reaction. However, in the presence of exogenous ferrous ion we found that osteoarthritic synovial samples enhanced

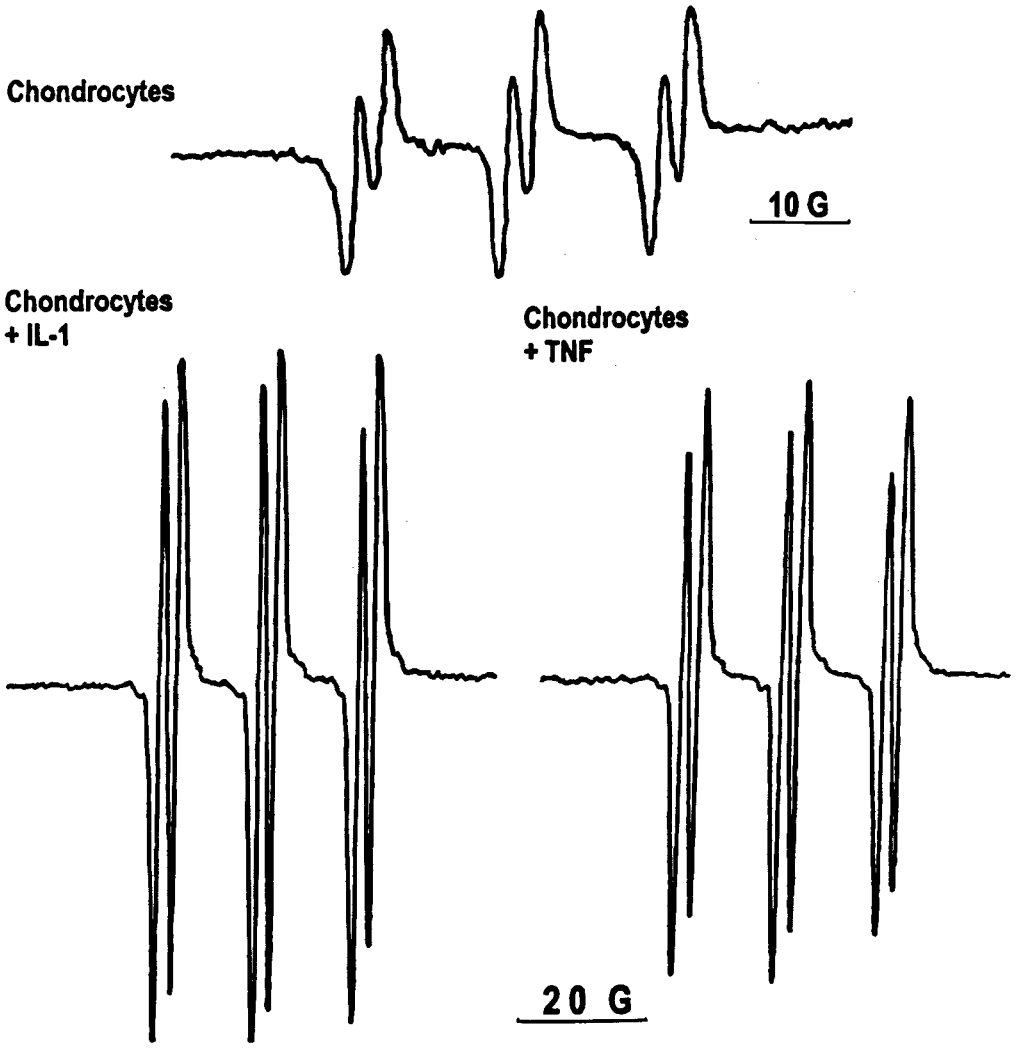



FIGURE 3 Effect of cytokine treatment on the hydroxyl radical formation by chondrocytes. EPR spectrum of rabbit articular chondrocytes ($1\times10^6$) in spin trapping reaction mixture of 4-POBN (10 mM), DTPA (0.1 mM) and ethanol (170 mM) in HBSS in presence exogenous ferrous sulfate (0.1 mM) 1 hour after addition of PMA (100 ng/ml). A, spectrum of PMA-stimulated chondrocytes in presence of supplemental iron. Bottom two tracings of chondrocytes pretreated with either TNF (100 mg/ml) or IL-1 (100 U/ml) and then PMA stimulated. Instrument settings were: modulation amplitude, 1.0 G; time constant; 0.03 sec; scan rate 50 G/min; receiver gain, $4\times10^3$; power, 10 mW. A representative cytokine treatment experiment repeated twice.

hydroxyl adduct signal formation in PMA-treated chondrocytes. In contrast, synovial fluid derived from rheumatoid joints decreased the EPR signal. These results clearly indicate that in addition to transition metals, factors present in normal or pathological synovial fluid could affect the oxidative response of chondrocytes.

### Hydroxyl Radical Formation by Fresh Cartilage Tissue

Since chondrocytes are the major cell type in cartilage tissue, we decided to examine whether hydroxyl radical formation can be directly detected in cartilage tissue with or without prior PMA treatment. Figure 5 shows that the α-hydroxyethyl

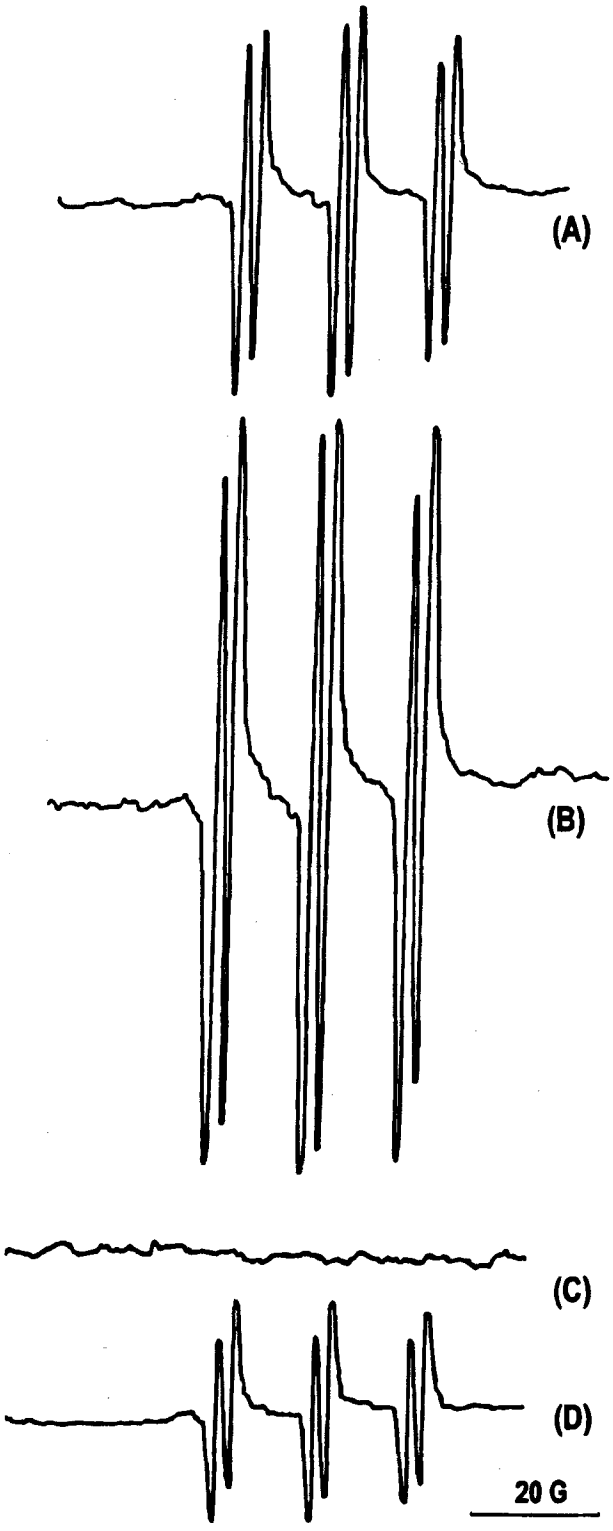
spin adduct of 4-POBN could be detected in fresh human and rabbit cartilage tissue pieces without any prior PMA treatment. Moreover, PMA treatment did not significantly affect the EPR signal (data not shown). This is the first demonstration that cartilage tissue alone has the potential to generate and accumulate hydroxyl radical. It is likely that chondrocytes are the source for this oxidative response in cartilage.

## DISCUSSION

Spin trapping agents react with short lived radicals such as hydroxyl radical to generate a long-lived adduct that can be detected by electron paramagnetic resonance spectroscopy. Thus the EPR/spin trapping technique has been used as a sensitive and definitive method for detecting hydroxyl radical formation in biological samples. Spin trapping agent, 5,5 dimethyl-1-pyrroline-*N*-oxide (DMPO) has been most frequently used to detect free radical production.[17–19] Recently a new spin trap system, 4-POBN/ethanol, has been shown to be more specific and sensitive as a hydroxyl trapping agent.[14] The hydroxyl radical is trapped in ethanol as α-hydroxyethyl adduct of 4-POBN, 4-POBN-CH($CH_3$)OH, with a rate at least one order of magnitude faster than the reaction with DMPO.[14]

Using the 4-POBN/ethanol as spin-trapping agent, we showed that chondrocytes, once stimulated with PMA, can generate hydroxyl radical, provided that ferrous ion is present (Figure 1). The hydroxyl radical was detected as α-hydroxyethyl adduct of 4-POBN. Hydroxyl radical in biological systems can be generated via different mechanisms.[20] Since the EPR signal was detected only in the presence of ferrous ion and catalase inhibits the formation of spin adduct, it is likely that hydrogen peroxide generated in PMA-treated chondrocytes is the precursor for hydroxyl radical. In contrast, we found that EPR signal of the α-hydroxylethyl 4-POBN adduct in thioglycollate-induced peritoneal exudate cells could be observed in the absence of supplemental iron (data not shown). Similarly, hydroxyl spin adducts formation in the absence of a supplemental source of iron has been reported for human neutrophils and monocytes shown to be myloperoxidase-dependent.[14] Candeias *et al.*, reported the formation of hydroxyl radical by the reaction of superoxide anion with hypochlorous in neutrophils.[21] Whether this reaction occurs in chondrocytes or not is unclear. However, the fact that chondrocytes can generate hydrogen peroxide in a burst manner following PMA treatment suggests that hydrogen peroxide may have some physiological role. This notion is also supported by the observation that cytokines can modulate the production of hydrogen peroxide in PMA-treated chondrocytes (Figure 3). In view of the prevalence of transition metals in body fluid, particularly synovial fluid, it is highly likely that hydrogen peroxide generated in chondrocytes can become hydroxyl radical under certain physiological or pathological conditions. Such conversion will have a profound effect on cartilage integrity and possibly arthritis.

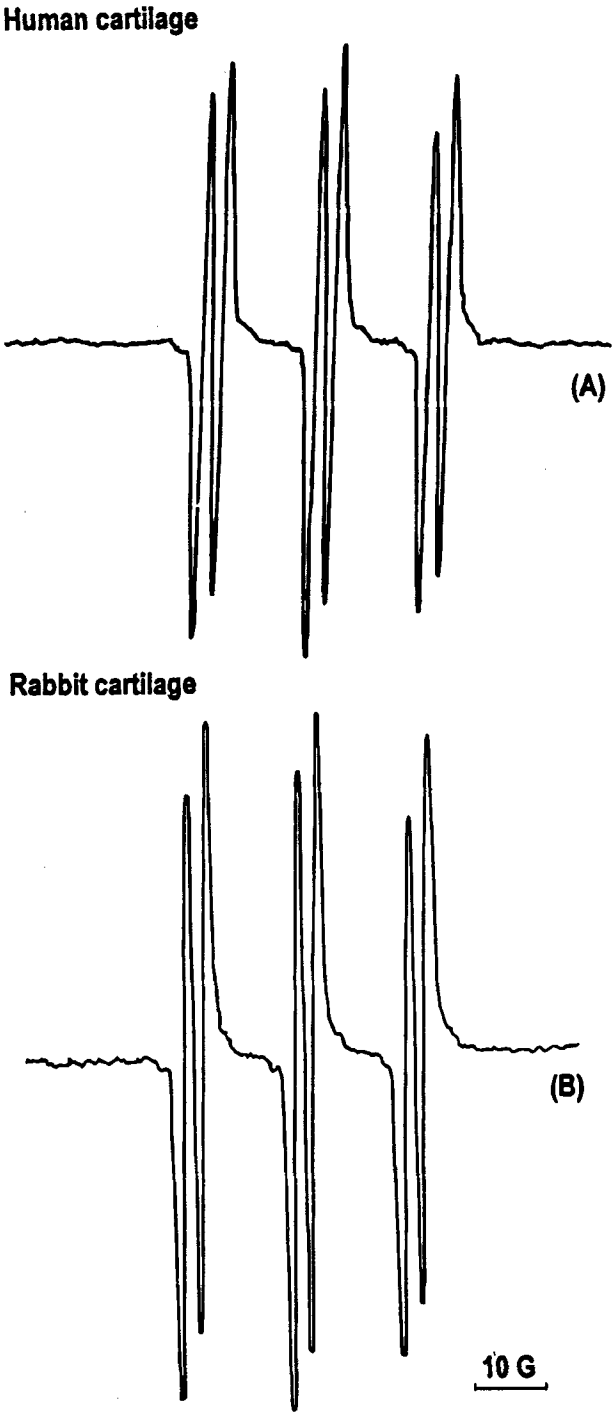
In addition to being a possible source of transition metal for converting hydrogen peroxide to hydroxyl radical, our data also showed that synovial fluid from pathological sources could further enhance the production of hydrogen peroxide, suggesting that synovial fluid may participate in the overall oxidative response of cartilage tissue *in vivo* (Figure 4). Since the spin trapping reaction mixture contained DTPA, a chelating agent, it is difficult to ascertain whether iron in synovial fluid can function catalytically in the formation of hydroxyl radical. DTPA has been shown to increase the efficiency of hydroxyl radical generation because of the nature of iron chelators (Fe(II)-DTPA).[22] On the other hand, the presence of DTPA (0.1 mM), EDTA (0.1 mM), or deferoxamine (0.1 mM) did not significantly affect the spin trapping of α-hydroxyethyl radical formation by neutrophils.[14] Taken together, DTPA may have facilitated hydroxyl formation. It is also possible that iron in synovial fluid may




be protein-bound and thus, like serum-bound iron, ineffective in catalyzing the Haber-Weiss reaction.[23] If this is the case, it will be an absolute protective mechanism for the prevention of oxidant-induced tissue damage.[13,23] In any event, it is of interest to note that there is a positive correlation between the presence of catalytic iron in synovial fluid and disease activity in rheumatoid arthritis.[16] Indeed, a number of clinical observations have suggested a correlation between iron availability and arthritis. For example, flare-up of arthritis is observed following parenteral injection of iron in rheumatoid arthritis patients and it may be caused by iron-induced oxidant stress.[24] In hematochromatosis, a disease of increased iron overload, there can be significant osteoarthritis, possibly related to excessive levels of iron allowing enhanced ROI production in cartilage. Together, these clinical observations implicate iron-induced oxidative mechanisms of matrix degradation in arthritis. Similarly, Wilson's disease, which is associated with enhanced storage of copper that acts as a transition metal in the Haber-Weiss reaction, results in osteoarthritis.

In previous studies we and others have shown that IL-1, TNF, and gamma interferon trigger and prime chondrocytes to increase production of ROI.[8,11,12] In the present study we confirmed that cytokines, IL-1 and TNF-treated

FIGURE 4 Effect of synovial fluids on hydroxyl formation by PMA-stimulated chondrocytes. EPR spectrum of articular chondrocytes ($1\times10^6$) in spin trapping reaction mixture of 4-POBN (10 mM), DTPA (0.1 mM) and ethanol (170 mM) in HBSS in presence or absence of exogenous ferrous sulfate (0.1 mM) 1.0 hours after addition of PMA (100 ng/ml). **A,** spectrum of PMA-stimulated chondrocytes in presence of supplemental iron. **B,** same as A but with osteoarthritic synovial fluid. **D** same as in A but with rheumatoid synovial fluid. **C,** spectrum of PMA-stimulated chondrocytes in absence of supplemental iron but with addition of synovial fluid. Synovial fluid concentration in B, C, and D was made to 25% v/v of 100 ul of reaction mixture distributed into capillary tube and then placed in the cavity of EPR spectrometer. Instrument settings were: modulation amplitude, 1.0 G; time constant, 0.3 sec; scan rate 50 G/min; receiver gain, $1.25\times10^4$; power, 10 mW. Synovial fluids were tested in a single experiment setting with positive and negative controls.




chondrocytes are primed to produce greater amounts of hydroxyl as compared to control (untreated) chondrocytes. The priming effect has been shown to be due to enhancement of NADPH-oxidase level and in chondrocytes similar mechanisms may be operative.[13,25] Cytokines and growth factors are implicated in the pathogenesis of cartilage matrix degradation.[26] Cytokines and growth factors could possibly cause increased matrix degradation by a mechanism that involves enhancement of the oxidative capacity of chondrocytes.

Chondrocytes are normally located as isolated cells or in small clusters and are immobile inside the cartilage. *In-vivo* chondrocytes function in a comparatively anoxic environment. In contrast, *in-vitro* chondrocytes are exposed to high concentration of oxygen which has been shown to modulate proteoglycan synthesis and aggregation.[27] The precise role of oxygen tension on formation of ROI by chondrocytes remains to be studied.

We found that both human and rabbit cartilage tissue allowed the formation of hydroxyl radical (Figure 5). Of note is that prior treatment of cartilage with PMA did not significantly affect the EPR signal, suggesting that chondrocytes in the cartilage were preactivated. It is possible that cutting and shaving cartilage into small pieces may activate chondrocytes. Also of note, is that human osteoarthritic chondrocytes showed, without PMA stimulation, hydroxyl radical formation, suggesting spontaneous production of hydrogen peroxide. In previous studies we observed luminol and lucigenin-dependent chemiluminescence in stimulated cartilage tissue.[11,12] Together, these observations indicate that respiratory burst activity occurs within the

FIGURE 5 Hydroxyl radical formation by rabbit or human cartilage tissue. EPR spectrum of unstimulated human and rabbit cartilage pieces in the presence of 4-POBN/ethanol spin trapping reaction mixture in presence of exogenous ferrous sulfate (0.1 mM) at 45 minutes. Instrument settings were: modulation amplitude, 1.0 G; time constant, 0.03 sec; scan rate 50 G/min; receiver gain, $4 \times 10^3$; power, 10 mW. A representative tracing; experiment repeated twice.

cartilage tissue and is indicative of *in-vivo* oxidative function of chondrocytes in the cartilage.

Hydroxyl radicals are one of the most powerful oxidants known.[13] Because of the highly reactive nature of hydroxyl radicals towards a range of cellular and extracellular targets, the demonstration of the formation of hydroxyl radical by chondrocytes/cartilage in this study implicates the involvement of hydroxyl radical in matrix damage. Increased production of free radicals has been directly correlated with oxidative damage.[5,6] It is possible that enhanced production of ROI in tridimensionally placed chondrocytes may contribute to matrix degradation. Hydrogen peroxide and other species of free radicals have been shown to destroy the biochemical integrity of matrix components of cartilage such as proteoglycan, hyaluronic acid, collagen and link protein.[28–30] Hydrogen peroxide inhibits the biosynthesis of proteoglycan and hyaluronic acid by chondrocytes.[31,32] It is possible that enhanced levels of chondrocyte-derived ROI along with the presence of available iron for catalysis in Haber-Weiss reaction results in formation of toxic hydroxyl radical which may damage matrix during unscheduled tissue remodelling and degradation associated with aging, osteoarthritis, and inflammatory arthritis. Our findings suggest strongly that chondrocytes can produce and accumulate hydrogen peroxide. In addition, our data suggest that synovial fluid may serve as a potential donor of transition metals. Taken together, these results point to the fact that synovial fluid not only can serve as potential donor of transition metals but also can further modulate ROI production. There is a need to further investigate the role of chondrocytes in the pathogenesis of arthritis.

## *Acknowledgements*

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