Activated PMNs lead to oxidative stress on chondrocytes

A study of swine knees

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ABSTRACT Using an in vitro model, based on primary cultured chondrocytes, we examined possible oxidative injury caused by activated polymorphonuclear neutrophil granulocytes (PMNs), which are thought to be part of the pathomechanism of hemarthrosis.

Chondrocytes were isolated from swine knee joints and divided into three groups. Pure chondrocytes acted as the control population (group I). PMNs from the systemic circulation, and hydrogen peroxide (as an artificial source of reactive oxygen species (ROS)) were added to groups II and III, respectively. All cultures were incubated for 6 hours. After the experiment, lipid membrane degradation by ROS was assessed by monitoring changes in the levels of malondialdehyde (MDA) and 4-hydroxyalkenal contents of the chondrocyte specimens. Changes in the endogenous scavenger status of the chondrocytes were characterized by measuring of reductions in glutathione (GSH) concentration and superoxide dismutase (SOD) activity.

Significant increases in MDA/4-hydroxyalkenal levels and SOD activity as well as an expressive reduction in intracellular GSH content were highlighted by comparing the control to the PMN- or H_2O_2-treated cell populations. These findings confirm previous suggestions that PMN-derived ROS contribute to degradation of cartilage in hemarthrosis.

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In intra-articular bleeding, synovial and blood-borne factors, such as inflammatory mediators and lysosomal enzymes, have been shown to cause joint destruction. Hoaglund (1967) suggested that leukocytes in joint fluid, derived from hemarthrosis, may generate sufficient proteolytic activity in chronic hemarthrosis to degrade cartilage. Because of its enhanced proteolytic activity, as well as its high concentrations in human hemophilic synovium and synovial fluid, Arnold and Hilgartner (1977) regarded Cathepsin D as the main cause of this process.

Roosendaal et al. (1998) reported that the synovial tissues obtained from hemarthrotic patients can produce large quantities of proinflammatory cytokines, namely IL-1, IL-6 and TNF-α, which inhibit the formation of human cartilage matrix.

The generation of reactive oxygen species (ROS) is one of the characteristic features of polymorphonuclear neutrophil granulocytes (PMNs) (Fridovich 1986). Many authors have studied the effects of ROS on cartilage tissue. Bates et al. (1984a, b) showed that oxygen-derived reactive species, generated enzymatically by the action of xanthine oxidase on hypoxanthine, can cleave proteoglycan-hyaluronate aggregates and inhibit proteoglycan synthesis in cultured cartilage. Using a similar experimental model, Burkhardt et al. (1986) showed that ROS are able to degrade intact cartilage matrix by direct action on matrix components and by activation of latent PMN-collagenase. Studies performed by Chung et al. (1984) and Bates et al. (1985) have confirmed that hydrogen peroxide also destroys matrix components and

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inhibits hyaluronic acid and proteoglycan synthesis in articular cartilage explants.

Oxidative stress is described as a condition in which excessive ROS production overwhelms the antioxidant defenses (Halliwell 1994). According to Jackson and Bushell (1999), reduced glutathione (GSH) is an integral element in this antioxidant defense, one that is depleted during oxidative stress (Marban et al. 1989). On the other hand, superoxide dismutase (SOD), another member of the endogenous antioxidant system, shows a compensatory increase in activity after exposure to bursts of superoxide radical (Fridovich 1986). Furthermore Halliwell and Gutteridge (1990) have proved that the detection of lipid peroxidation via measurements of malondialdehyde (MDA) and 4-hydroxy-2 (E)-nonenal (4-HNE) levels is a suitable indicator of ROS-mediated reactions. Thus monitoring the above parameters is regarded as appropriate for evaluating oxidative stress. Therefore, we describe our procedure in brief.

Animals and methods

Animal preparation. 3 healthy Yorkshire pigs (35–40 kg) were used as blood donors. Each animal was subjected to an overnight fast and sedated the following day with ketamine (15 mg/kg). Anesthesia was induced with pentobarbitone and maintained with 1.5% halothane. During the operation, a silicon catheter was inserted into the jugular vein. The drain was led subcutaneously to the posterior surface of the neck, thereby facilitating sampling of blood.

Chondrocytes were isolaged from 9 healthy Yorkshire pigs, weighing 20–25 kg. These animals were sedated with ketamine (15 mg/kg) and killed with an intra-cardiac injection of sodium pentobarbital (120 mg/kg). All procedures were done in accordance with the International Guiding Principles for Animal Research (Howard-Jones 1985).

Isolation and culturing of chondrocytes from pigs. Whole thickness cartilage was dissected under sterile conditions from the articular surface of the patella as well as the femoral and tibial condyles. Pieces of cartilage were placed in Petri dishes. To remove the extracellular matrix, these pieces were digested with 300 U/mL collagenase (Sigma, USA) and 10% fetal calf serum (FCS) in phenol red-free DMEM/F-12 media at 37 °C for 12 hours. After this, the supernatant was collected and filtered through a 70 μm nylon cell strainer (Becton Dickinson, USA). Finally, the cells were separated by centrifugation. Cell viability, determined by trypan blue exclusion, was 80–90% yielding 1–2 × 10^7 chondrocytes per gram wet weight of cartilage tissue. On the basis of viability, 20 ×10^6 healthy chondrocytes were seeded in 75 cm^2 plastic culture flasks (Iwaki, Japan) and incubated in a culture of phenol red-free DMEM/F-12 media containing 10% FCS.

Isolation of PMNs. This was done immediately before pretreatment of chondrocytes. EDTA anticoagulated blood was sedimented with 6% dextran at 37 °C. After aspiration of the supernatant, hypotonic hemolysis was performed with 0.8% NH4Cl to remove the erythrocytes. The white pellet obtained after repeated centrifugation was suspended in glucose containing modified Dulbecco's buffer (GDB) and spun against the gradient separator medium Histopaque 1077 (Sigma, USA) (Guarnieri et al. 1990). Finally, the pellet containing about 95% PMNs was suspended in cold buffer as above. After cell counting and estimation of viability, we used the number of cells needed for the experiments.

Treatment of chondrocytes. The culture medium was discarded and the cells gently washed with serum-free media to remove dead cells and undesirable metabolites. The flasks were filled with 40 mL of phenol red-free DMEM/F-12 to minimize the effect of decreasing amounts of nutrients during the experiment. Apart from the control populations (group I), these media were either supplemented with a suspension of isolated PMNs (20 × 10^7 PMN / 40 mL; group II), or freshly prepared hydrogen peroxide solution (final concentration 10–4 M; group III), as a controlled source of ROS. 8 μg phorbol 12-myrsistate 13-acetate (PMA) as the PMN stimulator agent (Sigma, USA) was added to each flask. Throughout the experiments, the flasks were incubated at 37 °C and continuously agitated at 60 rpm on a vibrating table.

Measurement of free radical-mediated changes. After incubation for 6 hours, the reaction media was discarded. Cells were washed twice with cold PBS buffer (Sigma, USA) to remove dead

chondrocytes and PMNs. The remaining cells were collected with a rubber cell-scraper. After centrifugation, the cells were resuspended in 300 μL PBS buffer, and homogenized by repetitive freezing/thawing and a plastic pestle. Samples of the cell homogenate were taken for biochemical analysis of free radical-mediated reactions.

Changes in the endogenous scavenging status were shown by finding a reduced glutathione (GSH) concentration and superoxide dismutase (SOD) activity in the samples with colorimetric assay kits (Calbiochem, USA). The results were expressed in μg of GSH per mg protein and units per mg protein, respectively.

The immediate harmful effect of ROS on cell membranes was evaluated by determining the degree of lipid peroxidation, using a relevant assay kit (Calbiochem, USA). Changes in the levels of malondialdehyde (MDA) and 4-hydroxyalkenals, such as the 4-hydroxy-2 (E)-nonenal (4-HNE) contents of the specimens, were assessed. The results were expressed as nmoles of MDA/4-HNE per mg protein. The total protein content of the homogenate was determined with Bradford's method.

Histological examinations. PMNs and chondrocytes were incubated together under similar conditions as above. At the end of the experiment, the flasks were washed twice with cold PBS buffer, fixed with 4% paraformaldehyde in PBS (pH 7.4) and stained with Leishman's stain. The effect of continuous agitation on PMN adherence to chondrocytes was studied by using light microscopy combined with an image analyzer system (Zeiss Image, Carl Zeiss Inc., USA).

Statistics

In each experiment, the populations of chondrocytes derived from the same animal were randomly divided into 3 groups (i.e., control and PMN- or H_2O_2-treated). The mean values of the various parameters and the standard errors are presented. The non-parametric Sign test was used to determine the significance of the differences between the mean values of the control and treated groups of chondrocytes. A p-value of less than 0.05 was considered statistically significant.

Results

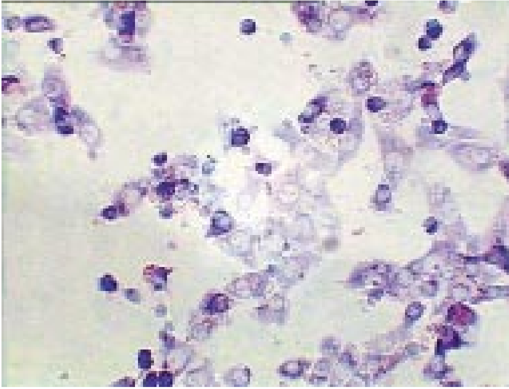
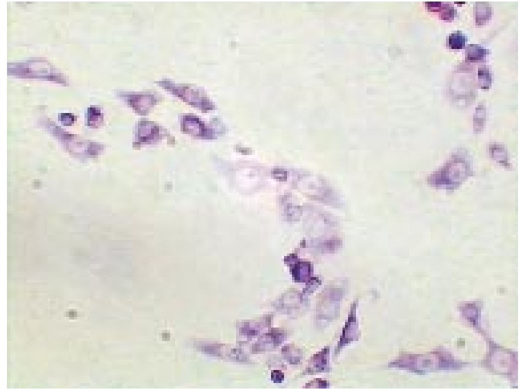
Exclusions. Chondrocytes were obtained from 9 healthy pigs, but in 2 cases, we had enough cells for additional sets of SOD and MDA. In another case, the samples for GSH measurement became contaminated during the biochemical analysis and they were then discarded. All sets of samples contained control, PMN- and H_2O_2-treated chondrocyte populations. GSH was therefore measured in 8 different sets of samples and MDA and SOD were measured in 11 sets of samples.

Monitoring the endogenous scavengers. Our findings show that the intracellular levels of GSH ($n = 8$) fell after co-incubation with H_2O_2 (1.36, SD 0.09) μg/mg, $p = 0.008$) or PMNs (1.13, SD 0.14 μg/mg, $p = 0.008$), as compared to the controls (1.86, SD 0.12 μg/mg). However, the differences between the differently-treated groups were not significant.

On the other hand, SOD activities ($n = 11$) in treated cells (PMNs: 25, SD 2.3 U/mg, $p = 0.001$; H_2O_2: 24, SD 1.8 U/mg, $p = 0.001$) were higher than in control cells (18, SD 1.8 U/mg). Although the effect of hydrogen peroxide was less than that of PMNs, the difference was also not statistically significant.

Measurement of lipid peroxidation. The data from the simultaneous measurement of MDA and 4-HNE ($n = 11$) indicated more lipid peroxidation in both groups of treated cells (PMNs: 2.6, SD 0.8 nM/mg, $p = 0.01$; H_2O_2: 3.1, SD 1.0 nM/mg, $p = 0.001$) than in the control group (1.7, SD 0.5 nM/mg), but the difference between the two treated groups was also not significant.

Histological examinations. Whole thickness cartilage was used to obtain chondrocytes. The populations could therefore have included all types of cells from the superficial layer to the tide mark zone. Polygonal and round cells with their basophilic nuclei were present on both the control and co-incubated (treated) slides. Marked reductions in the chondrocyte population were seen on slides of treated groups. Hardly any PMNs of similar size with their typical segmented nuclei were seen on the sections because of the shaking and rinsing procedures. Therefore they could not have interfered with the biochemical analysis of ROS-mediated chondrocyte damage (Figure).

Representative Leishman-stained sections from control (left) and PMN-treated (right) cell cultures. Histological examinations (400×) showed that only chondrocytes remained in the culture flasks and that their numbers had decreased far more in the treated groups than in the control. Note the absence of PMNs after removal during preparation of the slides.

Discussion

Studies have confirmed the importance of synovial changes in the pathogenesis of hemarthrosis (HA), and drawn attention to the fact that blood itself can harm the cartilage and that this harmful effect precedes the destructive processes caused by synovial inflammation (Fabry 1989, Roosendaal et al. 1997, 1999a). Moreover, ROS have been involved in these early changes (Borsiczky et al. 1998), which can be caused by even a single episode of HA (Zeman et al. 1991).

Leukocytes (mainly monocytes and lymphocytes) are the main normal cellular constituents of normal synovium, numbering less than 200–300 cells/mm^3 (Levick 1987). This cellular population dramatically changes during HA when PMNs increase markedly (about 5 × 10^6–10^7/mL). These cells produce ROS, when activated by various proinflammatory mediators (Røkke et al. 1989, Botha et al. 1995), which are released from endothelial cells and locally-recruited platelets after intra-articular vascular injury (Schwartz et al 1989). Several investigators have studied the damage to cartilage matrix and chondrocytes caused by activated PMNs, which are thought to augment the degradation of elastase (Weiss and Regiani 1984). Moreover, PMN-derived ROS have been shown to degrade cartilage proteoglycans and inhibit their synthesis (Bates et al. 1988 and Kowanko et al. 1989). However, little is known about the effect of PMN-evoked oxidative stress on chondrocytes. Although Rosendaal et al. (1997, 1999b) emphasized the importance of this role for mononuclear cells, based on the above, we suggest that PMNs are a major factor in the pathogenesis of HA.

Davies (1946) estimaed that the internal surface area of the human adult knee is about 4.3 cm^2. If one assumes that a single episode of HA in the knee joint would cause an intra-articular effusion of about 1411–3529 cells/mm^2 surface area 20 mL of blood (a moderate estimate), then PMNs would be present. According to Gilmore and Palfrey (1988), there are 250–350 chondrocytes/mm^2 in the articular cartilage of human femoral condyles—i.e., a chondrocyte: PMN ratio of 1:10, which we have used in our in vitro model. Since activated PMNs tend to adhere to their surroundings, we prevented adherence of the two cell populations by continuous agitatation on a shaking table at 60 rpm during the experiment. This ensured that the PMNs would not adhere to the base of the culture flasks, and facilitated their removal from the chondrocyte layer, which was subsequently confirmed on histological examination.

Since oxidative stress disrupts the balance between endogenous scavengers and antioxidants, studies of the changes in these systems would appropriately reflect the degree of stress caused by ROS. Reduced glutathione, due to its nucleophilic and reducing properties, plays a central role in the cellular antioxidant defense mechanisms. It has radical-trapping properties and acts as a substrate for glutathione peroxidase. This enzyme can eliminate hydrogen peroxide and inactivate organic peroxides (Guarnieri et al. 1980). Several authors

have studied the effect of oxidative stress on GSH content in various tissues. Evidence for a reduction of this antioxidant provides additional support for an increase in ROS activity during such processes (Jackson and Bushell 1999). We observed a significant decline in intracellular GSH concentration in treated samples, which indicated the presence of such reactive species.

Superoxide dismutase is an intracellular metalloprotein, which transforms superoxide anion into H_2O_2 and oxygen, thereby eliminating free radicals. Fridovich (1986) and others have reported that an increase in the production of superoxide radical activates SOD (Röth et al. 1998). Therefore we regard the significant increase in SOD activity in both of our treated groups as a response to the larger amount of O_2.-radical derived from either activated PMN or the reaction between H_2O_2 and .OH, which also results in superoxide radicals (Halliwell and Gutteridge 1990). Our findings are also in line with those of Sato et al. (1988) as regards SOD activity of synovial fluid in patients having rheumatoid arthritis and osteoarthritis.

Lipid peroxidation is a well-known mechanism of cellular injury in plants and animals. The importance of reactive oxygen species, such as hydroxyl radicals (.OH), H_2O_2, superoxide (O_2.-), and various transitional metal ions such as Cu^+, $Fe2^+$, $Fe3^+$, in this process has been well documented (Gutteridge and Halliwell 1990). On the basis of the immediate deleterious effects of ROS on chondrocytes, we studied the effects of lipid peroxidation in this model and found significant increases in MDA/4-HNE levels in both the PMN- and H_2O_2-treated groups, which would suggest an increase in ROS-mediated lipid membrane injury. This shows not only the presence of reactive species, but also their consequences—i.e., oxidative injury of chondrocytes.

Our findings provide evidence that short-term exposure to activated PMNs can cause oxidative injury of chondrocytes. It seems possible that in patients with hemarthrosis, PMNs and ROS may also contribute to damage of the cartilage.

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