Haemoglobin-derived iron-dependent hydroxyl radical formation in blood-induced joint damage: an in vitro study

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Objective. It has been reported that joint bleeds cause cartilage damage and that the combination of red blood cells (RBC) plus mononuclear cells (MNC) causes the adverse effects. The present study is to elucidate the mechanism by which blood, as present in whole blood, may cause this cartilage damage.

Methods. Human cartilage samples were cultured for 4 days in the presence of 50% whole blood, isolated MNC plus RBC, CD14+ cells (monocytes/macrophages) plus RBC, or lysed RBC with interleukin 1β (IL-1β; a major catabolic product of activated monocytes/macrophages). Antioxidants were used to investigate the involvement of oxidative stress. A subsequent 12-day culture period in the absence of additions is referred to as the recovery period. Changes in cartilage proteoglycan synthesis were determined at days 4 and 16.

Results. Cartilage cultured in the presence of whole blood, MNC plus RBC, or monocytes/macrophages plus RBC resulted in a prolonged inhibition of proteoglycan synthesis (>90% inhibition at day 16; all three P < 0.05). Lysed RBC together with IL-1β also induced prolonged inhibition of proteoglycan synthesis (>56% of controls, P < 0.05). Dimethylsulphoxide (DMSO), scavenging hydroxyl radicals, could reverse the inhibition of cartilage proteoglycan synthesis.

Conclusions. Based on these results we hypothesize that IL-1β produced by activated monocytes/macrophages increases the production of hydrogen peroxide by chondrocytes. This in combination with haemoglobin-derived iron from the RBC will result in the formation of hydroxyl radicals in the vicinity of chondrocytes. This mechanism may result in chondrocyte damage and as such be involved in blood-induced cartilage damage.

KEY WORDS: Interleukin-1β, Hydroxyl radical formation, Joint damage, Cartilage damage, Red blood cells, Mononuclear cells.
surgical procedure does not seem to prevent further degradation of cartilage [12].

We have recently reported that the initial and irreversible changes in cartilage matrix metabolism are independent of synovial inflammation [3]. The primary adverse changes in cartilage are induced by the combination of mononuclear cells (MNC) plus red blood cells (RBC) [13], which are subsequently followed by synovial changes [2, 3, 14]. The mechanism by which MNC plus RBC are involved in the pathophysiology of blood-induced cartilage damage, however, is still unclear.

The blood MNC in the joint cavity after a joint bleed, independent of an inflammatory response, can produce interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) [2]. The role of these cytokines in human cartilage degradation has been reviewed previously [7, 15]. Specifically, IL-1β is an important player in cartilage degradation [16]. However, this cytokine was found to give only transient adverse changes in cartilage matrix synthesis; when the source of IL-1β was removed, chondrocytes started to restore their cartilage matrix production [2, 17, 18].

The required concurrent presence of RBC with MNC suggests, besides the MNC, a role of RBC-derived toxicity. Haemoglobin-derived catalytic iron may result in oxidative stress, which may cause damage to chondrocytes [19, 20]. This is based on the fact that iron can act as a catalyst for the Fenton reaction in which hydroxyl radicals are formed from hydrogen peroxide [21]. Hydrogen peroxide production by chondrocytes is increased after stimulation with IL-1β [22]. IL-1β can be produced by the MNC as present in blood after a joint haemorrhage [2]. The monocytes/macrophages in the MNC population are able to phagocytose RBC and process the iron from haemoglobin. These iron compounds are then excreted into the extracellular surroundings of the macrophage [23]. The presence of iron has been shown in cartilage that has frequently been exposed to blood [24], and also in isolated chondrocytes after a short culture period with blood [25]. In addition, haemoglobin has been shown to be phagocytosed by chondrocytes and to have adverse effects on cartilage matrix turnover [26].

Based on the above, we investigated the role of iron-related oxidative stress in blood-induced articular cartilage damage, using an in vitro human articular cartilage culture system to which blood components were added.

Materials and methods

Cartilage culture technique

Healthy full-thickness articular cartilage was obtained at autopsy from human humeral heads within 24 h after death of the donor. The donors (n = 19, mean age 62.8 ± 14.9 yr, 17 males and two females) had no history of joint disorders. Slices of cartilage were cut aseptically from the articular surface excluding the underlying bone. Slices were kept in phosphate-buffered saline (PBS, pH 7.4) and cut into square pieces within 1 h of dissection. The square pieces were weighed aseptically (range 5–15 mg) and cultured individually in 96-well round-bottomed microtitre plates, in 200 ml of medium under 5% CO₂ in air at 37°C (pH 7.4). The culture medium consisted of Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with glutamine (2 mm), penicillin (100 IU/ml), streptomycin sulphate (100 mg/ml), ascorbic acid (85 mm) and 10% heat-inactivated pooled human AB⁺ serum.

Isolation of blood cells

For each individual experiment, fresh blood from healthy donors (n = 19, mean age 33.8 ± 8.6 yr, 11 males and eight females) was collected into heparinized vacuumer tubes (Becton Dickinson, 143 USP L-heparin/ml). Mononuclear cells (MNC) were isolated by density centrifugation (purity >95%), using Ficoll-Paque (Pharmacia, Sweden) and washed twice in cold DMEM (Gibco, The Netherlands) [27]. Isolation of monocytes/macrophages from the MNC population was performed using a magnetic cell separation technique (purity >85%; MACS/Monocyte isolation kit, Miltenyi, Germany). RBC from the same donor were isolated by filtration (purity >99%; Pall-filters, Belgium). RBC were lysed by freezing (liquid nitrogen) and thawing (room temperature) a volume of isolated RBC two times.

All cells were redistributed in culture medium to 50% of their numbers as present in whole blood, based on cell counts before and after isolation (Coulter Counter Z1, Coulter, USA).

Experimental set-up

Cartilage was cultured for 4 days in the presence of whole blood (50% v/v), MNC plus RBC, or monocytes/macrophages plus RBC. At day 4 the cartilage samples were washed twice (200 ml culture medium, 45 min under culture conditions). The sulphate incorporation rate as a measure for proteoglycan synthesis of the chondrocytes was determined. To measure the reversibility of the effect of additions after a 4-day exposure, cartilage was washed twice (200 ml culture medium, 45 min under culture conditions) and subsequently cultured for an extra 12 days without any additions (referred to as the recovery period). The medium was refreshed every 4 days. At day 16 the sulphate incorporation rate as a measure of proteoglycan synthesis of chondrocytes was determined.

Proteoglycan synthesis

As a measure of chondrocyte activity, proteoglycan synthesis was measured as the rate of sulphate incorporation during the last 4 h of the 4- or 16-day culture period, according to standard procedures [28]. In short, Na₂³⁵SO₄ (37 MBq/ml, NEX-041-H, carrier-free; DuPont, The Netherlands) was used as a tracer and added in 10 ml aliquots (~74 kBq) to the 200 ml culture medium. In the 4-day cultures, cartilage samples were placed in fresh medium before addition of ³⁵SO₄⁻ without additions (to prevent interference of the cells with the assay). After 4 h, the explants were rinsed twice in ice-cold PBS, digested with 2% papain (Sigma, The Netherlands), and the glycosaminoglycans were precipitated with cetylpyridinium chloride (Sigma, The Netherlands). The amount of ³⁵SO₄⁻ incorporated was analysed by liquid scintillation analysis. The ³⁵SO₄⁻ incorporation rate was normalized to the specific activity of the medium and is expressed as nanomoles of sulphate incorporated per hour per gram wet weight of cartilage tissue (nmol h⁻¹ g⁻¹).

Interleukin-1β measurements

Supernatants from control cartilage cultures and cartilage co-cultured with whole blood or MNC plus RBC were harvested and rendered cell-free by centrifugation (500 g, 10 min). Enzyme-linked immunosorbent assay was used to measure
concentrations of IL-1β, according to the manufacturer’s instructions (Medgenix, The Netherlands). The detection limit was 20 pg/ml.

**Interleukin 1β addition**

IL-1β was added to cultures to stimulate the production of hydrogen peroxide by chondrocytes. Human recombinant IL-1β (rIL-1β BioSource, USA) was added up to a concentration of 1.0 ng/ml, according to the concentration found in the cartilage and blood cell co-cultures.

**Antioxidant addition**

Several antioxidants were tested, all of which were added to the co-cultures in aliquots of 10 ml during the first 4 days of culture. Dimethylsulphoxide (DMSO; Merck, Germany), a scavenger of hydroxyl radicals, which are expected to be involved in the haemoglobin-derived iron-mediated oxidative stress, was used in concentrations up to 700 mM. Catalase (Sigma, The Netherlands), which acts by reducing hydrogen peroxide to water and oxygen, was dissolved in DMEM shortly before use (0–10 kU/ml). As additional controls, manganese superoxide dismutase (Mn-SOD; 0–300 U/ml; Sigma, The Netherlands), a scavenger for superoxide anions, and ebselen (Alexis Corporation, USA), a scavenger for peroxyxinitre anions (ON-OO, the toxic end-product after increased nitric oxide and superoxide formation), both not expected to be involved, were added to the co-cultures.

**Calculations and statistical analysis**

Each cartilage culture experiment was performed with cartilage from a single donor with at least 10 tissue samples for each culture condition, each taken randomly and handled individually. The mean value of the 10 explants was taken as representative for that condition per experiment. For the co-cultures of cartilage with MNC plus RBC, repeated experiments were performed with cartilage and blood from at least four different donors, of which mean values ±SEM are given. Data were analysed with a non-parametric test for independent samples (Mann–Whitney U-test), using SPSS v. 10.0 software. P values <0.05 were considered statistically significant.

**Results**

**Inhibition of proteoglycan synthesis by blood cells**

Almost complete inhibition of proteoglycan synthesis was found after a 4-day in vitro exposure of cartilage to whole blood (50% v/v) or isolated MNC plus RBC (50% v/v, equivalents of whole blood). This effect was identical when the CD14+ monocytes/macrophages were isolated from the MNC fraction and together with RBC added to cartilage for 4 days (Fig. 1, open bars). Inhibition of proteoglycan synthesis was observed directly after 4 days of exposure to monocytes/macrophages plus RBC (>99% inhibition), as was observed for whole blood and the MNC plus RBC. Moreover, the effect was still evident after a 12-day recovery in the absence of monocytes/macrophages plus RBC; 83% inhibition of proteoglycan synthesis at day 16 (P <0.01), not statistically significantly different from the inhibition at day 4. Also, this effect was not different from that of whole blood or MNC plus RBC (80 and 79% inhibition at day 16, respectively). No statistically significant differences were found between the effects of the CD14+ cells plus RBC, whole blood or the addition of MNC plus RBC, at day 4 and day 16 of culture.

**The role of IL-1β and haemoglobin-derived iron**

IL-1β is one of the most important mediators in cartilage destruction [5, 7, 16]. Structural changes of the RBC membrane have been described to initiate monocyte/macrophage activation [29] leading to up-regulation of IL-1β production by these cells, a phenomenon independent of inflammation. The levels of IL-1β measured after a 4-day exposure of cartilage to whole blood or MNC plus RBC were 339 ± 60 and 677 ± 160 pg/ml, respectively. Supernatants of MNC plus RBC without cartilage had equal amounts of IL-1β production (830 ± 255 pg/ml), and cartilage without additions had no detectable IL-1β production at day 4 of culture, demonstrating the IL-1β to be produced by the MNC and not by the chondrocytes.

Addition of similar amounts of IL-1β (up to 1000 pg/ml) to cartilage cultures appeared to be able to reduce proteoglycan synthesis on day 4 by more than 50% (Fig. 2, IL-1β, open bar). However, this amount of IL-1β was unable to induce a prolonged inhibition of proteoglycan synthesis. At day 16 (12 days recovery), proteoglycan synthesis was inhibited by less than 10%, not statistically significantly different from control cultures (IL-1β, hatched bar).

A lysate of RBC, as a source of catabolic/catalytic iron, was also not capable of inhibiting proteoglycan synthesis for a prolonged period. After 4 days of culture the synthesis was statistically significantly inhibited by 63%, but this inhibition was restored within 12 days after the lysate was removed from the culture (Fig. 2, lysed RBC, hatched bar) and there was no significant statistical difference from the controls.
It appeared that a 4-day exposure of cartilage to the combination of IL-1β plus lysed RBC resulted in a prolonged inhibition. A 4-day exposure of IL-1β plus lysed RBC showed a significant inhibition (68%) of proteoglycan synthesis (Fig. 2, lysed RBC + IL-1β, open bars). After a 12-day recovery period the proteoglycan synthesis was still statistically significantly inhibited, resulting in less than 44% proteoglycan synthesis compared with controls (Fig. 2, lysed RBC + IL-1β, hatched bar; P < 0.05).

Effects of antioxidants
As expected, no effects of SOD and ebselen were found when they were added to the cultures during the first 4 days. According to our hypothesis, when a scavenger of hydroxyl radicals, i.e. DMSO, was added during the first 4 days of culture, a significant proportion of proteoglycan synthesis could be restored within the 12-day recovery period (Fig. 3A). The recovery of proteoglycan synthesis was concentration dependent (Fig. 3B, at 700 mM, 58% of control synthesis was obtained; P < 0.05). In cartilage–DMSO control cultures no effects on proteoglycan synthesis were found (data not shown). Catalase, reducing hydrogen peroxide to water and oxygen, was not able to prevent the lasting inhibition of proteoglycan synthesis within the first 4 days. Proteoglycan synthesis was still inhibited after a 12-day recovery period (Fig. 3A). This indicates that hydrogen peroxide production by monocytes/macrophages was not involved in the inhibition of proteoglycan synthesis.

Discussion
The present results suggest a possible mechanism by which RBC in combination with MNC, as present in whole blood, induce a lasting inhibition of cartilage proteoglycan synthesis. Based on these results we
hypothesize that haemoglobin-derived iron, together with an enhanced hydrogen peroxide production by chondrocytes owing to IL-1β stimulation, causes an increased formation of hydroxyl radicals, permanently damaging the chondrocytes.

In our test system the monocytes/macrophages were activated, illustrated by the increased production of IL-1β. As a result of exposure to this cytokine, chondrocytes are stimulated to increase their production of hydrogen peroxide [20]. Under normal conditions hydrogen peroxide is a by-product of redox cycling in healthy cells. We suggest that owing to the activation with IL-1β, in our culture system as well as in vivo, chondrocytes are stimulated and secrete large amounts of hydrogen peroxide, which diffuses into the pericellular matrix.

The presence of iron in joints frequently affected by joint bleeding has been described before [14]. The possible harmful effects of iron and iron compounds in cartilage have been described as well [25, 29–31]. It is known that iron can be released from macrophages after processing opsonized, damaged or senescent RBC [23, 32]. Iron can be released in the form of haemoglobin and ferritin, but also as non-protein-bound low-molecular-weight iron. We found that RBC are phagocytosed in vitro, as analysed by FACS analysis according to Bratosin et al. [33] and by microscopy, using May–Grünwald staining (data not shown). When the macrophages have processed RBC, different forms of iron can be released and become toxic to cartilage in our system. The toxicity of redox cycling metals, such as iron and copper, is based on Fenton-like reactions, i.e. the catalytic reduction of hydrogen peroxide into hydroxyl radicals [34].

Our results demonstrate that chondrocytes participate in an active manner in the induction of such adverse effects since no other cell type was present when we added IL-1β and lysed RBC to the cartilage. So, the added IL-1β could only affect the chondrocytes. Whole RBC (with spontaneous lysis during culture) or lysed RBC, as a pool of iron, are on their own not able to induce permanent adverse changes in chondrocyte activity. The lysed RBC in the presence of the IL-1β-stimulated chondrocytes induced a statistically significant prolonged inhibition of proteoglycan synthesis. The effect was less prominent than the effect of MNC plus RBC or whole blood. This may be explained by the fact that other catabolic factors are involved, such as TNF-α, which is known to have adverse effects on cartilage matrix turnover and adds to the increase in hydrogen peroxide production by chondrocytes [6, 7, 35]. Another issue might be the process in which macrophages handle the degradation of RBC and release iron compounds. This process might be much more efficient and result in a more continuous release of iron compounds, compared with our one-time addition of lysed RBC. Catalytic ferrous iron has a relatively short half-life.

In our culture system it is possible that the hydrogen peroxide, produced by the chondrocytes, is transformed into hydroxyl radicals under the catalytical conditions created by ferrous ions [36]. The results showing that DMSO was capable of restoring the proteoglycan synthesis corroborated that hydroxyl radicals are involved. By scavenging hydroxyl radicals, DMSO prevents damage to chondrocytes and with that the lasting inhibition of cartilage matrix turnover by chondrocytes. The fact that catalase, which reduces hydrogen peroxide, a precursor molecule for hydroxyl radicals, does not restore the proteoglycan synthesis implies that the hydrogen peroxide formed outside the cartilage matrix (i.e. by the monocytes/macrophages) is of no importance to the adverse changes in chondrocytes. It is not reasonable to expect catalase to penetrate the cartilage matrix owing to its size, which makes it unable to act on the hydrogen peroxide formed by the chondrocytes. The point that hydrogen peroxide formation by monocytes/macrophages is not harmful to the chondrocytes is supported by the fact that these molecules, in the presence of catalytic iron, will be reduced to hydroxyl radicals that have an extremely short half-life and thus have to be formed in the vicinity of the chondrocytes to be toxic.

We were unable to demonstrate actual levels of hydroxyl radicals, hydrogen peroxide and the direct involvement of catalytic iron in our culture system. The present data only provide indirect evidence for the involvement of hydroxyl radicals. We tried to use a specific analytical method for direct oxidative stress measurement by electron-paramagnetic spin trap resonance spectrometry (EPR spectrometry). The low density of cells in the dense extracellular matrix made it impossible to obtain reliable results. Other techniques such as biochemical or histochemical analysis of, for instance, thiobarbituric acid reactive material (TBAR) might be explored to demonstrate hydroxyl radicals directly, but these approaches will also encounter the difficult properties of cartilage tissue.

Owing to the fact that the release of haemoglobin, either macrophage-derived or by RBC lysis, interferes with the current methods of analysis (personal observations), we were unable to measure the actual levels of hydrogen peroxide. The analysis is based on substrate conversion by hydrogen peroxide, which can be detected photospectrometrically, in which haemoglobin interferes with the absorbance spectrum. Also, the direct measurement of hydrogen peroxide after IL-1 stimulation may be hampered by the tissue characteristics. However, for isolated chondrocytes, the direct effect of IL-1 on hydrogen peroxide production has been clearly demonstrated [22].

We also investigated the possibilities of interception of the catalytical iron. To this end we tested whether chelation of iron cations with the iron chelators deferoxamine (DFO) and deferiprone (L1) was possible. However, the amounts of DFO and L1 to be used for chelation were toxic to chondrocytes (personal observations). Thus, unfortunately, iron chelation was impossible to investigate in our tissue culture technique.

In summary, we hypothesize that haemoglobin-derived iron from RBC, together with an enhanced hydrogen peroxide production by chondrocytes as a
result of stimulation by IL-1β produced by activated monocytes/macrophages, causes an increased formation of hydroxyl radicals, permanently damaging the chondrocytes and thus causing the prolonged inhibition of cartilage proteoglycan synthesis as observed after blood exposure. Although we feel that this mechanism is important in blood-induced cartilage damage, we cannot rule out the involvement of other mediators. For example, the production of nitric oxide (NO), known to induce chondrocyte apoptosis [37], might be involved. However, the ineffectiveness of ebselen in our culture system, scavenging the product (peroxynitrite) of NO with superoxide (which are concurrently produced [38]), is not supportive in this respect. However, a number of other mechanisms, not studied so far, may still take part in blood-induced joint damage.

The present findings may have clinical implications for haemophiliacs, but also for patients who have suffered from traumatic bleeding. The hydroxyl radical-mediated toxicity may already have altered the metabolic state of the chondrocytes or may have induced chondrocyte death after a very short period of intra-articular blood exposure. We found a significant increase in chondrocyte toxicity after 4 days of co-culture of cartilage with whole blood or MNC plus RBC (manuscript under revision). However, because cartilage damage is a very slow process, it will take many years before such damage becomes clinically evident. Therefore, prevention of free radical damage might be evaluated as a new tool in the treatment of blood-induced joint damage.

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References


