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RECOMBINANT HUMAN INTERLEUKIN-1 INHIBITS PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1) PRODUCTION BY HUMAN ARTICULAR CARTILAGE AND CHONDROCYTES

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Human articular cartilage and chondrocyte monolayers in culture constitutively produced plasminogen activator inhibitor-1 (PAI-1) protein and mRNA, as assessed by a specific enzyme-linked immunosorbent assay and Northern blotting analysis, respectively. Recombinant human interleukin-1 (IL-1) invoked a dose-dependent inhibition of PAI-1 production in both cartilage and chondrocyte cultures. The inhibitory effect of IL-1 was observed between 2-8h after addition of the cytokine, while the optimal dose was between 10-100U/ml IL-1α (57-570pM IL-1α). Results obtained by Northern analysis of chondrocyte total RNA reflected those found for the PAI-1 antigen, namely, that nontreated chondrocytes showed PAI-1 mRNA which was reduced by IL-1 treatment. To our knowledge, this is the first report where IL-1 has been found to inhibit PAI-1 expression. Since IL-1 has been shown before to cause human cartilage destruction and a correlated change in plasminogen activator activity, it could be that a concomitant reduction in PAI-1 levels by IL-1 may be significant in the control of these changes in cartilage.

Plasminogen activators (PAs) , urokinase-type PA (u-PA) and tissue-type PA (t-PA), are serine proteinases which convert plasminogen to the broad spectrum proteinase, plasmin, and as such they have been implicated in tissue destruction and remodelling (1,2). A role has been proposed for PA in the cartilage destruction observed in arthritis on account of its presence in rheumatoid synovial fluids (3) and in the culture fluids of joint cells (i.e. chondrocytes and synoviocytes) (4,5). The activity of PA can be modulated by its level of biosynthesis, secretion,

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, heat-inactivated fetal calf serum; IL-1, interleukin-1; PA, plasminogen activator; PAI, plasminogen activator inhibitor; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

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activation, by the actions of specific and fast-acting inhibitors and, for u-PA, by binding to cell surface receptors (6,7).

The cytokine, interleukin-1 (IL-1), is a product of activated monocyte/macrophages (8), is also found in rheumatoid synovial fluids (9), and can cause cartilage destruction through chondrocyte activation (4,10,11). IL-1-mediated cartilage destruction may well involve u-PA and/or t-PA since their levels of activity are elevated in IL-1-treated cartilage cultures (4).

At least two specific inhibitors of PA (PAIs) have been described, from a variety of sources, some of which could be important in controlling PA activities in diseased joints (12). These include PAI-1 (endothelial-type PAI) and PAI-2 (placental or macrophage/monocyte-derived PAI). PAI-1 is the major intravascular inhibitor of tissue-type PA and is also produced by endothelial cells in culture (13). PAI-2 is the primary PAI of macrophages, neutrophils and other cells of hemopoietic origin (14). PAI-1 and PAI-2 are secreted proteins regulated by a large variety of hormones, cytokines and growth factors (for reviews, see 12,15).

Variations in PAI activity in biological samples may be due to not only variations in the concentrations of the inhibitors but also of the PAs. In addition, inhibition assays and neutralizing antibodies do not allow ready detection of one type of inhibitor if the other type is present in large excess. These difficulties have been overcome by the development of specific and sensitive enzyme-linked immunosorbent assays (ELISA).

A study by Yamada *et al.* (16) has reported the presence of PAI-1 in human chondrocyte cultures by the neutralization of PAI activity with specific antisera. The object of the present study was to monitor the production of PAI-1 in human cartilage and chondrocyte cultures and to determine whether IL-1 modulates PAI-1 levels in these cultures. For this purpose we have utilized a specific ELISA which uses a combination of monoclonal and polyclonal antibodies raised against human PAI-1 for the detection of PAI-1 levels in the culture fluids of human cartilage and chondrocytes cultured with and without IL-1. In addition, our findings were verified at the level of mRNA by Northern analysis. It is suggested that IL-1 effects an increase in chondrocyte PA activity by inhibiting PAI-1 production in addition to its stimulation of u-PA and t-PA synthesis.

MATERIALS AND METHODS

Reagents: Reagents were obtained as gifts from the following sources: recombinant human IL-1α, (Hoffmann-La Roche, Nutley, NJ); recombinant human IL-1β (Dr. A. Shaw, Glaxo, Geneva, Switzerland); human PAI-1 cDNA (Dr. D. Loskutoff, La Jolla).

Cartilage and cell culture: Macroscopically normal human articular cartilage was obtained from the femoral condyles of above-knee amputation specimens which were surgically removed as a result of lower limb ischemia or trauma. Cartilage and primary chondrocyte monolayer cultures were prepared and established as previously described (4), chondrocytes being isolated from the

cartilage by sequential enzymic digestion. All cultures (tissue and cells) were performed in 24-well plates initially containing 1ml/well of Dulbecco's modified Eagle's medium (DMEM), further modified as described (17), supplemented with 5%(v/v) heat-inactivated fetal calf serum (FCS), and were maintained in a humidified incubator gassed with 5% CO_2/95% air. Wells contained approximately 50mg (wet weight) tissue or 4 x 10^5 chondrocytes. Only high density, primary chondrocyte cultures were used in these studies as such culture conditions are conducive to the retention of the chondrocyte phenotype; we have previously found that, under these conditions, the cells synthesize type II (chondrocyte) but not type I (fibroblast) collagen, as assessed by Northern analysis of extracted RNA and by SDS-polyacrylamide gel electrophoresis of conditioned culture media (4 and unpublished observations).

IL-1-stimulation of cartilage/cells: Following 3-4 days incubation, the tissue/cells were washed, changed to DMEM + 1%(v/v) plasminogen-depleted acid-treated FCS and stimulated (triplicate wells) for usually 48h with the IL-1. The use of the above FCS permitted the measurement of the PA activity in the conditioned media - a concurrent study which has been reported elsewhere (4). At termination, the conditioned media were harvested, the cartilage pieces were weighed and the cell monolayers, once washed, were removed in 0.2%(v/v) Triton X-100. Spent media and cell lysates were stored at -20°C until subsequent assay. Polymyxin B, which inhibits the action of lipopolysaccharide (18), was preincubated (15min/37°C) with the IL-1 prior to their addition to cultures, where it had a final concentration of 1μg/ml - this was to reduce the likelihood of any possible effects of endotoxins on the target cells. For experiments examining chondrocyte PAI-1 mRNA synthesis, chondrocytes were seeded into 6-well plates at 1.5 x 10^6 cells/well in 4ml DMEM + 5%(v/v) FCS. After 4 days the cells were washed, given 2ml/well of fresh DMEM + 1%(v/v) plasminogen-depleted acid-treated FCS containing either 50U/ml IL-1α or no IL-1 (control) and the culture continued for 7h before the total RNA was extracted.

ELISA for PAI-1: PAI-1 concentrations in conditioned culture fluids and cell extracts were measured by a sandwich ELISA using a combination of monoclonal and polyclonal antibodies, as previously described (19). Briefly, 96-well plates (Nunc-Immuno Plate IIU, Nunc, Denmark) were coated with monoclonal anti-PAI-1 IgG by overnight incubation at 37°C. Samples or affinity purified PAI-1 standard were then applied to the wells followed by polyclonal rabbit anti-PAI-1 IgG and finally peroxidase-conjugated swine antibodies against rabbit Ig. The detection limit of the ELISA was ~1ng/ml and the absorbance was linearly related to the PAI-1 concentration up to 5ng/ml. The PAI-1 ELISA recognizes free PAI-1, as well as PAI-1 in complex with u-PA or t-PA (19).

Northern analysis of total extracted RNA: The procedures for Northern analysis and hybridization were performed as described by Sambrook *et al.* (20). Following culture, cells were immediately lysed and the total RNA extracted by the guanidine isothiocyanate/cesium chloride method. The RNA from duplicate wells was pooled, size fractionated by electrophoresis through a formaldehyde-containing 1.4%(w/v) agarose gel and transferred to Hybond nylon membranes (Amersham). Hybridizations were conducted at 42°C overnight in a buffer containing 50%(v/v) formamide (20) with >2 x 10^6 cpm/ml heat-denatured [^{32}P]PAI-1 cDNA fragments (21), which were labelled by the random primer method (20). After hybridization the membranes were washed several times, finishing with two washes in 0.2x SSC/1%(w/v) SDS at 65°C for 30min each, and were then exposed to Kodak XAR-5 film, overnight at -70°C.

Assay for IL-1 activity: These were performed as detailed elsewhere (17,22). One unit/ml IL-1 activity was equivalent to 5.7pM IL-1α and 2.3pM IL-1β.

Statistics: Results are expressed as the mean ± SEM for n=3 observations. Where applicable data were compared using Student's *t*-test (two-tailed) where $P<0.05$ was considered significant.

RESULTS

Both organ cultures of human articular cartilage and high density, primary monolayer cultures of chondrocytes, isolated by enzymic digestion of such tissue, produced basal levels of PAI-1, as assessed by ELISA of the conditioned culture media (Figs.1 & 2). Since we have previously reported the stimulation of u-PA and t-PA activities in chondrocyte and cartilage cultures by IL-1 (4), and also obtained evidence of enzyme/inhibitor complexes containing PA in these cultures by zymography of conditioned media on both fibrin and casein (4,22), we were interested in examining the modulation of PAI-1 by IL-1. We found that recombinant human IL-1α inhibited in a dose-dependent manner the synthesis of PAI-1 by both organ and cell cultures (Figs.1 & 2, respectively). The two culture procedures yielded similar results, with optimum doses for IL-1α ranging between 10-100U/ml and effects being observed with as little as 0.1U/ml IL-1α. Although Figure 1 shows only partial inhibition (73%) of PAI-1, in two out of three other cartilage experiments, and in all of three chondrocyte cultures, complete inhibition was attained when IL-1 was used at its optimal dose, as observed in the chondrocyte culture in Figure 2. IL-1β elicited a similar response to IL-1α at comparable doses of IL-1 activity (data not shown). No PAI-1 could be detected in the cell lysates of either control or IL-1-treated chondrocyte cultures (data not shown). Kinetics studies showed that differences in the levels of PAI-1 synthesized by nontreated and IL-1-treated chondrocytes could be detected as early as 2-

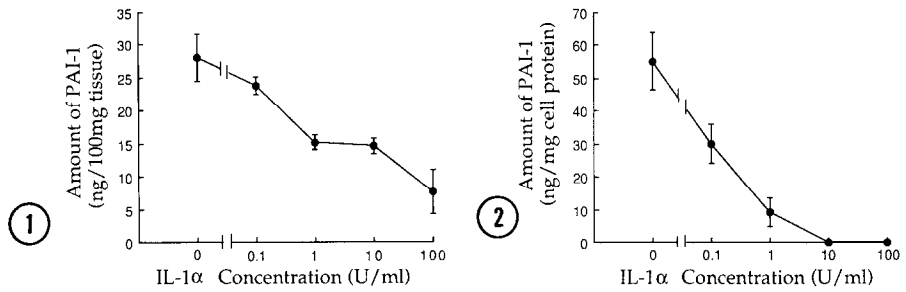
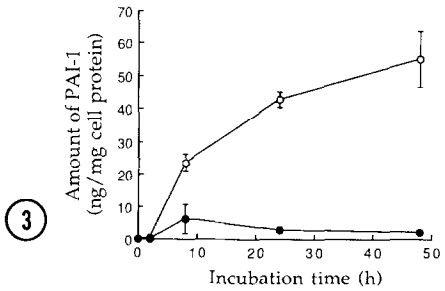


Figure 1. Effect of IL-1α concentration on PAI-1 production by human articular cartilage in culture.

Cartilage organ cultures, prepared and established as described in Materials and Methods, were incubated for 48h with various concentrations of IL-1α (1U/ml is equivalent to 5.7pM). The amounts of PAI-1 accumulated in the culture media were then determined by ELISA. Data represent the mean ± S.E.(n=3); where absent, S.E. < symbol size. The IL-1-treated cultures contained significantly less ($P<0.05$) PAI-1 levels compared with the control for IL-1 concentrations ≥1U/ml.

Figure 2. Effect of IL-1α concentration on PAI-1 production by human articular chondrocytes in monolayer culture.

High density, primary chondrocyte monolayer cultures, prepared and established as described in Materials and Methods, were incubated for 48h with various concentrations of IL-1α (1U/ml is equivalent to 5.7pM). The amounts of PAI-1 accumulated in the culture media were then determined by ELISA. Data represent the mean ± S.E.(n=3); where absent, S.E. < symbol size. The IL-1-treated cultures contained significantly less ($P<0.01$) PAI-1 levels compared with the control for IL-1 concentrations ≥1U/ml.



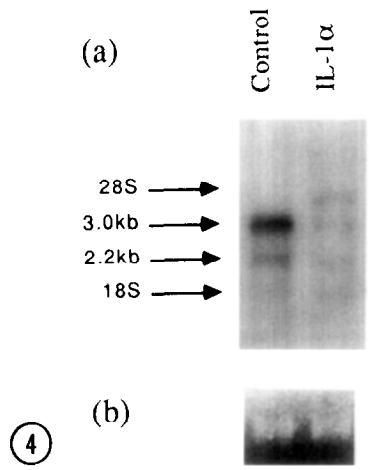


Figure 3. Kinetics of chondrocyte PAI-1 production and its inhibition by IL-1α.
Chondrocyte monolayers were incubated for different times with either 0.17nM IL-1α (30U/ml) (●) or without stimulus (○). The amounts of PAI-1 accumulated in the culture media were then determined and are expressed as the mean ± S.E.(n=3); where absent, S.E. < symbol size. The IL-1-treated cultures contained significantly less ($P<0.05$) PAI-1 levels compared with the controls for incubation periods ≥8h.

Figure 4. The effect of IL-1α on PAI-1 mRNA levels in chondrocyte monolayer cultures.
(a) Three microgram of total cellular RNA from human articular chondrocytes, incubated for 7h with control media or 0.29nM IL-1α (50U/ml), was analyzed by Northern blotting and hybridization using a [^{32}P]-labelled cDNA specific for human PAI-1. (b) The lower panel shows ethidium bromide staining of total RNA (28S band) under U.V. light.

8h after exposure of the cells to IL-1α (Fig.3). The range of effective doses of IL-1 and the time-course of its effect is comparable to that which we have previously reported in our cartilage and chondrocyte cultures for the stimulation of u-PA, t-PA and prostaglandin E_2 synthesis by IL-1 (4,17).

To determine whether the IL-1-induced inhibition of PAI-1 levels in the cultures is associated with reduced levels of PA-1 mRNA, equal amounts of total RNA extracted from control cultures and chondrocytes treated for 7h with 50U/ml IL-1α were analyzed by Northern analysis. Figure 4(a) shows that human articular chondrocytes have detectable PAI-1 mRNA and that this is down-regulated by IL-1α. The size of the PAI-1 mRNA is consistent with previously reported findings of a 2.2kb mRNA and a more abundant, poly-adenylated 3.0kb species (21). Even loading of RNA was verified by ethidium bromide staining under U.V. light (Fig.4b).

DISCUSSION

This study has employed a specific ELISA to demonstrate that human articular cartilage and chondrocytes constitutively produce PAI-1 in culture and that its levels are down-regulated by IL-1α and β. Furthermore, we have shown that the reduced PAI-1 antigen levels observed

in IL-1-treated cultures is also reflected at the level of the mRNA. PAI-1 has been previously reported as a minor component of PAI activity in human chondrocyte cultures although its synthesis was not monitored (16). The present findings are also unique in that, until now, IL-1 has been reported only to up-regulate PAI-1 expression in cells such as human endothelial cells (23) and human lung fibroblasts (24).

IL-1, which is present in rheumatoid lesions, can cause cartilage destruction *in vitro* (4,10) and *in vivo* (11) and is considered to be a key cytokine involved in mediating the cartilage destruction of inflammatory arthritis. While it is generally agreed that the tissue destruction observed in arthritis and in IL-1-treated cartilage cultures is mediated by proteolytic enzymes, the pathway involved remains unclear. In view of its presence in rheumatoid synovial fluids (3), and in cartilage cultures undergoing resorption in response to IL-1α and β, tumor necrosis factor α and β and all-trans retinoic acid (4,22), PA could be involved. Plasmin, generated by the proteolytic cleavage of plasminogen by PA, can degrade cartilage proteoglycans (25) as well as activate latent collagenase (26) and stromelysin (27). Chondrocyte-derived PAI-1 could therefore have an important regulatory role in maintaining the normal turnover rate of cartilage matrix through its inhibition of PA. We would also like to suggest that down-modulation of PAI-1 expression in human cartilage by IL-1 could be contributing to the increased PA activity in the cultures and hence to the enhanced cartilage breakdown.

The mechanism by which IL-1 mediates a reduction in chondrocyte PAI-1 mRNA requires further investigation. Although the simplest explanation would involve a direct inhibition of PAI-1 gene transcription, in view of the fact that IL-1, when effective, generally elevates mRNA levels (e.g. 24,28-31), one could postulate the presence of an inhibitory transcriptional factor, which is positively regulated by IL-1. We are currently investigating these possibilities using transcriptional and translational inhibitors.

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