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Tumor necrosis factor and interleukin 1 activate phospholipase in rat chondrocytes

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Tumor necrosis factor (TNF) and interleukin 1 (IL-1) are both cytokines of macrophage origin with similar activity on several cell types. We investigated whether TNF can, analogously to IL-1, stimulate phospholipase activity of chondrocytes. Addition of each of these cytokines to cells, isolated from the xiphisternum of adult rats, resulted in a time- and dose-dependent increase in phospholipase activity in both secreted and membrane-associated form. Moreover, TNF and IL-1 both induce a transformation of chondrocyte morphology. In conclusion, TNF stimulates chondrocyte phospholipase activity and extends the long list of actions shared by IL-1 and TNF in a diversity of cellular systems.

Tumor necrosis factor; Interleukin 1; Phospholipase; (Rat chondrocyte)

1. INTRODUCTION

Inflammation of joints is associated with degradation of connective tissue; this is believed to be mediated by macrophage-derived polypeptides, such as IL-1, produced after stimulation. This monokine exerts a wide range of biological activities, some of which lead to bone resorption and cartilage destruction [1,2]. These activities are at least partly mediated by an IL-1-induced activation of PLA_2 resulting in production of PGE_2 in cultured chondrocytes [2] and bones [3]. Two distinct human IL-1-cDNAs have been cloned and expressed [4]. The corresponding monokines, IL-1α and IL-1β, both bind to the same receptor, apparently without strict species preference [5].

Tumor necrosis factor is another macrophage- and monocyte-derived protein that has been described to exert multiple effects on different biological systems [6]. Its most promising action is a specific cytotoxic activity towards certain tumor cells, both in vivo and in vitro [7]. TNF cDNA has been cloned by several groups; its expression in *Escherichia coli* results in biologically active, recombinant TNF [8-10]. Fiers et al. [11] reviewed the many activities shared by TNF and IL-1, including some possibly involved in inflammation and tissue damage. TNF, analogous to IL-1, stimulates collagenase and PGE_2 production by synovial cells and fibroblasts [12]. Furthermore, both TNF and IL-1 stimulate phospholipid hydrolysis in synovial cells [13].

These data led us to investigate whether TNF can activate phospholipase activity in chondrocytes.

2. MATERIALS AND METHODS

2.1. *Isolation and cultivation of chrondrocytes*

Chondrocytes were released by enzymatic digestion of xiphisternum slices of adult Fischer rats by a procedure slightly modified after Benya et al. [14]. Cells were grown in Dulbecco's modified minimal essential medium (DMEM, Gibco Europe, Ghent, Belgium), supplemented with 5% heat-inactivated (30 min at 56°C) newborn calf serum (Gibco Europe), 100 U/ml penicillin and 0.1 mg/ml streptomycin.

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Abbreviations: IL-1, interleukin 1; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; TNF, tumor necrosis factor

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2.2. *Phospholipase extraction*

Cells grown in 10 cm^2 Petri dishes to confluency were rinsed twice with DMEM and incubated in the presence of TNF or IL-1 in 1 ml serum-free medium. At appropriate times supernatant was extracted as follows. Cells were rinsed twice with ice-cold buffer (10 mM Hepes + 0.5 mM $MgCl_2$, pH 7.5) and scraped off with a rubber policeman in 300 μl of the same ice-cold buffer. After 30 min incubation on ice the cell suspension was dounced 30 times; the membranes were isolated by centrifuging at $2000 \times g$ for 5 min, followed by a second centrifugation of the supernatant at $10000 \times g$ for 10 min. Phospholipase was then differentially extracted in 0.18 N H_2SO_4.

2.3. *Assay for phospholipase activity*

Phospholipase activity was measured by the hydrolysis of [^{14}C]arachidonic acid-labeled *E. coli* phospholipids [15]. 50 μl of phospholipase extract and 2.5×10^8 labeled *E. coli* bacteria (10000 cpm) were adjusted to a total volume of 0.5 ml with 100 mM Tris, pH 7.5, and 5 mM $CaCl_2$ buffer. Incubation took place for 15 min at 37°C. The reaction was stopped by addition of 1.5 ml of chloroform/methanol (1:2; v/v), followed by vigorous shaking for 15 min at 37°C. Finally, 1 ml of chloroform and 1.5 ml of water were added and mixed; the lower chloroform/lipid phase was removed and evaporated. The dry residue was redissolved in 50 μl of chloroform/methanol (9:1, v/v) and spotted on silica gel thin-layer chromatography plates (Merck, Darmstadt, FRG). These were developed in a solvent system containing petroleum ether:diethyl ether:acetic acid (80:20:1, v/v). Radioactive spots were localized by fluorography according to Bonner et al. [16] and cut from the plate for liquid scintillation counting. The percentage of hydrolysis was calculated as follows (all values in cpm):

$$\frac{\text{free fatty acid}}{\text{total phospholipid} + \text{free fatty acid}} \times 100.$$

The values were corrected for spontaneous release of [^{14}C]arachidonic acid from *E. coli* (ranging from 3.8 to 6%).

2.4. *Monokine preparations*

Recombinant human TNF was produced by *E. coli* and purified to at least 99% homogeneity [10]. The preparation used had a specific activity of 2 to 3×10^7 U/mg protein (units as defined by Ostrove and Gifford [17] in a cytotoxicity assay on L929 cells). The preparation contained less than 13 ng/mg endotoxin; it was stored in aliquots in phosphate-buffered saline at −70°C. Recombinant human IL-1β was a gift from Dr A. Shaw (Biogen SA, Geneva, Switzerland); the specific activity was 2.7×10^8 U/mg protein (units as defined by Erard et al. [18] in a cytotoxicity-induction assay with PC60 cells).

3. RESULTS AND DISCUSSION

Treatment of chondrocytes in culture in serum-free medium with TNF or IL-1 resulted in an increase in extracellular phospholipase activity. This increase was dose dependent and still increasing in the range of monokine concentrations tested (table 1). Furthermore, the increase in enzyme activity was caused by stimulation of neither chondrocyte proliferation nor non-specific secretion of protein, as there was no difference in the amount of extracellular protein between cells treated or untreated with TNF or IL-1.

Table 1

Dose-dependent increase in phospholipase activity in the supernatant of rat condrocytes in the presence of increasing concentrations of IL-1 or TNF for 72 h

U/ml of monokine used	Phospholipase activity (% hydrolysis) after treatment with	
	TNF	IL-1
0	7.0	7.0
1	7.9	9.8
3	7.3	11.4
10	9.1	12.8
33	11.2	12.0
100	16.0	15.0
330	15.0	15.9
1000	18.1	16.9
3300	21.4	17.8

In spite of some background activation in the untreated control, it was clear that secreted phospholipase activity also increased according to the duration of TNF or IL-1 treatment (fig.1). In agreement with the results obtained by Chang et al. [2] we found that the increase in monokine-mediated phospholipase release requires a relatively long period of TNF or IL-1 treatment.

As it is known that PLA_2 is a membrane-bound enzyme [19], we investigated the effect of TNF on cell-associated phospholipase activity. Treatment of chondrocytes for 48 h with various concentrations of TNF or IL-1 resulted in a dose-dependent stimulation of membrane-associated phospholipase activity (fig.2). In these (and previous) experiments we did not determine the specificity of the phospholipase involved. It is, however, likely that the enzyme is PLA_2, because (i) most arachidonic acid added to *E. coli* is incorporated in the C_2 position of phospholipids [2]; (ii) IL-1 induces PLA_2 [2]; (iii) we previously found evidence for an involvement of PLA_2 in TNF-action in mouse fibrosarcoma cells [20].

Cultured chondrocytes have a typically flat morphology. After treatment with TNF, and especially with IL-1, the cells acquire a more contracted and

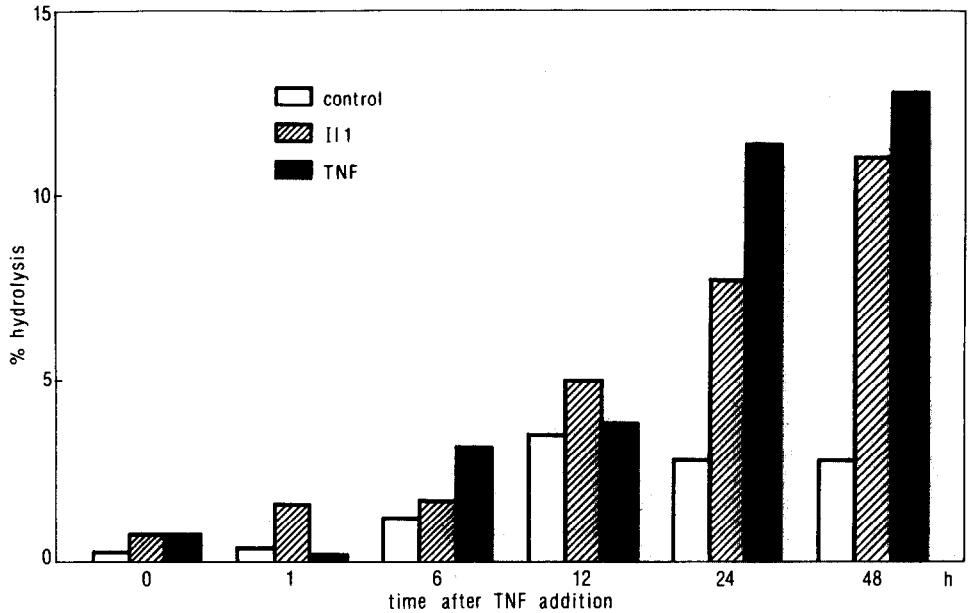

Fig.1. Effect of the addition of 5000 U TNF/ml or 5000 U IL-1/ml on phospholipase activity in the supernatant of rat chondrocytes as a function of time.

refractile morphotype (fig.3). We do not know if there is a correlation between the increased phospholipase activity and this change in morphology. It was, however, found that the effect of IL-1 on cell morphology is much more pronounced than that of TNF, at a concentration at which both monokines stimulate phospholipase activity equally well. TNF induces phospholipase activity in mouse fibrosarcoma cells [20–22]; this seems, however, not to be the only mechanism that regulates the sensitivity of a given cell line towards TNF or IL-1-induced cytotoxicity. Indeed, chondrocytes – which also show an increased phospholipase activity after treatment with these monokines – are not sensitive to the cytotoxic action of TNF or IL-1.

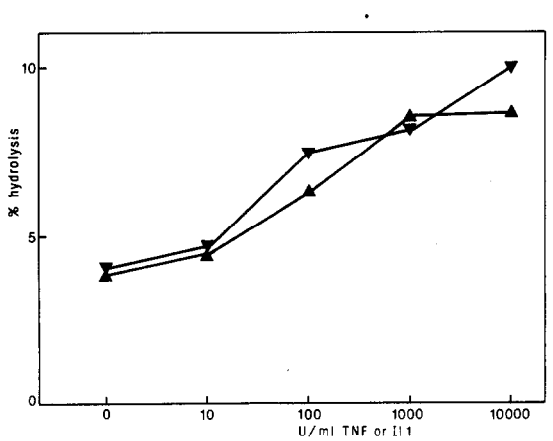

Fig.2. Dose-related induction of membrane-bound phospholipase activity of rat chondrocytes after treatment with increasing concentrations of TNF or IL-1. The cultures were treated for 48 h with TNF (▲) or IL-1 (▼).

The mechanism by which TNF and IL-1 activate phospholipase still needs to be determined. Possible explanations are: modulation of lipocortin activity, modulation of phospholipase activity protein, regulation of Ca^{2+} inflow, proteolytic activation of phospholipase, induction of membrane changes or de novo synthesis (review [19]). We do not yet know whether both TNF and IL-1 activate phospholipase through the same mechanism. Both could, however, be important mediators of some diseases such as rheumatoid arthritis [23], considering that soluble phospholipase has been detected in rheumatoid synovial fluid [24]. Phospholipase induction in chondrocytes, as reported here, is one more example of the biological activities shared by TNF and IL-1. Consequently, it is the more striking that these two

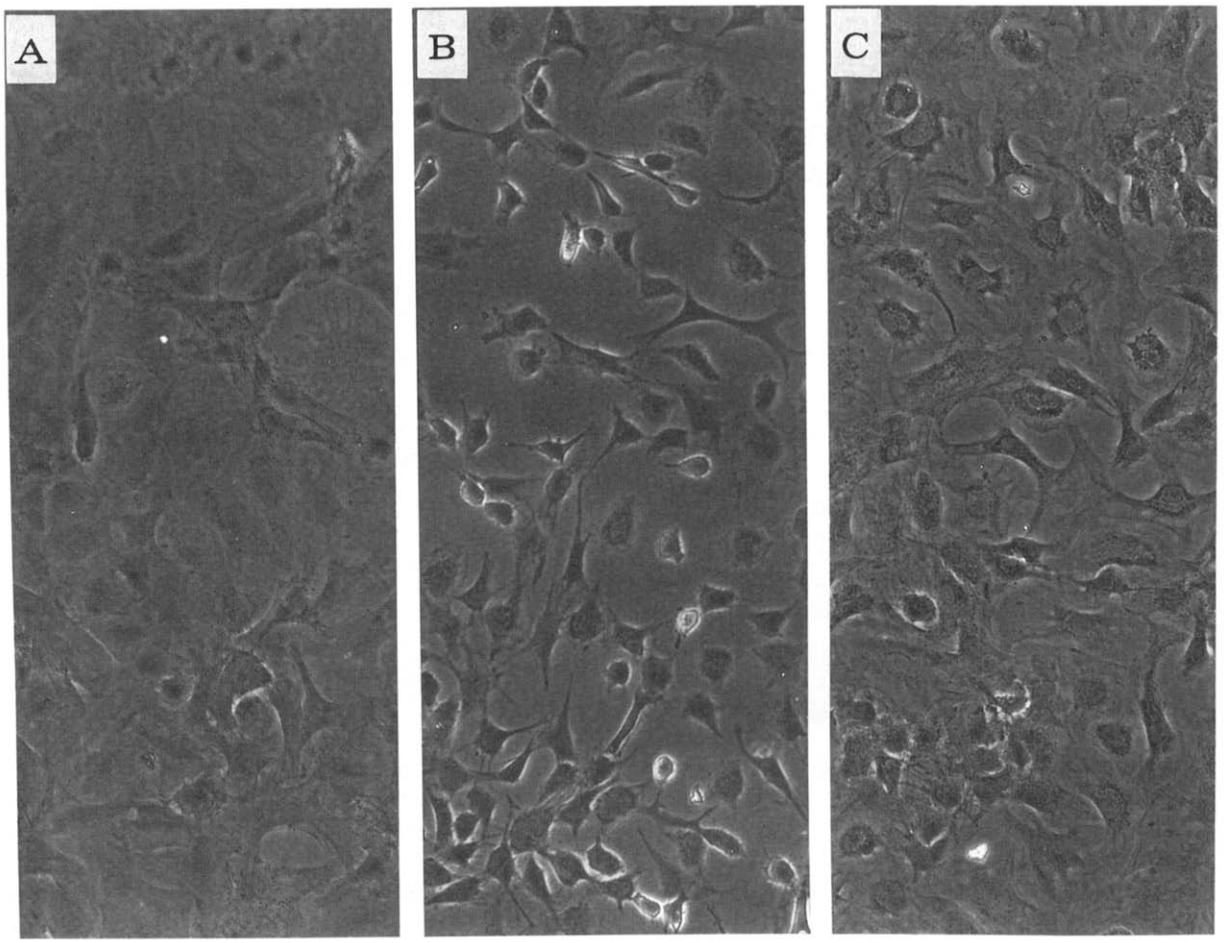

Fig.3. Morphotype of rat chondrocytes influenced by treatment with TNF or IL-1. A confluent monolayer of chondrocytes was either untreated (A), or stimulated with 5000 U/ml IL-1 (B) or 5000 U/ml TNF (C) for 72 h. Phase contrast micrographs using a 25 × objective.

monokines exert, generally speaking, dissimilar cytotoxicity-inducing activities on malignant cells [25].

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