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Release of proteases from cartilage cells as a result of activation by a macrophage factor—effects of some anti-inflammatory drugs

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An important facet of the pathology of rheumatoid arthritis is the degeneration of articular cartilage due to the active release of proteases by hypercellular synovial tissue and infiltrated mononuclear cells in the joint [1–5]. The extent of participation of chondrocytes in this process has not been determined. The clinical status of a typical osteoarthritic joint is different. The synovium exhibits little or no inflammation, only a few monocytes and lymphocytes have infiltrated into the joint, the levels of proteases in the synovial fluid are not significantly altered, and the degradation of cartilage-matrix occurs progressively over a long time period. Recent reports indicate that normal human articular cartilage contains low levels of metalloproteases [6]. The activities of cathepsin D, collagenase and other neutral proteases are high in osteoarthritic cartilage [7–9], although the mechanisms underlying these changes are not known. We have demonstrated recently that the chondrocytes from normal rabbit articular cartilage secrete very small amounts of proteases in their culture medium. Nevertheless, they can be activated repeatedly by a macrophage-derived inducer to produce high levels of these degradative enzymes [10]. The present study deals with the effects of various anti-inflammatory drugs on the induction of chondrocytic enzyme release using this *in vitro* model system.

The chondrocytes were isolated from articular cartilage of 2-month-old rabbits by enzymatic digestion, plated at a density of 3×10^5 cells/25 cm^2 flask, and grown to confluency in Ham's F-12 nutrient mixture, containing fetal calf serum (10% v/v) and antibiotics, in an atmosphere of 5% CO_2 in air [10].

The induction of cellular infiltration in the peritoneum of rabbits was carried out by an intraperitoneal injection of sterile light mineral oil. After 96 hr, the peritoneum was washed with Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), antibiotics, 2 mM glutamine and 2 units/ml of heparin. The lavage was centrifuged, the cells were washed twice with the same medium (without heparin), and then plated at a density of 1×10^6 cells/cm^2. After 4 hr, the medium and floating cells were removed. The adherent cells were mostly (at least 95 per cent) macrophages, as judged from their capability to phagocytoze oil droplets or latex particles. These cells were incubated overnight with 4 ml of DMEM (without serum), containing 30 μg/ml of lipopolysaccharide fraction (LPS) from *Escherichia coli* (Difco; 055:B5). The next day, the conditioned medium of macrophages (MCM) was removed and stored frozen [10].

The confluent chondrocytes were treated with MCM, diluted 1:5 with DMEM. Each chondrocyte-culture flask received 4 ml of the diluted medium. After a 48-hr incubation, the medium was dialyzed against 50 mM Tris-HCl buffer (pH 7.5), containing 200 mM CaCl, 5mM $CaCl_2$ and 0.02% NaN_3. Aliquots of the medium were treated with trypsin (100 μg/ml) for 10–15 min at 25° and then with soybean trypsin inhibitor (300 μg/ml) for 20 min at 25°, to convert any latent enzymes to their active forms. The collagenase activity in the medium was assessed by incubation with reconstituted fibrils of [^{14}C]glycine-labeled rat skin collagen (sp. act. 50,000–60,000 dis./min/mg) for 2 hr at 37°. The activities of other neutral proteases were estimated in the same manner, using [^{14}C]leucine-labeled globin as the substrate (sp. act. 15,000–20,000 dis./min./mg) [10].

The effects of various anti-inflammatory drugs on the release of these enzymes by chondrocytes were studied by adding them at various concentrations to the chondrocyte medium at the same time that MCM was added. Similar concentrations of the drugs were also added to the untreated chondrocytes as controls. After 48 hr at 37°, the media were dialyzed against Tris–HCl buffer to remove excess drugs. To assess the cytotoxic effects of the drugs, the total number of viable chondrocytes in each culture flask was estimated from their resistance for inclusion of trypan blue dye.

The results in Table 1 represent the inhibition of enzyme release by chondrocytes due to the presence of various drugs in the medium. As mentioned earlier, the confluent chondrocytes produced very small amounts of collagenase and other neutral proteases. The addition of MCM increased the enzyme secretion significantly during the 48-hr incubation period. To determine whether the chondrocytes synthesized a fraction of enzymes that was not released into the medium, the cells were isolated from the culture flasks with rubber policemen or by trypsin treatment. Trypsin was inactivated by the addition of excess soybean trypsin inhibitor or 10% FCS. The cells were frozen and thawed repeatedly, and the activities of collagenase and other neutral proteases in the cell-lysates were estimated after the treatment for conversion of any latent enzymes to the active forms. The levels of enzymes in the cell-lysates were undetectable, indicating that all the enzymes synthesized by chondrocytes were released into the medium.

Our previous findings had suggested that the optimum stimulatory effect on chondrocytes was observed when MCM was diluted 4- to 5-fold. Furthermore, the conditioned medium from the macrophages receiving no LPS showed minimal effect on the chondrocytes [10]. When LPS was added to the macrophages at various concentrations, 30 μg/ml of medium was found to the optimum concentration for maximum release of the stimulatory factor(s) (results not shown). The addition of varying amounts of LPS directly to the chondrocyte medium had no stimulatory or inhibitory effect on enzyme synthesis.

No significant effect was observed when the drugs were added to unstimulated chondrocytes. Paramethasone was extremely effective in suppressing the release of collagenase (approximately 70–80 percent inhibition) and, to a lesser extent, of neutral proteases by the MCM-treated chondrocytes. Aspirin was also very effective at 10^{-4} and 10^{-5} M

Table 1. Effects of drugs on the release of enzymes by chondrocytes

Drug	Conc. (M)	Collagenase Units	Collagenase % Inhibition	Neutral proteases Units	Neutral proteases % Inhibition
Chondrocytes + LPS*		0.33 ± 0.02		0.34 ± 0.04	
Chondrocytes + medium from unstimulated macrophages + LPS†		0.57 ± 0.05		4.11 ± 0.36	
Chondrocytes + MCM‡		14.41 ± 0.23		14.50 ± 1.17	
Paramethasone§	10^{-6}	2.37 ± 0.46	84	7.81 ± 0.84	46
	10^{-8}	4.71 ± 0.27	67	9.39 ± 0.03	35
Indomethacin	10^{-5}	9.49 ± 0.87	34	11.54 ± 0.78	21
	10^{-6}	9.21 ± 0.55	36	11.25 ± 1.03	22
	10^{-7}	11.49 ± 1.32	20	12.99 ± 0.79	10
	10^{-8}	12.91 ± 0.70	10	14.04 ± 0.82	3
Colchicine	10^{-6}	Cytotoxic		Cytotoxic	
	10^{-7}	7.89 ± 0.58	45	11.11 ± 0.96	23
	10^{-8}	8.78 ± 0.63	39	11.28 ± 0.62	22
Aspirin	10^{-4}	2.37 ± 0.46	84	8.42 ± 0.26	42
	10^{-5}	3.48 ± 0.66	76	9.06 ± 0.75	38
	10^{-6}	7.72 ± 0.23	46	10.77 ± 1.16	26
	10^{-7}	9.84 ± 0.54	32	12.92 ± 0.35	11
	10^{-8}	12.23 ± 0.68	15	14.56 ± 0.98	
D-Penicillamine	10^{-5}	10.18 ± 0.97	29	11.95 ± 0.87	18
	10^{-6}	11.42 ± 0.94	21	13.81 ± 1.13	5
	10^{-7}	12.94 ± 0.60	10	14.36 ± 0.95	1
Gold Na thiomalate	10^{-5}	11.00 ± 0.52	24	12.17 ± 0.59	16
	10^{-6}	11.87 ± 1.00	18	14.07 ± 0.82	3
	10^{-8}	14.45 ± 0.37		14.48 ± 0.78	
Phenylbutazone	10^{-6}	12.62 ± 1.41	12	12.05 ± 0.28	17
	10^{-8}	13.06 ± 1.45	9	12.91 ± 1.15	11
Fenoprofen	10^{-6}	13.36 ± 1.08	7	13.70 ± 0.78	6
	10^{-8}	14.73 ± 0.74		14.28 ± 0.65	2

* Chondrocytes were treated with LPS (6 μg/ml of medium).

† Oil-induced macrophages were not activated with LPS in culture. The conditioning medium was added to chondrocytes, undiluted or diluted 1:5 with DMEM, along with LPS (6 μg/ml).

‡ Chondrocytes were treated with the macrophage-conditioned medium (MCM), diluted 1:5 with DMEM (see text).

§ All the drugs were added simultaneously with MCM to the culture flasks. One unit of collagenase activity represents μg of [^{14}C]collagen degraded/min/total medium from one flask (1.8 to 2.0×10^6 cells). One unit of neutral protease activity represents μg of [^{14}C]globin degraded/min/total medium (4 ml) from one culture flask. The values are mean ± S.D.: N = 4. None of the drugs (except colchicine) had a cytotoxic effect on chondrocytes at the concentrations used in these experiments.

concentrations. Colchicine was cytotoxic at a concentration of 10^{-6} M, but inhibited the release of collagenase by approximately 40–45 percent at 10^{-7} and 10^{-8} M concentrations. Indomethacin (10^{-5}, 10^{-6} M) suppressed the release of collagenase by about 35 percent. D-Penicillamine and gold Na thiomalate were less effective in this respect, while little or no inhibition was observed in the presence of phenylbutazone and fenoprofen. In general, the suppression by these drugs of the release of neutral proteases was significantly lower than of collagenase. The potent drugs may inhibit the release of enzymes by affecting the cellular receptors or by other intracellular mechanisms rather than by directly inhibiting the enzymes in the medium. When added to the activated enzymes in the cell-free medium, these drugs showed a very different pattern of inhibition from that reported in Table 1 (results not shown).

The effects of some of the anti-inflammatory drugs have been studied with various cell culture or organ culture systems. Harris and Krane [11] have reported that, at 2.5×10^{-7} or 2.5×10^{-8} M concentration, the presence of colchicine in the medium increases the synthesis of collagenase by human rheumatoid synovial explants. This effect of colchicine was not due to the change in mitotic activity of the cells but to an overall increase in protein synthesis. In the present studies, colchicine did not stimulate collagenase production by chondrocytes, and the effect on mitotic activity of the chondrocytes could not be estimated because the cells were used at the confluency stage. Dayer *et al.* [12] have shown that dexamethasone, at 10^{-6} and 10^{-8} M concentrations, caused a marked inhibition of the synthesis of collagenase and prostaglandin E_2 by human synovial fibroblasts in culture. Furthermore, indomethacin inhibited the production of prostaglandin E_2, but stimulated collagenase synthesis by these synovial cells. In contrast, the production of collagenase by endotoxin-stimulated guinea pig peritoneal macrophages was inhibited significantly by indomethacin, and the inhibitory effect was overcome by the addition of excess exogenous prostaglandin E_2, indicating an involvement of prostaglandins in the release of collagenase by macrophages [13]. Werb *et al.* [14] have demonstrated the binding of glucocorticoids to specific receptors on the surface of mouse peritoneal macrophages, rabbit alveolar macrophages and human peripheral blood monocytes.

The oil-induced peritoneal macrophages from rabbits do

not produce any significant amounts of collagenase or other neutral proteases in culture. Nevertheless, when activated with LPS, they release a factor in the medium which induces the chondrocytes to synthesize proteases. The chondrocytes respond to this factor for 48–72 hr, after each addition of MCM [10]. The synovial cells obtained from normal or inflamed rabbit joints synthesize proteases in culture, after activation with latex particles or *Mycobacterium butyricum*. Nevertheless, these cells do not release any activators that will stimulate chondrocytic protease synthesis (K. Phadke *et al.*, unpublished data).

The osteoarthritic condition is associated frequently with a low grade inflammation and influx of some mononuclear cells into the joint cavity. The infiltrated macrophages, although few in number, may be activated by degradative products of the damaged tissue, local immune complexes, activators produced by the lymphocytes, etc., and may, in turn, stimulate the chondrocytes to produce proteolytic enzymes. As mentioned earlier, the synovium plays a minimum role in the process of cartilage destruction. The intrinsic enzymes, therefore, may be primarily responsible for the slow, but progressive cartilage degradation. The interference with the release of the majority of the enzymes may be an important factor in the treatment of osteoarthritis.

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Effects of deanol, choline and its metabolites on binding of [^{3}H]quinuclidinyl benzilate to rat brain membranes

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Choline and its analog, dimethylaminoethanol (deanol), elicit a variety of pharmacologic effects in both humans and animals which can be attributed to the activation of cholinergic neurons in the brain [1, 2]. However, neither compound is as active as acetylcholine (ACh) at cholinergic receptors in the peripheral nervous system, their potencies being 1000–100,000 times less than that of ACh [3–6]. Nevertheless, the possibility exists that these compounds, or one of their metabolites, stimulate cholinergic receptors in the brain. To test this hypothesis, we have compared deanol, choline and some of the metabolites of choline with cholinergic drugs for their ability to displace radiolabeled quinuclidinyl benzilate (QNB) from muscarinic receptors in rat brain membrane preparations.

Male Sprague–Dawley rats, weighing 120–150 g, were used. Binding of [^{3}H]–QNB to rat brain S_1 (whole brain minus debris and nuclei) fractions was measured by the method of Yamamura and Snyder [7]. Dimethylaminoethanol acetamidobenzoic acid (Deaner) was obtained from Riker Laboratories, Northridge, CA. All other compounds were purchased from the Sigma Chemical Co., St. Louis, MO.

Choline displaced [^{3}H]–QNB from its muscarinic binding site with an IC_{50} of approximately 1800 μM (Table 1). Choline was less potent than the other muscarinic receptor agonists by a factor that ranged from 20 for carbachol to 4000 for oxotremorine. The IC_{50} value for scopolamine, a muscarinic antagonist, was similar to that reported by Yamamura and Snyder [7], and more than 100,000 times as potent as choline.

Choline was more than three times as potent as its analog, dimethylaminoethanol, and was two to more than five times as potent as any of its metabolites, including phosphorylcholine, betaine aldehyde, CDP–choline or glycerylphosphorylcholine.

The finding that choline and deanol displace [^{3}H]–QNB from its muscarinic binding site in brain is consistent with results of previous studies showing that these compounds stimulate cholinergic receptors at high concentrations [3–5]. However, the fact that choline and its metabolites are considerably less potent than cholinomimetic drugs in displacing [^{3}H]–QNB from the mammalian brain muscarinic cholinergic receptors would indicate that the central cholinergic actions of choline cannot be attributed entirely to direct activation of these receptors.

In humans treated with therapeutically active doses of choline, the concentration of the compound in the cerebrospinal fluid is only 3 μM [8]. In studies in which choline has been administered to elicit an increase in the concentration of ACh in brain, the level of choline did not exceed 59 μmoles/kg (65 μM, assuming that 80 per cent of the tissue was aqueous) [9]. The concentration of choline needed to displace half the [^{3}H]–QNB from muscarinic receptors is more than 25 times this amount (Table 1.)