Vol. 154, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1988 Pages 421-428

INHIBITION OF CARTILAGE PROTEOGLYCAN SYNTHESIS BY INTERLEUKIN I

Hilary P. Benton and Jenny A. Tyler

Strangeways Research Laboratory,
Worts Causeway, Cambridge CB1 4RN, U.K

Received May 25, 1988

We have investigated the mechanism of inhibition of cartilage proteoglycan by interleukin 1. Proteoglycan synthesis was inhibited using lower doses of interleukin 1 than those required to cause cartilage resorption. There was no significant effect on DNA or total protein synthesis. Gel electrophoresis showed a direct inhibitory effect on core protein synthesis while pulse-chase experiments using radiolabelled sulphate showed no alteration in the rate of intracellular transport and secretion of completed proteoglycan. Chondrocytes incubated with cycloheximide showed a first-order decrease in rate of uptake of radiolabelled sulphate ($t_{1/2}$ = 25 mins) but interleukin 1 induced inhibition showed a delay of at least 1 hr, consistent with a requirement to deplete intracellular pools of protein before effects on post-translational events could be observed. Foetal and neonatal cartilage responded to the cytokine in a similar way to adult cartilage.

There is now substantial evidence to suggest that the macrophage-derived cytokine, interleukin 1 (IL1) is an important regulator of cartilage matrix metabolism. Many of these observations have been made in cell and tissue culture systems but IL1-like activity has also been detected in human joint effusions (1) and has been shown to induce leucocyte infiltration and degradation after injection into rabbit synovial joints (2). This suggests a possible physiological or pathological role for this mediator in human joint diseases. It is apparent that chondrocytes themselves express the genes encoding IL1 (3) and bear specific IL1 receptors on their surface (4).

IL1 and macrophage-derived IL1-like factors have been shown to modulate cell activity in articular joints in a number of ways. These include stimulation of collagenase and stromelysin production (5) and induction of transcription from the stromelysin promoter (6). IL1 also stimulates prostaglandin E_2 production (7) and plasminogen activator production by synovial cells (8,9), causes an increased proteoglycan (PG) release from cartilage explants (10,11,12) and decreased production of [^{35}S]-sulphate

Abbreviations used in this paper: IL1, interleukin 1, fcs, foetal calf serum, SDS, sodium dodecyl sulphate, GuCHl, guanindium chloride, PG, proteoglycan, GAG, glycosaminoglycan, SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

0006-291X/88 $1.50

labelled molecules (13). In cartilage, the majority of incorporated [^{35}S]-sulphate is found in the large aggregating PG (14). These are high molecular weight glycoproteins (15) which form large aggregates by binding to hyaluronic acid (16). The intracellular assembly and subsequent secretion of these molecules is a complex multi-stage process. The PG core protein is synthesised in the rough endoplasmic reticulum (17) where it is maintained as an unglycosylated pool (18,19). A series of transferase enzymes catalyse the elongation and sulphation of the glycosaminoglycan (GAG) chains (20) in the Golgi apparatus and the completed molecule is rapidly secreted. This synthetic and secretory pathway may therefore be regulated at a number of different stages. The aim of the current study was to determine the mechanisms by which IL1 decreases the production of cartilage PG, and to determine whether foetal and neonatal cartilage responds in the same way as adult cartilage.

METHODS

Cartilage explant cultures were set up as previously described (11,13) from adult, foetal and neonatal pigs. For chondrocyte cultures, cartilage was incubated overnight in serum-free medium (10ml/g of cartilage) containing 1mg/ml collagenase and 50µg/ml gentamycin. Chondrocytes were pelleted by centrifugation at 100g for 5 mins. The pellet was washed by resuspending in Iscoves medium containing 5% fcs and 5µg/ml gentamycin. Cells were counted, and plated at 10^6 cells/well into 24 well plates or 3 x 10^6 cells/dish into 35mm dishes. Cultures were incubated for 48 hr at 37°C in a humidified atmosphere of 5% CO_2/95% air before addition of IL1. Human recombinant IL1α was from Roche, U.K. Newly synthesised GAG and total GAG were determined as previously described (13). Total protein synthesis was determined as uptake of [^{35}S]-methionine and DNA synthesis as uptake of [^{3}H] thymidine incorporated into TCA precipitable material. For secretion experiments, 35mm plates of chondrocytes were incubated with and without IL1 for 24 hr prior to receiving a 5 min pulse of 50µCi/ml [^{35}S]-sulphate in sulphate-free medium. They were then washed with PBS and chased in 1ml medium containing 1mM non-radioactive sulphate. IL1 was present in experimental plates throughout the pulse and chase periods. At various chase times ranging from 0-60 mins, medium was removed from 3 control and 3 IL1-treated plates and frozen. 1ml of 4M GuHCl, 0.05M sodium acetate pH 5.8 was added to the plates and incubated for 15 mins at 4°C. The remaining residue fraction was digested in 1ml papain solution and analysed for labelled GAG content as previously described (13).

Synthesis of GAG in the presence of cycloheximide was determined by incubating chondrocytes with 0.5µg/ml cycloheximide for various times (0-240 mins) before receiving a pulse of 20µCi/ml [^{35}S]-sulphate. Pulse medium was removed, cell layers washed in PBS and chased in 1ml medium for a further 60 mins. Experimental plates contained cycloheximide throughout the pulse and chase periods. The incubations were terminated by addition of an equal volume of 8M GuHCl, 0.1M sodium acetate pH 5.8 and agitated for 1 hr at 4°C to extract GAG from a combined cell and medium fraction.

Gel electrophoresis was performed on a continuous system using 4% SDS-polyacrylamide gels. Samples were loaded in buffer containing SDS and 8M Urea to prevent aggregation of PG (21). Gels were processed for fluorography, dried and exposed to preflashed X-ray film at -80° (22).

RESULTS AND DISCUSSION

Previous experiments demonstrated that natural porcine IL1 caused a reversible,dose-dependent inhibition of PG synthesis in explants of cultured

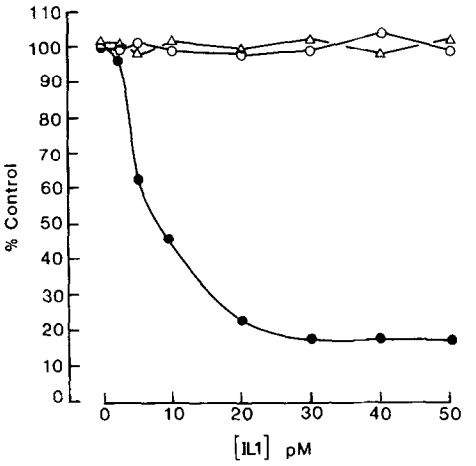

Figure 1. Chondrocytes were cultured for 48 hr and then for a further 24 hr in the presence and absence of IL1. Incorporation of [^{3}H]-thymidine into DNA synthesis (○), [^{35}S]-methionine into total protein (△) and [^{35}S]-sulphate into GAG (●) was assessed and expressed as the percentage of control values.

cartilage (13). We show here that this inhibition is selective using high density chondrocyte monolayers cultured with recombinant human IL1. Fig.1 shows the effect of human recombinant IL1 on GAG synthesis, total protein synthesis and DNA synthesis over a period of 24 hr in chondrocytes derived from adult pigs. GAG synthesis was decreased at concentrations of IL1 which had no significant effect on either total protein synthesis or DNA synthesis. An inhibition of GAG synthesis was seen at concentrations of IL1 as low as 2pM and at 50pM a maximal inhibition of over 80% was seen. The inhibition was fully reversible, synthesis returning to control values by 48 hr after removal of IL1 from the medium.

In order to examine PG core protein synthesis directly, cartilage explants were cultured for 3 days, then for a further 24 hr with or without 5pM human recombinant IL1, an approximally half maximal dose. 50μCi/ml [^{35}S]-methionine was added to in the medium for the final 6 hr. Labelled PG were separated from other components of a GuHCl extract by electrophoresis in 4% SDS-PAGE gels before and after digestion with chondroitinase ABC. An equal number of TCA precipitable counts were loaded into each lane of the gel. Electrophoresis of crude cartilage extracts (Fig.2) showed that a proportion of the radiolabelled material was unable to enter the gel. After digestion with chondroitinase ABC this material entered the gel and migrated as a single band (Fig.4) which co-migrated with purified [^{125}I]-labelled bovine nasal cartilage PG. Densitometer traces showed that human recombinant IL1 (5pM for 24 hr) caused a 46.5% reduction in the amount of [^{35}S]-methionine-labelled core protein, comparable with inhibition of sulphate incorporation into GAG.

It was previously established that IL1 reversibly inhibited PG synthesis without altering the average hydrodynamic size of the molecules synthesised or

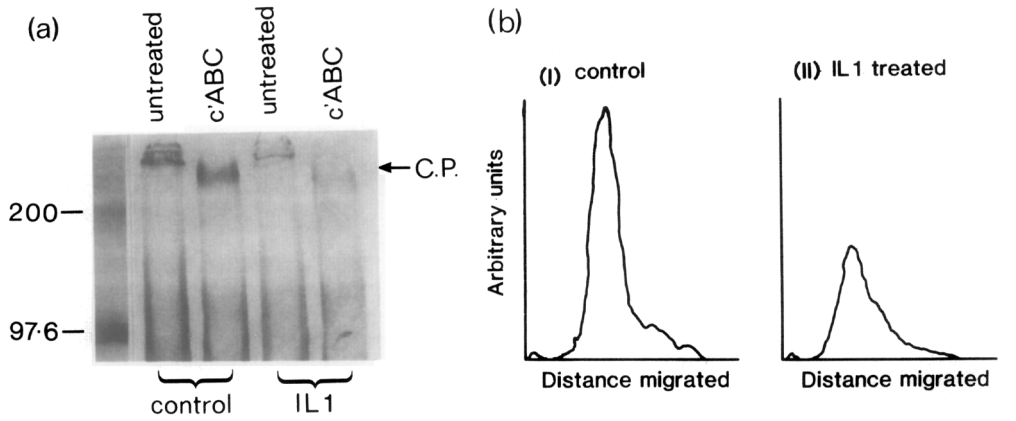

Figure 2. Adult pig cartilage cultured in the presence and absence of IL1 was labelled with [^{35}S]-methionine and extracted in 4M GuHCl. An equal number of counts from each preparation was either subject to chondroitinase ABC (c'ABC) digestion or run directly on 4% SDS/PAGE gels. (a) CP indicates the position of c'ABC treated [^{125}I]-labelled PG. (b) represents densitometer traces of c'ABC digests of control and IL1-treated cartilage.

impairing the mechanism of GAG elongation and sulphation (13). Here, pulse-chase experiments were performed to determine the rate of intracellular transport and secretion of PG in the presence and absence of IL1. There was no significant increase in the total amount of [^{35}S]-labelled GAG during the chase, indicating that removal of free [^{35}S]-sulphate was efficient (Fig.3a). Treatment with 4M GuHCl for 15 min without agitation efficiently separated intracellular from pericellular pools. The rate of secretion of [^{35}S]-labelled PG was rapid in both control and IL1-treated cultures. Over 70% of the labelled macromolecules were extractable from intracellular stores after 15 min and by 60 min, 35% were secreted into the medium (Fig.3b). No significant change in the rate of extraction and secretion was seen in the presence of IL1 (Fig.3c).

The continued production of PG in the presence of protein synthesis inhibitors reflects the existence of one or more intracellular pools of precursor PG core protein (18,19). Fig.4a shows decay of [^{35}S]-sulphate incorporation into GAG in primary pig cultures in the presence of cycloheximide. A $t_{1/2}$ of 25 min was calculated from a semi-log plot of the data (see insert, Fig.4a). The time course of inhibition of GAG synthesis in the presence of IL1 was determined (Fig. 4b). No significant inhibition of [^{35}S]-sulphate incorporation was seen before 3 hr of culture using a half maximal dose of IL1. Even very high doses of IL1 (up to 500pM) had no inhibitory effect before 1hr of culture. The delay in inhibition by IL1 may be assumed to be, at least in part, due to the time taken to deplete the intracellular supply of core protein demonstrated by the cycloheximide experiment.

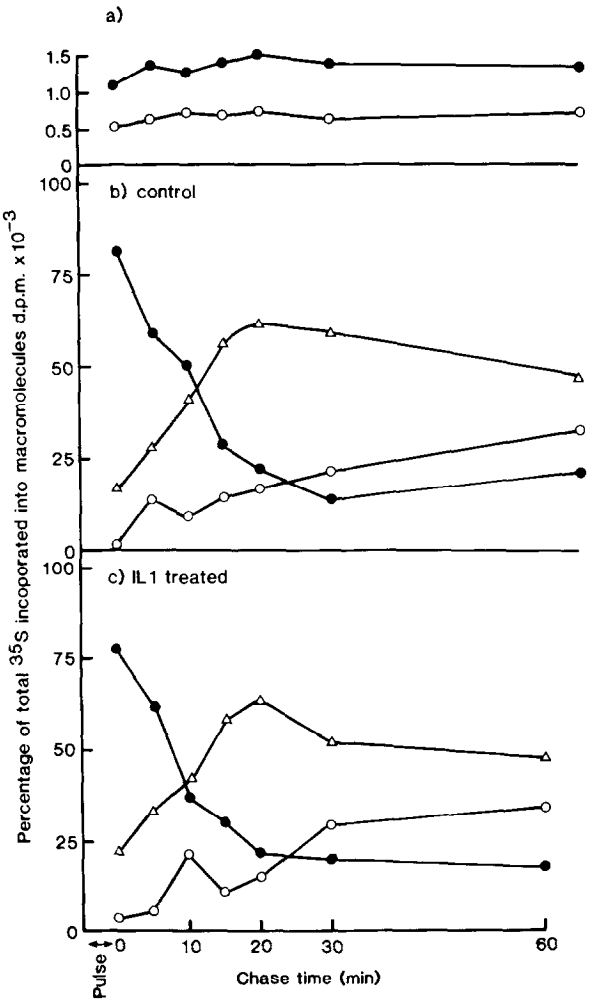

Figure 3. Chondrocytes were cultured in the presence or absence of IL1 for 24 hr followed by a pulse-chase experiment as described in the methods a) total GAG synthesis in control cultures (●) and IL1 (5pM) treated cultures (○). Percentage of labelled GAG in the papain digest (●)), GuHCl extract (△), and medium (○) in b) and c) IL1-treated cultures.

As well as the effects on adult cartilage, IL1 also inhibited GAG synthesis in cartilage obtained from foetal and neonatal pigs (Table 1). Foetal material was obtained from 110 day old foetuses (gestation period = 115 days). Neonatal material was either obtained at birth or from 6 day old animals. Labelled GAG extracted from explant cultures of neonatal pig cartilage were analysed by elution from columns of Sepharose CL2b under associative conditions (Fig. 5). IL1 did not alter either the average molecule size of the molecules synthesised or their ability to aggregate to hyaluronic acid as was previously found in adult cartilage (13). These observations add a new dimension to the interaction of IL1 in cartilage

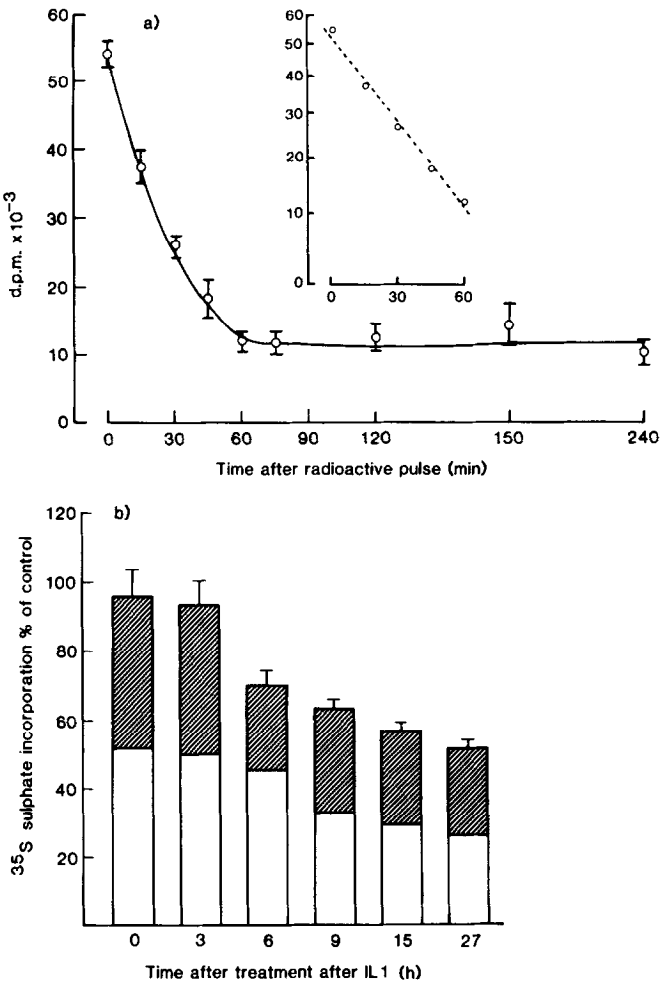

Figure 4. (a) Chondrocytes were cultured with cycloheximide followed by a pulse-chase experiment as described in the methods. Results represent the mean ± S.E. of 3 separate determinations. The $t_{1/2}$ value was obtained from a semi-logarithmic plot of the data as shown in the insert.
(b) Chondrocytes were cultured with IL1 for increasing lengths of time, as shown and then received a 15 min pulse of [^{35}S]-sulphate. □ cell layer, ▨ medium.

metabolism, adding a possible role for this cytokine in development and differentiation of a normal cartilage matrix.

When adult cartilage is maintained in explant cultures, IL1 not only decreases the synthesis of GAG but also increases the rate of release of PG components into the medium (10,11,12). Table 1 compares the dose of IL1 required to release GAG into the medium with that which will decrease the synthesis of GAG, in adult, neonatal, and foetal cartilage. These data show that, in all age groups of cartilage tested, IL1 inhibits GAG synthesis at lower doses than required to release matrix GAG into the medium. At IL1

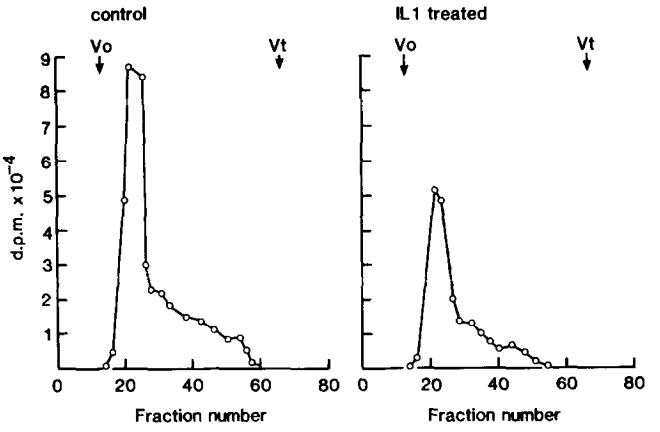

Figure 5. PG extracted from control and IL1-treated neonatal cartilage were eluted from a column of Sepharose CL-2B under associative conditions after mixing with hyaluronate.

concentrations of 5pM there is no detectable stimulation of PG release over a period of 24hrs but synthesis has been inhibited by almost 40%.

Our results show that IL1 begins to reduce PG biosynthesis prior to initiating depletion of existing matrix PG, by directly inhibiting core protein synthesis and with no significant effect on the rate of glycosylation and secretion of completed proteoglycan.

ACKNOWLEDGMENTS

This work was supported by a grant from the Arthritis and Rheumatism Council of Great Britain and the Medical Research Council. We would like to

Table 1. Effects of IL1 on release of GAG from explant cultures of cartilage and on incorporation of [^{35}S]-sulphate into newly synthesised GAG. Figures in brackets represent the percentage of total GAG.

	Glycosaminoglycan (µg)			[^{35}S]-glycosaminoglycan	
	Released in medium	Cartilage extract	Total	dpm x 10^{-3}	% of control
Adult Cartilage					
Control	74 (11.2%)	585 (88.8%)	658	84.5	100%
5pM IL1	69 (10.5%)	654 (89.5%)	723	53.4	63.1%
50pM IL1	245 (35.1%)	452 (64.9%)	697	27.9	33.0%
500pM IL1	314 (48.8%)	329 (51.2%)	643	10.4	12.3%
6 day Neonatal Cartilage					
Control	79 (18.3%)	351 (81.7%)	430	115.3	100%
5pM IL1	88 (19.5%)	271 (80.5%)	452	79.2	68.7%
50pM IL1	125 (31.6%)	271 (68.4%)	396	43.8	38.0%
110 Day Foetal Cartilage					
Control	79 (17.4%)	375 (82.6%)	454	127.4	100%
5pM IL1	101 (21.3%)	371 (78.7%)	472	78.3	61.4%
50pM IL1	134 (32.5%)	278 (67.5%)	412	52.1	40.9%

thank David Tivey and Greer Wilson from the AFRC Animal Physiology Institute, Babraham, Cambridge, for supplying the trotters from foetal and neonatal pigs and Lynne Woodfine for technical assistance.

REFERENCES

1. Wood, D. D., Ihrie, E. J., Dinarello, C. A. and P. L. Cohen. 1983. Arth. Rheum. 26, 975-983.
2. Pettipher, E. R., Higgs, G. A. and B. Henderson. 1986. Proc. Natl. Acad. Sci. U.S.A. 83, 8749-8753.
3. Olivierre, F., Gublier, W., Toole, C. A., Laurencin, C. and B. V. Treadwell. 1986. Biochim. Biophys. Res. Commun. 14, 904-911.
4. Bird, T. A. and J. Saklatvala. 1986. Nature 324, 263-268.
5. Murphy, G., Hembry, R. M. and J. J. Reynolds. 1986. Collagen Rel. Res. 6, 351-364.
6. Frisch, S. M. and H. E. Ruley. 1987. J. Biol. Chem. 262, 16300-16304.
7. Mizel, S. B., Dayer, J-M., Krane, S.N. and S. E. Meregenhagen. 1981. Proc. Natl. Acad. Sci. U.S.A. 78, 2474-2477.
8. Hamilton, J. A. 1986. Biochem. Biophys. Acta. 886, 195-202.
9. Mochan, E., Uhl, J. and R. Newton. 1986. Arth. Rheum. 29, 1078-1083.
10. Saklatvala, J., Curry, V.A. and S. J. Sarsfield. 1983. Biochem. J. 215, 385-392.
11. Tyler, J. A. 1985. Biochem. J. 225, 493-506.
12. Ratcliffe, A., Tyler, J. A. and T. E. Hardingham. 1986. Biochem. J. 23, 571-580.
13. Tyler, J. A. 1985. Biochem. J.. 227, 493-506.
14. Campbell, M. A., Handley, C. J, Hascall, V. C., Campbell, R. A. and D. A. Lowther. 1984. Arch. Biochem. Biophys. 234, 275-289.
15. Hardingham, T. E. 1981. Biochem. Soc. Trans. 9, 489-497.
16. Hardingham, T. E. and H. Muir. 1972. Biochem. Biophys. Acta. 279, 401-405.
17. Fellini, S. A., Hascall, V. C. and J. H. Kimura. 1984. J. Biol. Chem. 259, 7883-7889.
18. Mitchell, D. and T. Hardingham. 1981. Biochem. J. 196, 521-529.
19. Kimura, J. H., Caputo, C. B. and V. C. Hascall. 1981. J. Biol. Chem. 256, 4368-4376.
20. Helting, T. and L. Roden. 1969 J. Biol Chem. 244, 2799-2805.
21. Kimura, J. H., Thonar, J-M., Hascall, V. C., Reiner, A. and A. R. Poole, 1981, J. Biol. Chem. 256, 7890-7897.
22. Bonner, W. M. and R. A. Laskey. 1974. Eur. J. Biochem. 46, 83-88.