Inverse correlation between tyrosine phosphorylation and collagenase production in chondrocytes

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Collagenase production by chondrocytes appears to play a major role in the development of osteoarthritis. Although the mechanisms regulating collagenase production by chondrocytes are not known, incubation of bovine chondrocytes in serum markedly decreases collagenase production. Since serum has been demonstrated to increase levels of phosphotyrosine (*P*-Tyr) in several cell types, we determined the effect of altering intracellular levels of *P*-Tyr on collagenase production. Both orthovanadate, a potent inhibitor of tyrosine phosphatases, and serum caused a marked increase in tyrosine phosphorylation. The increase in *P*-Tyr was associated with a decrease in the production of collagenase, suggesting that the two processes may be linked. Orthovanadate caused an increase in *P*-Tyr in the absence of serum, suggesting that *P*-Tyr levels in resting chondrocytes are regulated through activity of both tyrosine kinases and phosphatases. Orthovanadate and serum induced a synergistic increase in *P*-Tyr levels, suggesting that serum functions through increasing kinase activity rather than decreasing phosphatase activity. In the absence of serum, concentrations of orthovanadate which maximally inhibited collagenase production primarily increased phosphorylation of a 36 kDa protein, suggesting that the phosphorylation of this protein may play a major role in regulating collagenase production. Orthovanadate had limited effects on chondrocyte proteoglycan synthesis, morphology or viability in the presence or absence of serum, suggesting that the decrease in collagenase production was not due to non-specific inhibition of protein synthesis or cellular toxicity. Inhibition of tyrosine phosphatases by orthovanadate or activation of tyrosine kinases by addition of serum correlated with the inhibition of collagenase production.

INTRODUCTION

The cartilage matrix macromolecules consist mainly of proteoglycan, collagens and minor amounts of non-collagenous proteins (Howell, 1986). In osteoarthritis these components undergo proteolytic degradation leading to cartilage erosion and loss of joint function (Howell, 1986). Neutral metalloproteinases, including collagenase and proteoglycanase, are involved in the breakdown of these components in cartilage in the human disease and in experimental models of osteoarthritis (Ehrlich *et al.*, 1978; Pelletier *et al.*, 1983; Pettipher *et al.*, 1986; Dingle *et al.*, 1987; Dean *et al.*, 1987; Duff, 1988). Mammalian collagenase is capable of degrading native collagen (Murphy *et al.*, 1988) and has been found to be elevated in osteoarthritic cartilage (Ehrlich *et al.*, 1978). The levels of collagenase parallel the severity of the disease until the late stages (Pelletier *et al.*, 1983). The loss of collagen and eventual cartilage erosion appear to be due to an increase in collagenase production by chondrocytes coupled with their inability to replace collagen lost from the matrix (Fell *et al.*, 1976). To date there is very little information on the mechanism regulating collagenase production by chondrocytes.

It has been shown that monolayer chondrocyte cultures produce collagenase constitutively in the absence of fetal bovine serum (FBS) (Ehrlich *et al.*, 1977; Malesud *et al.*, 1981; Cartwright *et al.*, 1983). Chondrocytes grown in the presence of FBS produce little or no collagenase constitutively (Kandel *et al.*, 1987). Recently, we have shown that FBS contains a factor which inhibits collagenase production (Kandel *et al.*, 1990). Several growth factors, which are known to be present in FBS, have also been shown to modulate collagenase production in other cell types (Chua *et al.*, 1985; Bauer *et al.*, 1985; Edwards

et al., 1985; Kohno, 1985) raised the possibility that tyrosine phosphorylation–dephosphorylation reactions may be involved in the regulation of collagenase production. In the present investigation we examined whether orthovanadate, a potent inhibitor of phosphotyrosine phosphatases (Swarup *et al.*, 1982; Chasteen, 1983; Klarlund, 1985), had an effect on collagenase production. Orthovanadate both increased phosphotyrosine (*P*-Tyr) levels and inhibited collagenase production by chondrocytes, suggesting that increases in *P*-Tyr may be involved in the regulation of collagenase production.

MATERIALS AND METHODS

Materials

Goat anti-(type I collagen) antibodies were obtained from Southern Biotechnology Associates Inc., Birmingham, AL, U.S.A. Alkaline-phosphatase-conjugated rabbit anti-goat antibodies and the alkaline phosphatase kit used for determination of collagenase activity were purchased from Bio-Rad. Anti-*P*-Tyr monoclonal antibodies PY20 and PY69 were obtained from ICN. The alkaline phosphatase reaction kit used for Western blotting was purchased from Promega. Human recombinant interleukin 1 β was generously supplied by Ciba-Geigy, Basel, Switzerland. Phorbol 12-myristate 13-acetate, bacterial collagenase type II, Pronase type XIV from *Streptomyces griseus*, and low endotoxin BSA (fraction V) were purchased from Sigma. Highly purified BSA was purchased from Calbiochem. Tissue culture dishes were obtained from Becton Dickinson. Nunc-Immulon IF titre plates and FBS were obtained from Gibco/BRL. All other reagents were of analytical grade.

Abbreviations used: *P*-Tyr, phosphotyrosine; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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Isolation of bovine articular chondrocytes

Bovine articular chondrocytes were isolated by the procedure previously described by Kuettner *et al.* (1982) with some modifications. Briefly, cartilage fragments were washed three times in Ham's F12 medium, and incubated for 1 h with 15 ml of the same medium containing 0.5 % Pronase and 5 % FBS. The cartilage was washed and then incubated overnight with 0.04 % bacterial collagenase in the same volume of medium containing 5 % FBS. The cells were washed and plated in 6-well plates at a density of 4×10^6 cells/well in 2 ml of Ham's F12 medium containing 5 % FBS. The cells were allowed to recover for 24 h at 37 °C in a humidified atmosphere supplemented with 5 % CO₂. They were then pre-incubated in medium containing the appropriate concentration of FBS for 1 h before setting up the experiment.

Effects of FBS and orthovanadate on collagenase production

The cells were incubated in 1 ml of medium containing the indicated concentrations of FBS and orthovanadate for 24 h at 37 °C in a humidified atmosphere supplemented with 5 % CO₂. The cell-conditioned medium was removed and assayed for collagenase activity.

Determination of collagenase activity

The cell-conditioned medium was removed from each well and assayed for collagenase activity by a modification of a procedure described previously (Kandel *et al.*, 1990). A 96-well plate was coated with 250 ng of acid-extracted purified rat tail type I collagen per well at 4 °C. The plates were washed with Buffer A (50 mM-Tris/HCl, pH 7.8, 150 mM-NaCl, 5 mM-CaCl₂) containing 0.04 % Tween 20. Just before use, BSA in Buffer A was added to each well to a final concentration of 2 %.

Chondrocyte medium (200 µl) was treated with trypsin/Tos-Phe-CH₂Cl for 5 min at 37 °C to activate the collagenase. The amount of trypsin used varied with the amount of FBS in the sample; 10 µg of trypsin was added to samples containing 5 % FBS or less and 100 µg of trypsin was added to samples containing 15 % FBS. The trypsin was inactivated by the addition of 5-fold (by weight) of soybean trypsin inhibitor. Volumes of samples ranging from 12.5 µg to 100 µl were added to the collagen-coated wells. Following a 3 h incubation at 37 °C, the sample was removed and the plates were washed. Goat antibody to type I collagen [100 µl; 1:200 dilution in phosphate-buffered saline (PBS) containing 1 % BSA] was added to each well. The plates were incubated for 16 h at 4 °C. The plates were washed and then 100 µl of alkaline-phosphatase-conjugated rabbit anti-goat antibody (1:2250 dilution) was added to each well. Following a 2 h incubation at room temperature, the plates were washed as above and 100 µl of substrate (*p*-nitrophenyl phosphate; Bio-Rad alkaline phosphatase kit) was added to each well. The colour was allowed to develop for 20–30 min, 50 µl of 1 M-NaOH was added and the absorbance was determined at 405 nm using a Titertek spectrophotometer. Medium containing FBS that had not been exposed to cells served as a control for no enzymic activity and medium containing high levels of synovial collagenase served as a control for maximal activity and allowed for the correction of the background absorbance. Incubation with 0.0025 % trypsin digested less than 15 % of the total collagen available for digestion on the plate. All samples were done in duplicate and values obtained were within 10 % of each other. The specific activity of collagenase is expressed as units/ml; one unit is defined as the amount of cell-conditioned medium required to inhibit 50 % of the binding of the anti-collagen antibody to the e.l.i.s.a. plates in 1 h at 37 °C.

Measurement of tyrosine phosphorylation in chondrocytes

After incubation of cells, the cell-conditioned medium was removed and the cells were washed once with cold PBS. Cells were solubilized by the addition of 0.3 ml of Laemmli (1970) buffer containing 100 µM-orthovanadate and 5 % β-mercaptoethanol. The extract was removed and boiled for 5 min. The samples were stored frozen at –70 °C. The proteins were separated on a 10 % polyacrylamide gel. After gel electrophoresis, the gel was incubated for 10 min in the transferring buffer. The proteins were transferred to nitrocellulose sheets by electrophoresing for 16 h at 200 mA. To ensure that all of the proteins were transferred, the gels were stained with Coomassie Blue following transfer. The nitrocellulose blots were blocked with 5 % BSA in PBS containing 0.02 % NaN₃ overnight at room temperature. The blots were incubated with 1 µg of anti-*P*-Tyr antibody in 10 ml of 1.5 % BSA/PBS for 16 h at room temperature with constant shaking. The nitrocellulose sheet was washed twice with 150 ml of PBS for 10 min, then once with 1 % Nonidet P-40, and then twice with PBS. The anti-*P*-Tyr antibody bound to the proteins on the nitrocellulose was detected using one of two methods: alkaline phosphatase or ¹²⁵I-labelled Protein A. For the alkaline phosphatase method, the washed nitrocellulose sheets were treated with 20 ml of alkaline-phosphatase-conjugated sheep anti-mouse antibody (1:3000) in 1.5 % BSA by incubating for 4 h at room temperature with constant shaking. The sheets were washed as described above and incubated with the colour reagent consisting of 20 ml of 20 mM-Tris/HCl, pH 9.5, 100 mM-NaCl, 5 mM-MgCl₂, 132 µl of Nitro Blue Tetrazolium and 66 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega kit) until colour developed. The reaction was stopped by removing the substrate and adding 20 ml of 20 mM-Tris/HCl, pH 8.0, containing 5 mM-EDTA.

For detection of anti-phosphotyrosine antibody with labelled Protein A, the sheets were incubated with 10 ml of 1.5 % BSA in PBS containing 2 µg of rabbit anti-mouse IgG by incubating for 4 h at room temperature with shaking. The sheet was washed as described above and then treated with 10 ml of 1.5 % BSA in PBS containing 20 µl of ¹²⁵I-Protein A (Amersham; 50 µCi/500 µl) for 2 h at room temperature. The sheet was washed and set up for autoradiography. Similar results were obtained with either developing system, with PY20 and or PY69 (obtained from ICN) and with a polyclonal antibody (obtained from Dr. M. Kamps and Dr. B. Sefton, Salk Institute, San Diego, CA, U.S.A.).

Measurements of keratan sulphate levels

The amount of keratan sulphate in the conditioned medium was determined using an inhibition e.l.i.s.a. method described previously (Cruz *et al.*, 1989). Monoclonal antibody D1B2 (kindly provided by Dr. M. A. Adams, University of Calgary, Calgary, Canada) was used to measure keratan sulphate. All samples were assayed in triplicate. The amount of keratan sulphate present in each sample was estimated from a standard curve using purified bovine keratan sulphate.

Determination of [³H]thymidine incorporation

Chondrocytes were plated at 3.5×10^5 cells/well in a 96-well tissue culture plate (equivalent to 4×10^6 cells/cm²). Following the 24 h recovery period and the 1 h pre-incubation, medium containing concentrations of orthovanadate ranging from 0 to 100 µM, 1 µCi of [³H]thymidine (40 Ci/mmol) and 1 % or 15 % FBS was added to the cells and incubated for 24 h. The cells were harvested using an automated cell harvester. The glass filters were dried and suspended in 5 ml of scintillation cocktail. The amount of radioactivity incorporated was determined using an LKB scintillation counter.

Fig. 1. Identification of orthovanadate- and FBS-induced tyrosine phosphorylation by immunoblot analysis

Chondrocytes were incubated with different concentrations of orthovanadate (0–100 μM) as indicated in Ham's F12 medium alone (c) or supplemented with 1% (b) or 15% (a) FBS. After a 24 h incubation, the conditioned medium was removed and the cells were solubilized by adding 0.3 ml of Laemmli (1970) buffer containing 100 μM -orthovanadate. The samples were boiled and 20 μl samples were run on 10% polyacrylamide gels as described in the Materials and methods section. The proteins containing *P*-Tyr were identified with an anti-*P*-Tyr antibody (PY 20) by Western analysis as described in the Materials and methods section. The stained molecular mass markers run with each experiment included: a, phosphorylase B (110 kDa); b, BSA (84 kDa); c, ovalbumin (47 kDa); d, carbonic anhydrase (33 kDa); e, soybean trypsin inhibitor (24 kDa); and f, lysozyme (16 kDa).

Assessment of cell viability

Cells were incubated in the presence and absence of 100 μM -orthovanadate exactly as described for experiments examining its effect on collagenase production. After a 24 h incubation, the cells were incubated in medium containing 1 μM -carboxyfluorescein diacetate for 30 min. The medium was removed and the cells were washed and incubated for a further 1 h in fresh medium. The cells were examined for fluorescence and photographed on a Wild Leitz Inverted Fluorovert Microscope.

RESULTS

Serum and orthovanadate increases tyrosine phosphorylation in chondrocytes

As demonstrated by Western blotting using three separate antibodies specific for *P*-Tyr, incubation of chondrocytes with serum increased the content of *P*-Tyr in a number of cellular proteins. The results for antibody PY20 are presented in Fig. 1; PY69 and a polyclonal antibody from Dr. M. Kamps demonstrated similar results (results not shown). Irrespective of the presence or absence of serum, orthovanadate at concentrations greater than 25 μM induced a marked increase in *P*-Tyr content. In the absence of serum, orthovanadate increased *P*-Tyr primarily in a 36 kDa protein. This is most clearly indicated in Fig. 1 (100 μM -orthovanadate; –FBS) where background levels are at a minimum and the alkaline phosphatase substrate was unlikely to be limiting. Orthovanadate and serum produced a synergistic increase in tyrosine phosphorylation, suggesting that they altered different processes. Considering their reported modes of action, it is most likely that the orthovanadate effect was due to inhibition of tyrosine phosphatases, whereas serum, probably through specific growth factors, was increasing tyrosine kinase activity. Since orthovanadate increased the *P*-Tyr content in cells cultured in the absence and presence of serum, these cells contain both activated tyrosine kinases and phosphatases, with the net level of *P*-Tyr determined by the balance between these two processes.

Table 1. Effects of orthovanadate and FBS on collagenase production

Chondrocytes (2×10^5 cells/cm²) were incubated in the presence or absence of 50 μM -orthovanadate in Ham's F12 medium supplemented with various amounts of FBS. Following a 24 h incubation at 37 °C, the conditioned medium was removed and analysed for collagenase activity as described in the Materials and methods section. The results are expressed as percentages of constitutive collagenase activity, which was 3.2 units/ml. Values represent the means $\pm$ S.E.M. from four separate experiments.

FBS (%)	Collagenase activity (% of control)	
	–Orthovanadate	+Orthovanadate
None	100 $\pm$ 9	13 $\pm$ 6
1.0	86 $\pm$ 11	5 $\pm$ 6
2.5	48 $\pm$ 18	2 $\pm$ 4
5.0	7 $\pm$ 10	6 $\pm$ 8
15	2 $\pm$ 6	4 $\pm$ 7

Serum and orthovanadate decrease collagenase production

As indicated in Table 1, an overnight incubation of chondrocytes with either serum or orthovanadate induced a concentration-dependent decrease in collagenase present in media collected from chondrocytes. Serum (5%) in the absence of orthovanadate or 50 μM -orthovanadate in the absence of serum induced maximal inhibition of collagenase production. In the presence of suboptimal concentrations of serum (1%), orthovanadate also decreased collagenase production (Table 1).

The concentration-dependent inhibition of collagenase production was similar to that required to induce increases in *P*-Tyr content (compare Fig. 1 and Table 2). Maximal inhibition of collagenase was achieved with the accumulation of low levels of *P*-Tyr (25–50 μM -orthovanadate in 1% FBS, Fig. 1 and Table 2) as compared with the levels of *P*-Tyr that could be obtained when the concentrations of serum and orthovanadate were

Table 2. Effect of orthovanadate concentration on the inhibition of collagenase production

Chondrocytes were incubated in Ham's F12 medium supplemented with 1% FBS containing concentrations of orthovanadate ranging from 0 to 100 μM . Following a 24 h incubation at 37 °C, the conditioned medium was analysed for collagenase activity as described in the Materials and methods section. The results are expressed as a percentage of constitutive collagenase activity. Values represent the means $\pm$ S.E.M. of four separate experiments.

Orthovanadate (μM)	Collagenase activity (% of control)
0	100 $\pm$ 8
10	92 $\pm$ 11
25	23 $\pm$ 13
50	6 $\pm$ 8
100	8 $\pm$ 4

Table 3. Effect of orthovanadate on proteoglycan synthesis by chondrocytes

Cells were incubated in the presence or absence of 50 μM -orthovanadate in Ham's F12 medium alone or supplemented with 15% FBS. Following a 24 h incubation, the amount of keratan sulphate was measured by an inhibition e.l.i.s.a. as described in the Materials and methods section. The results are expressed as μg of keratan sulphate/ml and the values represent the means $\pm$ S.E.M. from three separate experiments.

FBS (%)	Orthovanadate (μM)	Keratan sulphate ($\mu\text{g/ml}$)
None	0	2.4 $\pm$ 0.4
	50	3.0 $\pm$ 0.5
15	0	15.4 $\pm$ 2.1
	50	14.0 $\pm$ 1.7

increased further (100 μM -orthovanadate in 15% FBS, Fig. 1). Therefore maximal inhibition of collagenase production was associated with relatively low accumulation of *P*-Tyr.

Effect of orthovanadate on proteoglycan synthesis

Since orthovanadate decreased the levels of collagenase production, we determined whether this represented a general decrease in protein biosynthesis by chondrocytes. The effect of orthovanadate on proteoglycan synthesis was examined by determining the amount of keratan sulphate in the conditioned medium. Chondrocytes were incubated for 24 h in Ham's medium alone or supplemented with 15% FBS in the presence and absence of 50 μM -orthovanadate. Table 3 shows the amounts of keratan sulphate in conditioned medium from cells incubated under the different conditions. This was 5-fold higher in medium of cells incubated with 15% FBS than in cells incubated in the absence of FBS. Addition of 50 μM -orthovanadate did not decrease significantly the amount of keratan sulphate in the medium under either of these conditions. When the matrix proteins were extracted and analysed for keratan sulphate, similar results were obtained (results not shown). Since 50 μM -orthovanadate does not decrease proteoglycan synthesis, the inhibition of collagenase production observed with orthovanadate is unlikely to represent a general decrease in protein synthesis.

Effect of orthovanadate on chondrocyte morphology and proliferation

Concentrations of up to 100 μM -orthovanadate did not have

Fig. 2. Effect of orthovanadate on chondrocyte morphology

Chondrocytes were incubated in Ham's F12 medium alone (a) or in medium containing 100 μM -orthovanadate (b) for 24 h as described in the Materials and methods section. The cells were analyzed by phase-contrast microscopy (magnification $\times$ 137.5).

detectable effects on chondrocyte morphology and viability. Fig. 2 shows the morphology of chondrocytes incubated for 24 h in the presence and absence of 100 μM -orthovanadate. Varying the concentration of FBS or orthovanadate also did not affect cell morphology (results not shown).

In order to examine cell viability following a 24 h incubation with orthovanadate, cells were loaded with carboxyfluorescein diacetate and then examined for fluorescence (results not shown). At 1 h after loading the cells, no differences were detected in the fluorescence of the cells incubated in the presence and absence of orthovanadate. These results indicate that concentrations of up to 100 μM -orthovanadate had no effect on cell viability.

Chondrocyte proliferation was determined by measuring [^3H]thymidine incorporation under the same culture conditions used to examine the production of collagenase (results not shown). Chondrocytes incorporated only negligible amounts of [^3H]thymidine even in the presence of FBS, indicating that very little proliferation occurs under the conditions examined. Concentrations of orthovanadate of up to 100 μM had no significant effect on the amount of thymidine incorporated by chondrocytes.

DISCUSSION

Tyrosine phosphorylation, although a relatively rare event, appears to regulate both cell proliferation and differentiation (Chou *et al.*, 1987; Glenn *et al.*, 1988; Chen *et al.*, 1988). Several growth factor receptors contain an intrinsic tyrosine kinase domain (Hunter, 1984; Bishop, 1985; Hanks *et al.*, 1988), and others appear to be associated with tyrosine kinases (Chou *et al.*, 1987; Deuel, 1987; Hanks *et al.*, 1988), implicating tyrosine phosphorylation in the regulation of cell proliferation. In addition, several of the proto-oncogenes encode for proteins with tyrosine kinase activity (Hunter, 1984; Bishop, 1985; Hanks *et al.*, 1988). However, brain, which contains few proliferating cells, is one of the richest sources of tyrosine kinase activity (Hanks *et al.*, 1988). Thus tyrosine phosphorylation probably plays a major role in the function of differentiated cells.

We have investigated the role of tyrosine phosphorylation in collagenase production by chondrocytes by altering the activity

of tyrosine kinases using serum and decreasing the activity of tyrosine phosphatases using orthovanadate. In both cases, the increase in tyrosine phosphorylation was associated with a marked decrease in collagenase production. In contrast, production of proteoglycans was markedly increased with serum and slightly increased or not affected by orthovanadate in the absence or presence respectively of serum, indicating that the changes in collagenase production did not reflect a generalized inhibition of protein synthesis or secretion.

Orthovanadate in the presence of serum induced a synergistic increase in tyrosine phosphorylation, suggesting that the two agents are acting through different mechanisms. Serum stimulates tyrosine kinase activity (Edwards *et al.*, 1985; Kohno, 1985) and orthovanadate is most probably inhibiting the activity of tyrosine phosphatases (Swarup *et al.*, 1982; Chasteen, 1983; Nelson & Branton, 1984; Spigelman *et al.*, 1987). In the absence of serum, orthovanadate also increased the *P*-Tyr levels in several proteins. This was probably the consequence of inhibition of tyrosine phosphatases in the presence of constitutive tyrosine kinase activity; however, it is not possible to eliminate a direct effect of orthovanadate on tyrosine kinase activity. Similarly, although orthovanadate has been demonstrated to inhibit ion-pumping ATPases (Josephson & Cantley, 1977; Chasteen, 1983) and to activate guanine-nucleotide-binding proteins (Bigay *et al.*, 1987), these processes appear to require much higher concentrations of orthovanadate.

In the absence of serum, orthovanadate increased tyrosine phosphorylation predominantly of a 36 kDa protein (Fig. 1). Although the level of tyrosine phosphorylation of a series of other proteins also increased, it is possible that the state of phosphorylation of the 36 kDa protein plays a role in the regulation of collagenase production. This is supported by the observation that the level of *P*-Tyr in the 36 kDa protein correlated closely with collagenase production, in that *P*-Tyr was markedly increased in the presence of orthovanadate alone and the levels of *P*-Tyr were not significantly further increased in the presence of concentrations of serum and orthovanadate above those which induced maximal inhibition of collagenase production.

In summary, increasing tyrosine phosphorylation by stimulating kinases with serum, by inhibiting phosphatases with orthovanadate or by a combination of the two correlated closely with a marked inhibition of collagenase production. Confirmation that the two processes are causally linked awaits identification of substrate(s) being phosphorylated and the regulatory mechanisms involved.

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