Research article

Chondrocyte response to growth factors is modulated by p38 mitogen-activated protein kinase inhibition

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Abstract

Inhibitors of p38 mitogen-activated protein kinase (MAPK) diminish inflammatory arthritis in experimental animals. This may be effected by diminishing the production of inflammatory mediators, but this kinase is also part of the IL-1 signal pathway in articular chondrocytes. We determined the effect of p38 MAPK inhibition on proliferative and synthetic responses of lapine chondrocytes, cartilage, and synovial fibroblasts under basal and IL-1-activated conditions.

Basal and growth factor-stimulated proliferation and proteoglycan synthesis were determined in primary cultures of rabbit articular chondrocytes, first-passage synovial fibroblasts, and cartilage organ cultures. Studies were performed with or without p38 MAPK inhibitors, in IL-1-activated and control cultures. Media nitric oxide and prostaglandin E₂ were assayed.

p38 MAPK inhibitors blunt chondrocyte and cartilage proteoglycan synthesis in response to transforming growth factor beta; responses to insulin-like growth factor 1 (IGF-1) and fetal calf serum (FCS) are unaffected. p38 MAPK inhibitors significantly reverse inhibition of cartilage organ culture proteoglycan synthesis by IL-1. p38 MAPK inhibition potentiated basal, IGF-1-stimulated and FCS-stimulated chondrocyte proliferation, and reversed IL-1 inhibition of IGF-1-stimulated and FCS-stimulated DNA synthesis. Decreases in nitric oxide but not prostaglandin E₂ synthesis in IL-1-activated chondrocytes treated with p38 MAPK inhibitors are partly responsible for this restoration of response. Synovial fibroblast proliferation is minimally affected by p38 MAPK inhibition.

p38 MAPK activity modulates chondrocyte proliferation under basal and IL-1-activated conditions. Inhibition of p38 MAPK enhances the ability of growth factors to overcome the inhibitory actions of IL-1 on proliferation, and thus could facilitate restoration and repair of diseased and damaged cartilage.

Keywords: chondrocytes, interleukin-1, nitric oxide, p38 mitogen-activated protein kinase, transforming growth factor beta

Introduction

Proinflammatory cytokines are responsible for much of the pathophysiology of both osteoarthritis and rheumatoid arthritis [1]. Activation of p38 mitogen-activated protein kinase (MAPK) has been implicated in the catabolic and anti-anabolic actions of both IL-1 and tumor necrosis factor alpha [2]. These cytokines are also induced in mechanically stressed [3,4] and damaged cartilage. The signal pathways they activate, including p38 MAPK, may thus influence the course of cartilage repair. It is therefore important to understand the consequences of p38 MAPK inhibition on cartilage/chondrocyte responses to the ana-

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p38 MAPK was recently identified, however, as part of the signal transduction pathway effecting transforming growth factor beta (TGF-β) stimulation of aggrecan gene expression by the chondrogenic cell line ATDC5 [10,11]. The relationship between TGF-β signaling through p38 MAPK and the Smad family was characterized in C2C12 cells. The conclusion was that the nuclear target of p38, ATF-2, and the Smad family was characterized in C2C12 cells. Similar signal synergy studies have not been carried out for chondrocytes. However, given the important anabolic and anticatabolic [13] actions of TGF-β, any maneuver that modifies responses to TGF-β and other anabolic growth factors could have critical consequences for maintenance and repair of cartilage. These studies were thus initiated to determine whether p38 MAPK inhibition affects chondrocyte responses to TGF-β, insulin-like growth factor 1 (IGF-1), and serum, and also whether p38 MAPK inhibition reverses the anti-anabolic actions of IL-1 on proliferative and synthetic responses of rabbit articular chondrocytes, cartilage, and synovial fibroblasts.

Materials and methods
Materials were obtained from the following suppliers: New Zealand White rabbits, 5–6 lb (Myrtle’s Rabbity, Thompson Station, TN, USA); modified Eagle’s medium (MEM), fetal calf serum (FCS), antibiotics, other tissue culture supplies, and protease inhibitor cocktail for use with mammalian cells (Sigma Chemical, St Louis, MO, USA); DuoSet IC for phospho-p38alpha (R&D Systems, Minneapolis, MN, USA); protein assay reagent (Bio-Rad, Hercules, CA, USA); type 1 collagenase and trypsin (Worthington Biochemical, Freehold, NJ, USA); 35S-sodium sulfate, 1 Ci/mmol (NEN, Boston, MA, USA); [methyl-3H]thymidine and prostaglandin E2 (PGE2) enzyme immunoassay kits (Amersham Pharmacia Biotech, Piscataway, NJ, USA); human TGF-β1, human IGF-1, recombinant human IL-1β (R&D Systems, Minneapolis, MN, USA); Sc-58125 (Cayman Chemical, Ann Arbor MI, USA); SB 203580 (SB) and SB 202190, hydrochloride, at 1 µM. The inhibitor concentrations used were kept below 2 µM as effects not related to p38 MAPK inhibition have been seen in other cell types with higher concentrations [16]. Seven hours later, 50 ng/ml IGF-1, 50 pM TGF-β or 5–10% FCS was added. Proteoglycan synthesis or proliferation was determined 24 hours after addition of growth factors.

Phosphorylated p38 MAPK was determined as an index of activation using a commercially available kit (R&D systems) on cell lysates collected 30 min after activation with IL-1 (2 ng/ml) and 60–120 min after addition of TGF-β (100 pM). Chondrocytes were grown to confluence in six-well plates, the serum reduced for 24 hours, fresh medium added, and the cells lysed after activation with IL-1 or TGF-β. After treatment the cells were washed twice with phosphate-buffered saline, lysed, the lysates analyzed as per kit instructions and the results normalized to the average protein content of 33 µg/ml (determined on 10-fold dilution of lysates as per Bio-Rad protein assay instructions).

Proteoglycan synthesis was measured as the incorporation of 35S-sulfate (6 hour pulse label) into molecules separated from unincorporated label using PD-10 columns as described for this laboratory [14]. Proliferation was measured as the incorporation of [3H]thymidine during a 2 hour pulse label into trichloroacetic acid precipitated material. NO was assayed as the nitrite concentration in conditioned media (CM) using the Griess reaction, and CM PGE2 was assayed using the ELISA kit from Amer sham Pharmacia Biotech.

Chondrocytes transduced with an adenoviral vector carrying the human inducible nitric oxide synthase gene (AdiNOS) were used in some studies to facilitate evaluation of the effects of NO independent of other actions of iNOS inducing cytokines on the cell. The adenoviral vector, previously described [14] with a titer of 1010 pfu/ml, was prepared by Dr Paul Robbins (University of Pittsburgh School of Medicine Human Gene Therapy Center). Transduction of chondrocytes was carried out as follows: monolayers of chondrocytes were washed with Gey’s Balanced Salt Solution, and 1 x 107 pfu virus in 0.2 ml Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1% bovine serum albumin, with or without 1 mM L-NMA added to each well. The transduction efficiency was 76% under these conditions [14]. The cells were washed after overnight incubation, and the culture continued for

[7]. SB also inhibits IL-1 induction of inducible nitric oxide synthase (iNOS) in bovine chondrocytes [8], and thus blocks nitric oxide (NO) synthesis. This effect may also protect cartilage from the damaging actions of NO [9].
24 hours in MEM, 0.5% fetal bovine serum, with or without L-NMA, agonists added, and conditioned media for determination of NO production collected 24 hours later. Proliferation was also evaluated at this time.

Experiments were performed at least three times, and data are presented as mean ± standard error. Statistically significant differences (\(P<0.05\)) were determined using Student’s \(t\) test.

**Results**

Figure 1 shows p38 MAPK is activated, as shown by increased phosphorylation, after exposure of chondrocytes to IL-1 or TGF-\(\beta\). There is detectable p38 MAPK phosphorylation under basal conditions in normal lapine chondrocytes, and this is consistent with the ability of p38 MAPK inhibition to modulate proliferation in the absence of, as well as in the presence of, cytokine activation. IL-1 increased phosphorylation ninefold after 30 min, consistent with prior observations in rabbit chondrocytes [17] and in human chondrocytes [18,19]. TGF-\(\beta\) caused a persistent activation of sixfold to eightfold, showing that this growth factor can activate this signal pathway in primary chondrocytes as well as chondrogenic ATDC5 cells [10,11].

IGF-1 and TGF-\(\beta\) stimulated lapine chondrocyte proteoglycan synthesis from 85 ± 7 pmol/10^5 cells to 202 ± 15 and 344 ± 50 pmol/10^5 cells, or by 2.4-fold and 4.0-fold, respectively (Fig. 2). Inhibition of p38 MAPK did not alter basal or IGF-1-stimulated proteoglycan synthesis. However, it did decrease the increase in response to TGF-\(\beta\) to one-half of that seen in the absence of SB. Inhibition of p38 MAPK did not prevent IL-1 inhibition of basal or stimulated proteoglycan synthesis by rabbit chondrocytes: IL-1, 65 ± 10 pmol/10^5 cells; IL-1 + SB, 65 ± 9 pmol/10^5 cells; IL-1 + TGF-\(\beta\), 227 ± 22 pmol/10^5 cells; and IL-1 + SB + TGF-\(\beta\), 223 ± 24 pmol/10^5 cells. Similar results were seen using a second p38 MAPK inhibitor, SB 202190.

To confirm the effect of p38 MAPK inhibition on chondrocyte proteoglycan synthesis in situ in the cartilage matrix, experiments using cartilage organ cultures were performed and are reported in Fig. 3. IL-1 at the low, but inhibitory, concentration of 0.1 ng/ml was used, and in this case there is a modest blunting of its action to diminish matrix proteoglycan synthesis by the p38 MAPK inhibitor SB 202190. However, the same concentration of inhibitor also decreased TGF-\(\beta\)-stimulated proteoglycan synthesis by 40%. p38 MAPK inhibition did not affect the ability of 5% FCS (Fig. 3) or of IGF-1 (data not shown) to stimulate cartilage proteoglycan synthesis. Similar results were found using SB 203580 (SB).

p38 MAPK inhibitors had significant effects on chondrocyte proliferation under basal, IL-1-activated, and growth factor-stimulated conditions. As shown in Fig. 4, IGF-1 (445%) and FCS (978%) stimulated chondrocyte proliferation. SB increased basal, IGF-1-stimulated, and serum-stimulated chondrocyte proliferation by 55%, 73%, and 45%, respectively. The relatively modest stimulation of proliferation by TGF-\(\beta\) (97%) was similar in the presence of and in the absence of p38 MAPK inhibition (data not shown).
IL-1 did not significantly inhibit basal chondrocyte proliferation. However, stimulation in response to IGF-1 or FCS was decreased by 54% and 87%, respectively, in IL-1-activated chondrocytes. When p38 MAPK was inhibited, however, growth factor-stimulated proliferation in the presence of IL-1 was significantly increased (IGF-1) or was completely restored (FCS). TGF-β stimulation of IL-1-activated rabbit chondrocyte proliferation (twofold) was less than that seen in response to IGF-1 or to FCS, but the modest increase in the presence of SB (70%) was significant (data not shown).

IL-1 induces iNOS and cyclooxygenase-2 (COX-2) in chondrocytes. The products of these enzymes, NO and PGE₂, have been shown to reduce chondrocyte proliferation [20,21]. SB inhibition of p38 MAPK decreased NO and PGE₂ syntheses by 50% and 76%, respectively, in IL-1-activated chondrocytes. When p38 MAPK was inhibited, however, growth factor-stimulated proliferation in the presence of IL-1 was significantly increased (IGF-1) or was completely restored (FCS). TGF-β stimulation of IL-1-activated rabbit chondrocyte proliferation (twofold) was less than that seen in response to IGF-1 or to FCS, but the modest increase in the presence of SB (70%) was significant (data not shown).

Figure 4 documented IL-1 inhibition of both IGF-1-stimulated and FCS-stimulated chondrocyte proliferation (by 54% and 87%, respectively). Figure 7 compares the ability of SB inhibition of p38 MAPK and L-NMA inhibition of NO synthesis to restore the proliferative response to growth factors in IL-1-activated cells. When NO synthesis in IL-1-activated cells was inhibited with 0.5 mM L-NMA, the restoration of basal and IGF-1-stimulated proliferation...
was similar to that seen in the presence of SB. The response to TGF-β was again potentiated by SB but not by L-NMA inhibition of NO synthesis. There was a significant 105% increase in FCS-stimulated proliferation in L-NMA-treated chondrocytes that was less than the 202% increase seen with p38 MAPK inhibition. The NO levels in CM in these experiments were: IL-1, 5 ± 0.2 µM; IL-1 + SB, 2.6 ± 0.13 µM; IL-1 + L-NMA, 1.4 ± 0.10 µM. These values were all significantly different from each other. The results suggest that the decrease in NO production by p38 MAPK inhibitors is responsible for some, but not all, of the restoration of proliferation in response to growth factors.

p38 MAPK inhibition also blunted IL-1-stimulated PGE₂ synthesis (Fig. 8). TGF-β alone significantly increased PGE₂ accumulation approximately threefold, from 47 to 170 pg/10⁵ cells per 24 hours. Inhibition of p38 MAPK completely blocked this increase. IL-1 increased PGE₂ to values 10-fold higher than did TGF-β, and the combination of IL-1 + TGF-β showed a striking synergy to increase PGE₂ accumulation to concentrations threefold higher that with IL-1 alone. In these later cases, p38 MAPK inhibition diminished PGE₂ but the medium concentrations were still greater than under basal conditions.

To test whether these concomitant changes in PGE₂ could contribute to the restoration of proliferative response in lapine chondrocytes, we compared the ability of Sc-58125, the specific COX-2 inhibitor, and SB to blunt the inhibitory actions of IL-1. Data from these series of experiments are shown in Fig. 9. As seen before, SB potentiates all growth factor-stimulated proliferation.

However, 0.5 µM Sc-58125, which decreased IL-1-stimulated PGE₂ in chondrocyte CM from 1073 ± 278 to 30 ± 13 pg/24 hours or in IL-1 + TGF-β-activated chondrocytes from 3153 ± 106 to 27 ± 16 pg/24 hours, failed to enhance basal, IGF-1-stimulated or TGF-β-stimulated proliferation. There was a modest but significant 43% potentiation of proliferation in the presence of FCS, which was far less than the 240% seen with p38 MAPK inhibition. In a separate series of experiments we compared the effect of SB alone with that of SB + Sc-58125 in IL-1-activated cells. Consistent with the data in Fig. 9, complete inhibition of PGE₂ synthesis with Sc-58125 in conjunction with
SB inhibition of p38 MAPK effected no significant differ-
ences in chondrocyte proliferation from those seen with
SB alone (data not shown).

We also tested the effects on lapine chondrocyte prolifer-
ation of exogenous PGE 2 at concentrations generated in
the previous experiments. Consistent with the results in
Fig. 9, there were no significant effects of PGE2 in this
concentration range (0.1–6 ng/ml) on chondrocyte prolif-
eration (data not shown).

Cartilage/chondrocyte metabolism may also be affected
by synovial hyperplasia and by the products secreted by
the synovial fibroblasts [22]. We therefore evaluated the
effects of inhibition of p38 MAPK on basal, growth factor-
stimulated, and IL-1-activated proliferation of lapine syn-
ovial fibroblasts (passage 1). The effects of p38 MAPK
inhibition in lapine synovial fibroblasts were modest in
comparison with those found in chondrocytes. SB had no
effect on basal proliferation and the 29% stimulation in
the presence of FCS is less than that seen in chondrocytes.
SB had no effect on proliferation in the presence of IGF-1
or TGF-β. SB did significantly stimulate proliferation of
IL-1-activated fibroblasts under both basal
(1629 ± 115 dpm/well versus 2970 ± 803 dpm/well) and
FCS-stimulated conditions (8614 ± 449 dpm/well versus
10372 ± 1104 dpm/well). IL-1 did not increase NO syn-
thesis in these preparations, and there were only modest
changes in PGE2 synthesis under the conditions evalu-
ated (data not shown).

Discussion
These studies evaluated the potential of p38 MAPK inhibi-
tion to modulate the response of lapine chondrocytes to
growth factors under basal and cytokine-activated condi-
tions. p38 MAPK activation (phosphorylation) in primary
lapine chondrocytes is documented. The studies show
that p38 MAPK inhibition can modestly enhance proteo-
glycan synthesis in cartilage organ cultures inhibited by
low concentrations of IL-1. On the contrary, p38 MAPK
inhibition blunted the synthetic response to TGF-β in both
isolated chondrocytes and cartilage organ cultures.
However, the response to IGF-1 and FCS is not affected.
Table 1 summarizes these findings.

The net effect of p38 MAPK inhibition on matrix protein
(proteoglycan) synthesis will thus depend on the growth
factor milieu effecting cartilage homeostasis. The partial
reversal of IL-1 inhibition in cartilage, and the lack of an
effect on the response to the complex mix of factors con-
tained in FCS, suggests that p38 MAPK inhibition in vivo
would positively affect cartilage proteoglycan synthesis.

p38 MAPK inhibition potentiates basal, IGF-1-stimulated
and FCS-stimulated chondrocyte proliferation with minimal
effect on the response to TGF-β. The inhibition modestly
increases proliferation of IL-1-activated chondrocytes,
enhances the response to IGF-1 in the presence of IL-1,
and restores the response to FCS completely. SB inhibi-
tion of IL-1-stimulated NO production accounts for some
of the restoration of response, but the action of SB to
diminish PGE2 is not critical to its effects on proliferation.
Table 2 summarizes these findings.

p38 MAPK inhibition had little effect on lapine fibroblast
proliferation under basal and IL-1-activated conditions,
precluding a concomitant synovial hyperplasia and poten-
tial increases in catabolic factors thereby secreted.
The data suggest a significant component of IL-1 inhibition of rabbit chondrocyte proliferation is effected through p38 MAPK-mediated actions. The role of p38 MAPK in the regulation of proliferation has been extensively studied [23]; however, the relationship varies with cell type. For example, p38 MAPK activation is linked with increased proliferation in vascular smooth muscle cells [24], and is necessary for the fibroblast growth factor 2 stimulation of fibroblasts [25], but it arrests proliferation of thymocytes [26]. The current studies show that, in lapine chondrocytes, inhibition of p38 MAPK enhances basal and growth factor-stimulated proliferation, and can restore proliferation in IL-1-activated cells. These data suggest that, in this cell type, p38 MAPK activation is associated with decreased DNA synthesis.

L-NMA inhibition of NO synthesis in IL-1-activated cells did increase IGF-1-stimulated and FCS-stimulated proliferation, but not as effectively as SB under some conditions. This suggests that some, but not all, of the potentiation of proliferation by SB in IL-1-activated cells may be secondary to the decrease in NO synthesis when p38 MAPK is blocked. The NO dose response (Fig. 6) shows that lapine chondrocyte proliferation is sensitive to NO over the concentration range found in the conditioned media of IL-1-activated cells, and is modulated by SB. The effects of NO in the context of IL-1-activated chondrocytes where multiple factors are altered may be different from that in cells where NO synthesis is enhanced in isolation from these factors. The data do suggest, however, that the diminution of NO synthesis by p38 MAPK inhibitors may contribute to their ability to blunt the anti-anabolic actions of IL-1. This pathway may or may not be relevant to human disease, as Badger and colleagues [27] found that SB 242235, another selective p38 MAPK inhibitor, did not decrease IL-1 induction of iNOS and NO synthesis in human chondrocyte cultures. However, p38 MAPK activation by NO has been demonstrated in several cell types [28, 29] and has been linked with NO induction of heme oxygenase 1 in HeLa cells [30]. The possibility that some of the pathophysiologic actions of NO in human chondrocytes may be mediated via p38 MAPK activation has not been evaluated, and thus remains a potential point of therapy by inhibitors of p38 MAPK in cytokine-activated human cartilage/chondrocytes.

SB also inhibited IL-1-stimulated increases in PGE2 synthesis/accumulation (Fig. 8). However, Sc-58125 inhibition of COX-2 and the resulting decreases in PGE2 had little effect on chondrocyte proliferation (Fig. 9). This suggests that the SB inhibition of PGE2 production in IL-1-treated and IL-1 + TGF-β-treated cells contributes minimally to the restoration of proliferation in rabbit chondrocytes. The effects of prostaglandins on chondrocyte proliferation have been variable. For example, Blanco and Lotz [21] concluded that NO inhibition of normal human chondrocyte proliferation was effected by concomitant changes in PGE2. Lowe and colleagues [31] showed that exogenous PGE2 had a dose-dependent, biphasic effect on rat chondrocytes with suppression at the lower concentrations tested (0.1 µM, or 35 ng/ml) and stimulation at higher concentrations (5 µM, or 1760 ng/ml). Schwartz and colleagues [32] found that PGE2 from 0.007 to 15 ng/ml increased the cell number and [3H]thymidine incorporation in chick costochondral cartilage cells.

Our data suggest that normal rabbit articular chondrocyte proliferation is relatively insensitive to the range of CM PGE2 attained subsequent to IL-1 activation. The relationship between chondrocyte proliferation and PGE2 thus seems highly species dependent. We recently reported that human chondrocyte proliferation is inhibited by PGE2 concentrations found in CM following IL-1 activation [33]. The mechanisms by which p38 MAPK inhibition restores proliferation in IL-1-activated/stressed human chondrocytes may thus be different from those described for lapine preparations (current studies) or for bovine preparations [27].

These studies were initiated, in part, to determine whether inhibition of p38 MAPK would blunt the actions of TGF-β on chondrocytes. In the case of proliferation, this was not
found to be so. Under basal conditions, SB did not modify the modest TGF-β-stimulated increases in DNA synthesis; in IL-1-activated cells, the response to TGF-β was actually enhanced. In contrast, both SB 203580 (SB) and SB 202190 blunted the ability of TGF-β to stimulate proteoglycan synthesis in bovine nasal septum cartilage in organ cultures. Ridley and colleagues [34] showed that even low concentrations of SB inhibited proteoglycan synthesis in bovine nasal septum cartilage in organ cultures in the presence of 10% FCS. We did not see a similar inhibition in rabbit cartilage. Furthermore, stimulation of proteoglycan synthesis by IGF-1 in lapine chondrocytes in monolayer culture was also unaffected by SB. Perhaps the difference between these results is related to species differences in the relative importance of the p38 MAPK pathway in maintaining proteoglycan synthesis. Regardless, our data do suggest that the signal pathways that effect TGF-β stimulation of chondrocyte proliferation and chondrocyte proteoglycan synthesis differ; the pathway activating proteoglycan synthesis appears to involve p38 MAPK, while that activating proliferation does not.

The data showing only minor modulation of IL-1-inhibited proteoglycan synthesis by SB (Figs 2 and 3) are consistent with prior studies of bovine cartilage [34]. However, even the ability to modestly reverse the anti-anabolic effects of IL-1 may be therapeutic under conditions of mild inflammation as is often seen in osteoarthritis, and in some stages of the repair of injured cartilage. Although proteoglycan synthesis responses to TGF-β are blunted, the responses to IGF-1 and FCS remain intact in the presence of p38 MAPK inhibitors. Coupled with the ability to reverse effects of low concentrations of IL-1 and the minimal effects on synovial fibroblasts, this suggests that p38 MAPK inhibition could have a positive effect on cartilage maintenance and repair.

Conclusions
Although activation of p38 MAPK has been observed in tissues from arthritic joints [1] and from mechanically stressed cartilage [2,3], and has been shown to be involved in IL-1 inhibition of collagen synthesis [8] and IL-1 induction of collagenases [19], this is the first report showing effects of basal levels of p38 MAPK activity on chondrocyte proliferation. Inhibition of p38 MAPK potentiates basal and stimulated proliferation. It thus appears to have a regulatory function in lapine chondrocytes under both normal and cytokine-activated conditions. p38 MAPK inhibition can partially reverse IL-1 inhibition of proteoglycan synthesis, and thus could contribute to maintenance of matrix proteins in cytokine-activated and stressed cartilage. Whether similar effects of p38 MAPK inhibitors on chondrocyte responses to cytokines and growth factors are found in human cartilage/chondrocyte preparations should be evaluated.

Competing interests
None declared.

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