OsteoArthritis and Cartilage (2007) **15,** 884–893

doi:10.1016/j.joca.2007.02.001

Osteoarthritis and Cartilage

International Cartilage Repair Society

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Evidence for JNK-dependent up-regulation of proteoglycan synthesis and for activation of JNK_1 following cyclical mechanical stimulation in a human chondrocyte culture model

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Summary

Objective: To examine the expression of mitogen-activated protein kinases (MAPKs) in human chondrocytes, to investigate whether selective activation of MAPKs is involved in up-regulation of proteoglycan (PG) synthesis following cyclical mechanical stimulation (MS), and to examine whether MS is associated with integrin-dependent or independent activation of MAPKs.

Methods: The C-28/I2 and C-20/A4 human chondrocyte cell lines were mechanically stimulated in monolayer cell culture. PG synthesis was assessed by [^{35}S]-sulphate incorporation in the presence and absence of the p38 inhibitor SB203580, and the extracellular-regulated kinase (ERK1/2) inhibitor PD98059. Kinase expression and activation were assessed by Western blotting using phosphorylation status-dependent and independent antibodies, and by kinase assays. The Jun N-terminal kinase (JNK) inhibitor SP600125 and the anti-β_1 integrin (CD29) function-blocking antibody were used to assess JNK activation and integrin dependence, respectively.

Results: Increased PG synthesis following 3 h of cyclic MS was abolished by pretreatment with 10 μM SB203580, but was not affected by 50 μM PD98059. The kinases p38, ERK1/ERK2 and JNKs were expressed in both stimulated and unstimulated cells. Phosphorylated p38 was detected at various time points following 0.5, 1, 2 and 3 h MS in C-28/I2, but not detected in C-20/A4 cell lines. Phosphorylation of ERK1 and ERK2 was not significantly affected by MS. Phosphorylation of the 54 and 46 kDa JNKs increased following 0.5, 1, 2 and 3 h of MS, and following CO_2 deprivation. MS-induced JNK phosphorylation was inhibited by SB203580 at concentrations $\geq$5 μM and activation of JNK1 following MS was blocked by SP600125 and partially inhibited by anti-CD29.

Conclusions: The data suggest JNK, rather than p38 or ERK dependent increases in PG synthesis, and selective, partially integrin-dependent, activation of JNK kinases in human chondrocyte cell lines following cyclical MS. JNK activation is also very sensitive to changes in CO_2/pH in this chondrocyte culture model.

Key words: Mechanical strain, Human articular chondrocytes, Proteoglycan, β1 Integrin, JNK, MAPK.

Introduction

The structural integrity of articular cartilage is critically dependent on mechanical loading as well as movement of joints[1,2]. Overloading[3] and unloading[4] of articular cartilage are both associated with proteoglycan (PG) depletion, while PG synthesis and articular cartilage thickness are increased by physiological exercise[2]. Joint loading and compression of articular cartilage results in deformation of chondrocytes which strain at contact points between the cells and the pericellular matrix; but compression of cartilage is also accompanied by hydrostatic pressure gradients, fluid flow and physical–chemical changes such as alteration in matrix hydration, fixed charge density, ion concentrations and changes in osmotic pressure[5].

Cyclical loading stimulates PG synthesis in cartilage explants[6–8] and chondrocytes in monolayer cell culture[9] or agarose culture[10], whilst static loading is associated with decreased PG synthesis[6,11]. The decrease in cartilage PG production that follows static compression is associated with down regulation of aggrecan gene expression[12], whilst cyclical cartilage compression results in transient stimulation of expression of both aggrecan[13] and matrix metalloproteinase genes[14]. Our group has previously demonstrated that cyclical mechanical stimulation (MS) (0.33 Hz, 3700 μstrain) of primary articular chondrocytes isolated from normal, but not from osteoarthritic, articular cartilage, results in an integrin and interleukin (IL)-4 dependent increase in aggrecan messenger RNA (mRNA) in monolayer cell culture[15]. Previous work in this laboratory has also provided evidence of integrin-dependent increase in PG synthesis following cyclical MS[16] and rapid

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Received 25 March 2005; revision accepted 4 February 2007.

integrin-dependent phosphorylation of focal adhesion kinase (p^{125} FAK), paxillin and β-catenin in primary human articular chondrocytes[16] and the human chondrocyte cell line C-20/A4[17,18]. Cyclical MS of human chondrocytes is also associated with signalling through phosphatidylinositol 3-kinase[19], which itself is regulated by integrins through integrin-linked kinases[20].

The mitogen- and stress-activated protein kinases are of critical importance in the signalling processes that lead to activation of transcription factors and gene expression in response to growth factors, cytokines and a wide variety of environmental stresses[21]. *Ex vivo* compression of cartilage explants results in the phosphorylation of extracellular-regulated kinases (ERK1/2), p38 mitogen-activated protein kinase (MAPK) and the stress-activated protein kinase (SAPK)/ERK kinase-1 (SEK) of the Jun N-terminal kinase (JNK) pathway[22]. It has recently been demonstrated that activation of ERK following cyclical loading of articular cartilage is stimulated by basic fibroblast growth factor (bFGF)[23] and it has been suggested that activation of ERK is integrin-independent and results from release of bFGF from a cartilage matrix pool following MS of cartilage[23]. However, integrin-mediated adhesion to extracellular matrix proteins is a requirement for the optimal activation of growth factor receptors in some situations[24] and the proliferation and differentiation of vascular endothelial cells in response to release of bFGF is integrin dependent[25]. Mechanical stretch is associated with activation of MAPKs in a variety of cell types such as patellar tendon fibroblasts[26], adult human mesenchymal stem cells[27] and myometrial smooth muscle cells[28], in culture systems where activation of MAPKs is likely to be independent of growth factor release. Immortalized human chondrocyte cell lines have been used as a model to examine signal transduction and transcriptional responses to cyclical mechanical stress[17,18], fluid shear, hydrostatic pressure, and cytokines[29–37], and the PGs synthesized and integrin profiles have been characterized[38,39]. We have therefore used these cell lines as a model to explore whether activation of MAPKs is involved in the changes in PG synthesis that follow MS and to investigate whether cyclical MS of chondrocytes results in integrin−dependent or independent activation of MAP kinases.

Materials and methods

ANTIBODIES, INHIBITORS AND CHEMICAL REAGENTS

Phospho-p38 MAP kinase antibody (Thr 180/Tyr 182 rabbit polyclonal immunoglobulin G (IgG), affinity purified), p38 MAP kinase antibody (rabbit polyclonal IgG, affinity purified), phospho-p44/p42 MAP kinase antibody (Thr 202/Tyr 204 rabbit polyclonal IgG, affinity purified), p44/p42 MAP kinase antibody (rabbit polyclonal IgG, affinity purified), phospho-SAPK/JNK MAP kinase antibody (Thr 183/Tyr 185 rabbit polyclonal IgG, affinity purified), SAPK/JNK MAP kinase antibody (rabbit polyclonal IgG, affinity purified), HRP-linked anti rabbit secondary antibody and LumiGLO chemiluminescent reagent were purchased from New England Biolabs (Hertfordshire, UK). The p38 inhibitor SB203580, the ERK1/2 inhibitor PD98059 and the JNK inhibitor SP600125 were obtained from Calbiochem (Nottingham, UK). Mouse anti-human CD 29 (clone number 3S3), which is a β_1-integrin function-blocking antibody, was obtained from Serotec (Oxford, UK). Unless otherwise stated, other chemical compounds and reagents were from Sigma (Poole, UK).

CELLS AND CULTURE

Human immortalized chondrocyte cell lines, C-28/I2 and C-20/A4, were derived by transduction with vectors bearing SV40 large T antigen from two different samples of juvenile human costal cartilage, as described[40]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4500 mg/l) and supplemented with 7.4 mg/ml $NaHCO_3$, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum in a humidified atmosphere incubator containing 5% CO_2 and 95% air at 37°C. The cells were grown in monolayer culture, medium was changed every 3 or 4 days and the cells were passaged weekly. The cells used were between passages 80 and 101. Cells were trypsinized at approximately 90% confluence, and seeded in 58-mm diameter tissue culture dishes (Nunc, Paisley, UK) at a density of 3.5×10^5 cells/dish in 3 ml DMEM, supplemented as above. For assays of PG synthesis, the cells were seeded at 5×10^5 cells/dish in 5 ml medium and grown to confluence for a further 3 days. Before each experiment, the cells were incubated in serum-free medium for 20 h. In order to examine the effects of MAPK inhibitors on the responses of chondrocytes to MS, C-28/I2 cells were incubated in the presence or absence of SB203580, PD98059 or SP600125 for 30 min, and C-20/A4 cells were incubated in the presence or absence of SB203580, and then subjected to cyclical MS. To investigate the role of β_1-integrin in the MAPK response, C-28/I2 or C-20/A4 cells were incubated with mouse anti-human CD 29, an anti-β_1 integrin function-blocking antibody, at a dilution of 1:500 for 30 min prior to 1 h cyclical MS.

MECHANICAL STIMULATION

Two types of apparatus were used for MS[41]. Some experiments were undertaken using a modification of a previously described method[42,43]. Cyclical mechanical strain (MS) was applied to chondrocytes in monolayer culture in 58 mm plastic dishes placed in a sealed pressure chamber with inlet and outlet ports. MS was generated by cyclical pressurization from above with nitrogen gas. Simultaneously pressurized but un-strained dishes served as controls [Fig. 1(a)]. In other experiments MS was applied to chondrocytes in monolayer cell culture in identical cell culture dishes cyclically stimulated from below [Fig. 1(b)]. In these experiments cells maintained either in the experimental incubator or in an adjacent CO_2 incubator served as controls. In most experiments cells were subjected to a standard regimen of cyclical pressurization of 0.25 atmosphere above atmospheric pressure (190 mmHg, 26.7 kPa) at a frequency of 0.33 Hz (2 s on, 1 s off) for 30 min, 1, 2 or 3 h. Cyclical pressurization in both apparatus (a) and (b) was accompanied by deformation of the base of the culture dish and the adherent cells. By use of strain gauges attached to the base of the cell culture dishes, it has been shown that a pressure of 26.7 kPa generates approximately 750 μstrain in apparatus (a) and 30,700 μstrain in apparatus (b)[44]. All MS was conducted in an incubator at 37°C.

[^{35}S]-SULPHATE INCORPORATION AND ASSAY FOR PG SYNTHESIS

PG synthesis was measured by incorporation of sodium [^{35}S]-sulphate into glycosaminoglycans (GAGs), using a method[45] adapted from Parkkinen *et al*[46]. Fresh medium containing 20 μCi/ml sodium [^{35}S]-sulphate (Amersham,

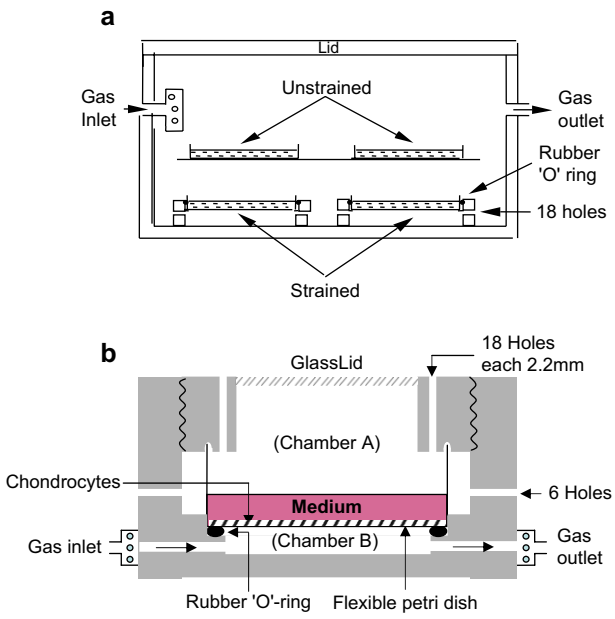

Fig. 1. Apparatus employed for MS. (a) Application of intermittent strain from above the cell culture dishes. (b) Application of intermittent strain from below the cell culture dishes.

UK) was added to cell culture dishes. After 3 h of MS, the cells were washed twice with phosphate-buffered saline (PBS) to remove free label and dried. PGs were extracted with 2 ml of a solution containing 4 M guanidine-HCl, 0.5% 3[(3-chloramidopropyl)-dimethylammoniol] 1-propane sulphonate, 3 mM Tris, and 10 μM ethylenediaminetetraacetic acid (EDTA). PGs were separated from free label using pre-packed size-exclusion columns of Sephadex G25 (PD10, Pharmacia, St Albans, Herts, UK). The columns were equilibrated with 2.0 M guanidine-HCl, 50 mM sodium acetate, pH 6. Aliquots of resulting fractions were counted in a Packard Minaxi Tricarb 4000 scintillation counter after addition of 5 ml Cocktail T (Merck, Lutterworth, Leics, UK). Experiments were performed in duplicate. DNA concentrations were measured using the fluorimetric dye Hoechst 33258 (Calbiochem, La Jolla, CA)[47] and a Perkin–Elmer LS-5B luminescence spectrometer. PG synthesis rates were expressed as cpm h^{-1} μg^{-1} DNA. In order to confirm that [^{35}S]-sulphate was incorporated into GAGs, samples were digested with ABC chondroitinase (Sigma, Poole, UK) and keratanases (ICN, Thame, UK). Treatment with all enzymes removed 86.3 ± 2.5% of incorporated [^{35}S]-sulphate from the labelled PGs[45].

STATISTICAL ANALYSIS

Rates of synthesis in treated and untreated cells were compared using a paired Student's *t* test.

P values less then 0.05 were considered significant.

PROTEIN EXTRACTION AND WESTERN BLOTTING

Immediately following MS, the cells were washed twice with ice cold PBS containing 100 μM Na_3VO_4 and then lysed on ice for 30 min with 400 μl/dish radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM NaCl, 50 mM Tris pH 7.4, 0.5% deoxycholate, 0.1% SDS, 1% NP40 (BDH), 1 mM ethylene glycol-bis(beta-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM NaF, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μM Na_3VO_4). Lysates were clarified by centrifugation and the supernatants were analysed for protein concentration using a Bio-Rad Protein Assay. Equal amounts of protein from each sample (50 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride membranes overnight at 4°C. After washing with tris-buffered saline (TBS) for 5 min and incubating with blocking buffer (1× TBS, 0.1% Tween-20, 5% non-fat milk) for 1–2 h, membranes were incubated with polyclonal rabbit phospho-specific antibodies to c-jun N-terminal kinase (SAPK/JNK), p38 MAPK, or extracellular-signal regulated kinase (ERK1/ERK2) at a dilution of 1:1000. Detection was carried out using horseradish peroxidase-linked secondary antibodies at a dilution of 1:2000 and an electrochemiluminescence kit. The membranes were then probed again using phosphorylation status independent antibodies or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) after stripping for 30 min at 50°C with a buffer containing 62.5 mM tris-B, 2% SDS and 100 mM β-mercaptoethanol at pH 6.7.

JNK KINASE ASSAYS[48]

Cells were mechanically stimulated for 1 h in the presence or absence of mouse anti-human β_1 integrin (CD29) antibody. In some experiments cells were pre-incubated for 1 h in the presence or absence of the JNK inhibitor SP600125 before MS. Stimulated cells were lysed for 10 min on ice in 400 μl of kinase lysis buffer (20 mM HEPES pH 7.4, 50 mM β-glycerophosphate, 2 mM EGTA, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM PMSF, 2 mM NaF, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, 10 μM E64, 1 mM Na_3VO_4). Samples were clarified by centrifugation at 4°C for 10 min at 12,000 × *g*. Following protein determination using the Bio-Rad protein assay, 1 mg of protein from each supernatant was immunoprecipitated with 1 μg (5 μl) of rabbit polyclonal anti-JNK1 antibody (Santa Cruz) at 4°C on a rotary mixer for 1 h. Complexes were then separated using protein G Sepharose Fast Flow beads (10% in kinase lysis buffer) (Amersham) at 4°C on a rotary mixer overnight. The following day, the immunocomplexes were washed four times in 1 ml of RIPA wash buffer (2 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 50 mM NaCl, 50 mM Tris pH 7.4, 0.5% Deoxycholate, 0.1% SDS, 1% NP40, 1 mM EGTA, 1 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) and twice in 1 ml of kinase wash buffer (20 mM HEPES pH 7.4, 10 mM NaF, 20 mM β-glycerophosphate, 0.5 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, 2 mM DTT, 10 mM $MgCl_2$, 0.05% BRIJ35, 1 mM Na_3VO_4). Samples were incubated in 30 μl of kinase assay buffer with 1 μg glutathione *S*-tranferase (GST) fusion protein containing human c-Jun (1–79) residues (GST-c-Jun) (Stress-Gen) as a substrate, with 20 μM adenosine 5′-triphosphate (ATP) and 4 μCi γ ^{32}P ATP for 20 min at room temperature with gentle shaking. The reaction was stopped by the addition of 6 μl of 5× SDS-PAGE sample buffer. Each sample was boiled for 5 min. The supernatants were separated by 12% SDS-PAGE and phosphorylation of the c-Jun (1–79)-GST was measured by exposure of the gel to auto-radiographic film (Kodak).

Results

THE EFFECTS OF SB203580 AND PD98059 ON THE RATE OF PG SYNTHESIS FOLLOWING MS

PG synthesis was assayed in the C-20/A4 cell line by measuring the incorporation of sodium [^{35}S]-sulphate into GAGs following 3 h of MS (apparatus (a) Fig. 1) with or without pretreatment with 10 μM of the p38 inhibitor SB203580 or 50 μM of the ERK1/2 inhibitor PD98059. PG synthesis increased by 25% after 3 h of MS. This increase was abolished by pretreatment with 10 μM SB203580 [Fig. 2(a)]. Pretreatment with 50 μM PD98059 had no effect [Fig. 2(b)].

EFFECTS OF MS ON MAP KINASE PHOSPHORYLATION

Both the C-28/I2 and C-20/A4 cell lines were studied to assess the effect of 0.33 Hz, 30,700 μstrain MS (apparatus (b) Fig. 1) on phosphorylation of MAP kinases. Both C-28/I2 and C-20/A4 cell lines express p38, ERK1/2 and 54 and 46 kDa JNK proteins before and following MS [Fig. 3(a–c)]. MS resulted in weak phosphorylation of p38, maximal at 3 h in C-28/I_2 cells, but phosphorylation of p38 was not observed in C-20/A4 cells [Fig. 3(a)].

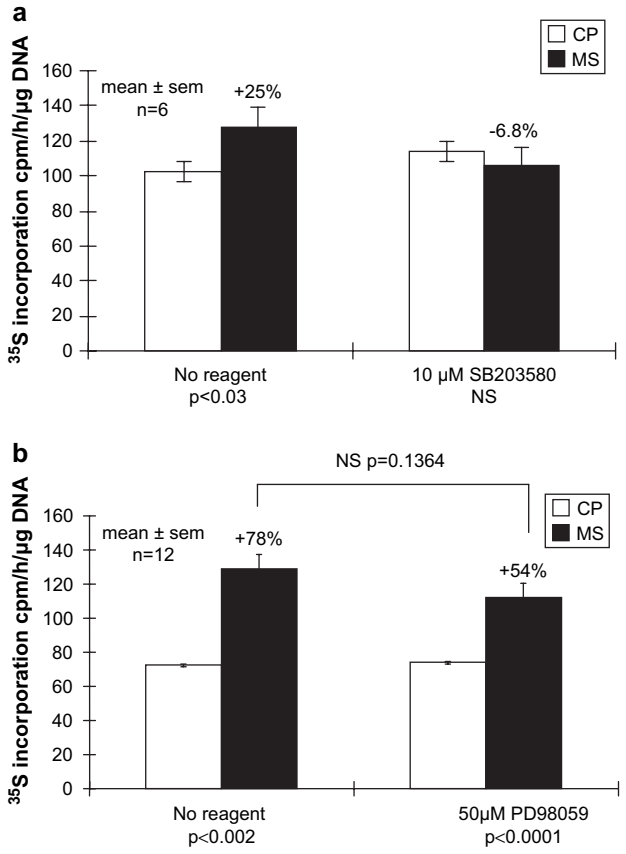

Fig. 2. Effects of MS and MAPK inhibitors on PG synthesis in C-20/A4 cells. (a) Effect of SB203580 on PG synthesis following MS. Mean (±S.E.M.) [^{35}S]-sulphate incorporation with or without 30 min pretreatment with 10 μM SB203580, followed by 3 h of MS ($n=6$). (b) Effect of PD98059 on PG synthesis following MS. Mean (±S.E.M.) [^{35}S]-sulphate incorporation with or without 30 min pretreatment with 50 μM PD98059 followed by 3 h of MS ($n=12$). CP: cyclical pressure control. MS: cyclical mechanical strain.

Phospho-ERK1/2 was detected in unstimulated cells, and phosphorylation was not significantly altered following MS [Fig. 3(b)]. In contrast both cell lines, when mechanically stimulated, showed increased phosphorylation of 54 and 46 kDa JNK throughout the time course of the experiment [Fig. 3(c)].

EFFECTS OF MAPK INHIBITORS ON JNK PHOSPHORYLATION FOLLOWING MS

C-28/I2 and C-20/A4 cells were incubated in the presence or absence of the p38 inhibitor SB203580 at concentrations of 1, 5, 10, 20 and 50 μM for 30 min prior to MS for 30 min (apparatus (b), Fig. 1). Western blotting demonstrated that phosphorylation of SAPKs/JNKs following MS was inhibited by SB203580 at concentrations from 5 to 50 μM (Fig. 4). Concentrations below 5 μM had no effect ($n=3$). PD98059, which inhibits ERK1/2 phosphorylation had no effect on phosphorylation of SAPKs/JNKs following MS at concentrations up to 50 μM in C-28/I_2 (data not shown).

MS INDUCES JNK1 ACTIVITY

To determine whether the phosphorylation of SAPK/JNK following MS was associated with activation of JNK, a kinase assay was used to detect the activity of SAPK/JNK. Phosphorylation of GST-c-Jun (1–79) was not seen in unstimulated controls, but was detected in both the C-28/I2 and C-20/A4 cell lines following MS for 1 h [Fig. 5(a)]. In C-28/I2 cells, SP600125, a specific JNK inhibitor, inhibited GST-c-Jun phosphorylation following MS at concentrations of 10 and 20 μM, but had no effect at lower concentrations [Fig. 5(b)]. β_1 integrins have been shown to act as a mechanoreceptor in chondrocytes[43]. To investigate whether the increase in JNK activity seen following MS is β1 integrin dependent C-28/I2 and C-20/A4 cells were subjected to 1 h of cyclical MS (apparatus (b) Fig. 1) in the presence of an anti-β_1 integrin, function-blocking antibody. As shown in Fig. 5c, the anti-β1 integrin antibody partially blocked MS-induced activation of JNK1.

THE EFFECTS OF CO_2 ON PHOSPHORYLATION OF JNK KINASE BEFORE OR AFTER CYCLICAL MS

As MAPKs can be activated by increases in CO_2 concentration in some cell lines[49] studies were undertaken to investigate whether phosphorylation of JNK before or after cyclical MS was influenced by CO_2. In some experiments one control dish was kept in the tissue culture incubator (C_t), while a second control was placed on the shelf of the stimulation chamber, but not mechanically stimulated (C_L). Additional dishes were stimulated for 0.5, 1, 2, and 3 h. Phosphorylation of JNK was detected in the C_L control without cyclical MS suggesting that JNK was activated by a lack of CO_2 and/or by pH change. Nevertheless, phosphorylation of JNK increased after 3 h of MS when compared with the control C_L (Fig. 6).

THE INFLUENCE OF THE CONTROL ENVIRONMENT ON JNK PHOSPHORYLATION

In order to establish optimum conditions for demonstrating JNK phosphorylation in the chondrocyte cell lines JNK phosphorylation was examined in four different controls without MS. A C_t control was left in the CO_2 incubator, a C_b control was sealed with parafilm and placed in a box

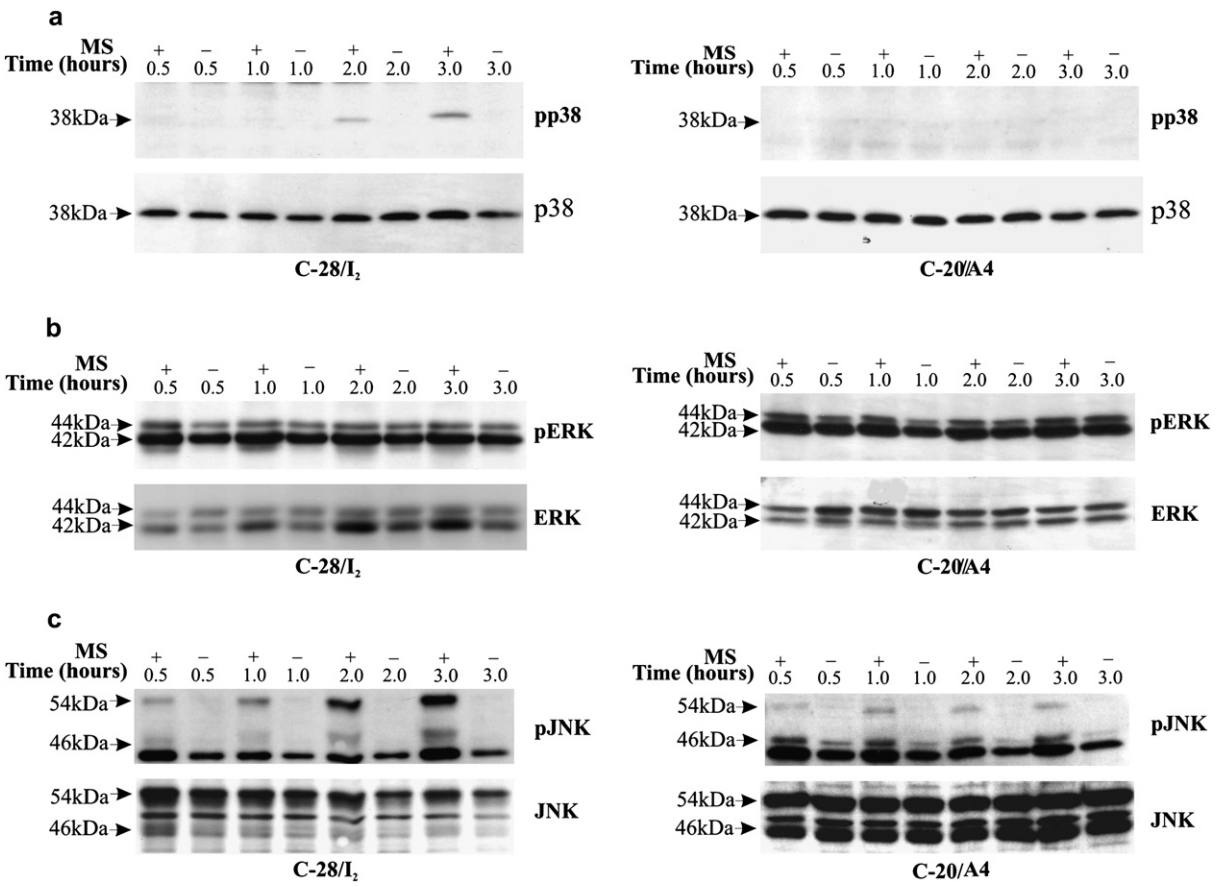

Fig. 3. MAPK expression and activation in C-28/I2 and C-20/A4 cells following MS. Western blots using phosphorylation status-dependent and independent antibodies against p38, ERK1/2 (44 and 42 kDa) and JNK kinases (54 and 46 kDa) following 0.5, 1, 2, and 3 h of cyclical MS. (a) Expression and phosphorylation of p38 following MS ($n = 3$). Western blots show phosphorylation of p38 in C-28/I_2 cells, but not in C-20/A4 cells following MS. (b) Expression and phosphorylation of ERK1/2 following MS. Western blots show that there is no significant change in phosphorylation following MS ($n = 3$). (c) Expression and phosphorylation of JNK following MS. Western blots show phosphorylation of 54 and 46 kDa JNK following 0.5, 1, 2 and 3 h of cyclic MS ($n = 3$).

on the shelf in the stimulation incubator, a C_L control was left on the shelf in the stimulation incubator, and a C_s control was placed in a pressure chamber [Fig. 1(b)] without application of MS. All controls were left for 0.5, 1, 2 and 3 h. Phosphorylated 54 and 46 kDa JNKs were detected only in the C_L control at 1 h. After leaving the cells for 2 and 3 h, phosphorylated 54 and 46 kDa JNK were detected in the C_s control, but were weaker than the corresponding C_L control (Fig. 7). The apparatus was then modified to introduce 5% CO_2 into the stimulation chamber. In the presence of CO_2 no phosphorylation of JNK could be detected (data not shown).

Discussion

Matrix synthesis increases in response to cyclical MS in articular cartilage *in vivo*[2] and in cartilage explants[6–8,50,51] and isolated primary chondrocytes in agarose[10,52] or monolayer cell culture[9,15,45] *in vitro*. The signal transduction

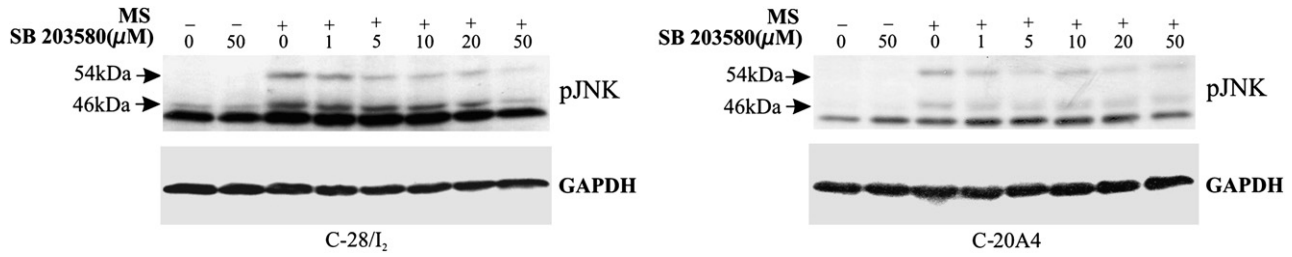

Fig. 4. The effects of SB203580 on phosphorylation of JNK in C-28/I2 and C-20/A4 cells. Western blots using phosphorylation dependent antibody show inhibition of JNKs by SB203580 at concentrations ≥ 5 μM.

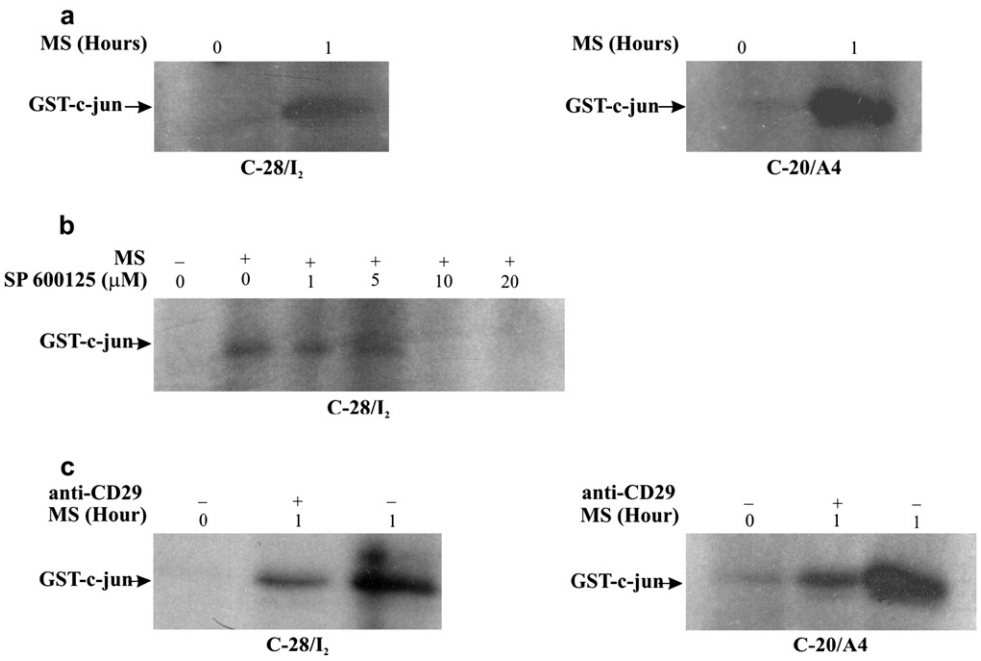

Fig. 5. The effects of MS, inhibition of JNK and an anti-β1 integrin antibody (anti-CD29) on JNK activation using a kinase assay. (a) JNK1 activation following MS. Activation of JNK1 following 1 h cyclical MS in the C-28/I2 and C-20/A4 cell lines ($n = 3$). (b) Effects of the JNK inhibitor SP600125 on JNK1 activity following MS. Pre-incubation with SP600125 blocks the activation of JNK1 in C-28/I2 following 1 h cyclical MS at concentrations $\geq$10 μM ($n = 3$). (c) Effects of anti-CD29 on JNK activation following MS. Mouse anti-human CD 29 partially blocked the activation of JNK1 following 1 h cyclical MS in C-28 I2 and C-20/A4 cells ($n = 3$).

mechanisms involved in these changes in chondrocyte metabolism in response to mechanical stimuli have been elucidated in part[42,53,54]. The $\alpha_5\beta_1$ integrin-dependent and IL-4-dependent increases in aggrecan mRNA and decreases in MMP-3 mRNA that follow cyclical MS of normal primary human articular chondrocytes in monolayer cell culture are not seen in chondrocytes from osteoarthritic articular cartilage[15]. Previous studies have shown that the SV40-immortalized human chondrocyte cell line C-20/A4 could be used to demonstrate integrin-dependent upregulation of sulphate incorporation into PGs following cyclical MS[16]. In the current studies, using two different human chondrocyte cell lines as models, we have shown that this stimulation of PG synthesis following cyclical MS can be inhibited completely in the presence of the pyridinyl imidazole compound SB203580[55] at a concentration of 10 μM but not 5 μM, while the MAPK kinase-1 (MEK-1), or ERK1/2, inhibitor PD98059[56] had no effect at concentrations as high as 50 μM. SB203580 was initially thought to be a specific inhibitor of the p38 MAP kinase[57]. Davies *et al*[58] showed that SB203580 inhibited SAPK 2a/p38 and SAPK 2b/p38β_2 with IC 50's of 50 nM and 500 nM. Lymphocyte kinase (LCK), glycogen synthase kinase (GSK), 3β and protein kinase C (PKCα) were also inhibited but at IC 50's that were 100- to 500-fold higher, and no other related protein kinase was inhibited by 10 μM SB203580. Others, however, found evidence that SB203580 could inhibit the phosphorylation of some isoforms of JNK[59,60] and it was subsequently shown that the 54 kDa JNK isoform could be inhibited significantly by 2 μM SB203580 although the 46 kDa isoform was unaffected[48]. Consistent with these published findings, our experiments showed activation of SAPK/JNK after 1 h of MS in two human chondrocyte cell lines (C-20/A4 and C-28/I2) by a kinase assay using GST-c-Jun as substrate. This was further confirmed by the inhibition of MS-stimulated GST-c-Jun phosphorylation with the JNK inhibitor SP600125[61] at concentrations of 10 and 20 μM. Although both cell lines contain ERK1/2, p38, 46 and 54 kDa JNK proteins in unstimulated conditions, only the JNK proteins were consistently phosphorylated by MS, whereas p38 phosphorylation was stimulated by MS in C-28/I2 cells, but not in C-20/A4 cells. Together these data support a role for JNK signalling in the response of chondrocytes to MS. Selective activation of JNK in response to cyclical mechanical strain has been demonstrated previously in cultured human osteoarthritic periodontal ligament cells[62].

The JNK family of stress-activated kinases constitutes a group of intracellular signalling enzymes that are activated by dual phosphorylation of threonine and tyrosine residues within a Thr-Pro-Tyr motif[66] in response to pro-inflammatory

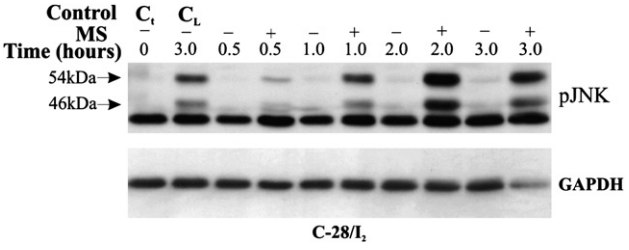

Fig. 6. The effects of CO_2 and MS on JNK phosphorylation in C-28/I2 cells. Western blots showing phosphorylation of 54 and 46 kDa JNK protein kinases when cells left in the pressure chamber without CO_2 for 3 h or following 0.5, 1, 2, and 3 h cyclical MS. C_t = Control in CO2 incubator. C_L = Control in stimulation chamber without CO2 ($n = 3$).

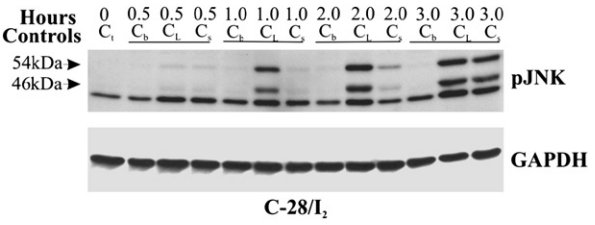

Fig. 7. The influence of the control environment on JNK phosphorylation in C-28/I2 cells. Western blots showing phosphorylation of JNK protein kinases (54 and 46 kDa) in controls. C_t = Control in CO_2 incubator. C_b = Control in sealed dishes in pressure incubator. C_L = Control in pressure incubator without CO_2. C_s = Control in pressure chamber without MS ($n = 3$).

cytokines and environmental stimuli such as osmotic stress, redox stress and radiation. The other stress-activated kinase pathway, involving p38 MAPK isoforms, is also activated by IL-1, TNF-α, and bacterial lipopolysaccharide, as well as by some additional cellular stresses[48]. The ERK group of MAPKs is activated typically by growth factors[66]. Static mechanical compression of cartilage *ex vivo*, with loads approximating to those that occur in weight-bearing joints *in vivo*, result in rapid strain-dependent phosphorylation of ERK and p38 MAPK followed by delayed activation of the JNK pathway via SEK1 and sustained phosphorylation of ERK1/2[22]. Unfortunately, it is not possible in such experiments to ascertain whether or not the kinase activation results indirectly from the release of cytokines or growth factors, or changes in charge density[67] or pH[65]. It is clear from our experiments that JNK phosphorylation can rapidly follow CO_2 deprivation and/or minor changes in pH in chondrocytes in culture, emphasising the importance of careful control of the physiological milieu when undertaking *in vitro* studies of MAPKs in chondrocytes. Significant rises in pH in HEPES-containing media as well as in bicarbonate-buffered media may occur within 15 min of exposure to air[63]. The C-20/A4 chondrocyte cell line has very low levels of carbonic anhydrase activity[64], limiting its capacity for cellular buffering, and there is evidence that the synthesis of cartilage matrix by chondrocytes is extremely pH sensitive[65]. Paradoxically, MAP kinases can also be activated by CO_2 or CO_2-induced acidosis in neural cell lines[49].

Activation of JNK can be mediated by integrin-dependent and integrin-independent mechanisms. Partial inhibition of the activation of JNK following 1 h of cyclical MS, with an anti-β_1-integrin function-blocking antibody [Fig. 5(c)], suggested that the activation of SAPK/JNK in these chondrocyte cell lines following MS was at least in part integrin dependent. This finding taken together with the data showing MAP kinase dependent up-regulation of sulphate incorporation into PGs following MS, the data demonstrating the activation of JNK kinases following MS and previous studies which demonstrated that up-regulation of PG synthesis in human chondrocyte cell lines was integrin dependent[45] lends credence to the hypothesis that integrin-mediated activation of JNKs may be involved in the signal transduction pathway that leads to up-regulation of PG synthesis in chondrocytes following MS.

Cutting articular cartilage is followed by sustained activation of ERK1/2 as a result of release of basic bFGF from an extracellular heparan sulphate bound matrix pool[68] and the rapid activation of the ERK1/2 following cyclical loading of porcine articular cartilage is also bFGF-dependent[23]. ERK activation in cartilage explants or isolated chondrocytes cannot be elicited with fibronectin- or RGD-containing peptides and RGD peptides fail to inhibit the release of bFGF after cutting the articular cartilage, suggesting that these effects may not be mediated by integrin mechanoreceptors. In other model systems, shear-induced release of bFGF from cultured vascular endothelial cells can be inhibited by blocking $\alpha_v\beta_3$ integrin with fibronectin[25] and static biaxial stretch of cardiac fibroblasts is associated with integrin-dependent activation of ERK2 and JNK1, but not p38[69]. Indeed, there is now a great deal of evidence to suggest that MAPK activation by flow in endothelial cells[70,71] and by MS in vascular smooth muscle cells[72] is integrin dependent. However, in our study, phosphorylation of ERK1/2 was apparently unaffected by MS, and this was consistent with the lack of effect of PD98059 on MS-stimulated PG synthesis.

In summary, these experiments provide evidence for JNK-dependent increase in proteoglycan synthesis and partially integrin-dependent selective activation of JNK kinases in human chondrocyte cell lines in response to cyclical mechanical strain.

Acknowledgments

Professor J. Saklatvala for helpful advice relating to the JNK kinase assay and AstraZeneca and the Arthritis Research Campaign for grant support. Dr Goldring's research was supported by National Institutes of Health grants R01-AR45378 and R01-AG22021.

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