p38 MAP kinase signalling is required for hypertrophic chondrocyte differentiation

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Longitudinal growth of endochondral bones is accomplished through the co-ordinated proliferation and hypertrophic differentiation of growth plate chondrocytes. The molecular mechanisms and signalling cascades controlling these processes are not well understood. To analyse the expression and roles of p38 mitogen-activated protein kinases in this process, we have established a micromass system for the reproducible hypertrophic differentiation of mouse mesenchymal limb bud cells. Our results show that all four mammalian p38 kinase genes are expressed during the chondrogenic programme, as well as their upstream regulators MKK3 (mitogen-activated protein kinase kinase 3) and MKK6. Treatment of micromass cultures with pharmacological inhibitors of p38 results in a marked delay in hypertrophic differentiation in micromass cultures, indicating a requirement for p38 signalling in chondrocyte differentiation. Inhibition of p38 kinase activity

leads to reduced and delayed induction of alkaline phosphatase activity and matrix mineralization. In addition, p38 inhibition causes reduced expression of hypertrophic marker genes such as collagen X, matrix metalloproteinase 13 and bone sialoprotein. The function of p38 in hypertrophic differentiation appears to be mediated, at least in part, by the transcription factor myocyte enhancer factor 2C. In summary, we have demonstrated a novel requirement for p38 signalling in hypertrophic differentiation of chondrocytes and identified myocyte enhancer factor 2C as an important regulator of chondrocyte gene expression.

Key words: chondrocyte, hypertrophic differentiation, Mitogen-activated protein kinase (MAP kinase), myocyte enhancer factor 2 (MEF2), p38.

INTRODUCTION

The growth of endochondral bones such as ribs, vertebrae and the long bones of vertebrate limbs is regulated through the highly controlled rates of proliferation and hypertrophic differentiation of growth plate chondrocytes [1–3]. Both the expansion in cell number and the volume increase associated with hypertrophy contribute to longitudinal growth. These processes are disrupted in a large number of growth anomalies, including chondrodysplasias [4,5] and endocrine diseases (such as growth hormone deficiency and hypothyroidism) [6,7]. In addition, deregulation of these processes appears to be involved in the pathogenesis of osteoarthritis [8–10] and the development of cartilaginous tumours [11]. The regulation of growth plate physiology is not completely understood. Several key regulators of chondrocyte proliferation and differentiation have been identified in recent years, including PTHrP (parathyroid hormone-related peptide), indian hedgehog, FGFs (fibroblast growth factors), and members of the transforming growth factor β family [12–14]. However, the intracellular signalling pathways mediating the actions of these extracellular factors are still largely unknown.

MAP (mitogen-activated protein) kinases play key roles in the transduction of extracellular signals to intracellular effectors such as transcription factors [15,16]. Among the various subfamilies of MAP kinases, p38 kinases have been the focus of much attention in the last few years [17]. Four different p38 kinases, encoded by four different genes, with overlapping substrate specificity have been described (p38 α , p38 β , p38 γ and p38 δ). Substrates encode transcription factors such as ATF-2 (activating transcription factor 2), MEF2C (myocyte enhancer factor 2C) and C/EBP homolo-

gous protein, kinases such as MAP kinase-activated protein 2 and p90 ribosomal S6 kinase, as well as heat shock proteins. Among these substrates, ATF-2-deficient mice have been shown to display multiple skeletal defects, including reduced proliferation of growth plate chondrocytes and altered cell-cycle gene expression in chondrocytes [18–21]. Whereas p38 kinases were originally cloned as mediators of stress and inflammatory signals, recent results demonstrate important roles of these proteins in growth and development. For example, p38 signalling controls terminal differentiation of myoblasts [22–24], proliferation and differentiation of osteoblasts [25–28] and maturation of osteoclasts [29–31].

During cartilage development, p38 signalling has been shown to be essential for the differentiation of mesenchymal precursor cells to chondroblasts [32–34] (reviewed in [35]). However, the roles of p38 in later stages of chondrocyte maturation (e.g. growth plate chondrocyte proliferation and hypertrophic differentiation), the identity of the p38 proteins involved and downstream components of p38 signalling in chondrocytes have been less studied. Watanabe et al. [36] have demonstrated a requirement for p38 signalling for the expression of aggrecan, the major proteoglycan of cartilage, in ATDC5 cells, a murine cell line capable of chondrogenic differentiation. In addition, we [37] and others [38] have shown that inhibition of p38 signalling in chondrocytes results in decreased expression of the collagen X gene, the classical marker of hypertrophic chondrocytes. However, no systematic investigations into the roles of p38 signalling in hypertrophic differentiation have been performed. Furthermore, no substrates involved in p38 signalling in chondrocytes have been identified.

Abbreviations used: ATF-2, activating transcription factor 2; BSP, bone sialoprotein; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; FGF, fibroblast growth factor; MAP, mitogen-activated protein; MEF2, myocyte enhancer factor 2; MKK, MAP kinase kinase; MMP13, matrix metalloproteinase 13; PTHrP, parathyroid hormone-related peptide; RT, reverse transcriptase.

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In the present paper, we demonstrate the use of a high-density micromass culture system for the reproducible hypertrophic differentiation of primary mouse mesenchymal cells. Inhibition of p38 activity by pharmacological inhibitors results in reduced and delayed hypertrophic differentiation. The transcription factor MEF2C appears to be involved in the regulation of chondrocyte gene expression by p38 signalling. These results suggest important roles of p38 and MEF2C in hypertrophic chondrocyte differentiation.

EXPERIMENTAL

Materials

Timed pregnant CD1 mice [at 11.5 d.p.c (days post coitum)] were purchased from Charles River Laboratories (St Constant, Quebec, Canada). All cell culture media components were from Invitrogen (Burlington, ON, Canada) unless stated otherwise. SB202474, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulphonylphenyl)-5-(4-pyridyl)-1H-imidazole], SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole] and PD169316 [4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole] were from Calbiochem (San Diego, CA, U.S.A.). All other reagents were of analytical grade from commercial suppliers.

Methods

Preparation of chondrocyte micromass cultures

Primary chondrocytes were isolated from forelimbs of CD1 mouse embryos at 11.5 d.p.c. and digested in 10 % (v/v) chick serum/Puck's solution A (0.4 g of KCl/l, 8 g of NaCl/l, 0.35 g of NaHCO_3 /l, 1 g of glucose/l) containing 10 mg of dispase/ml (1 unit/ml) for 1.5 h at 37 °C. The dispase activity was neutralized with 2:3 DMEM (Dulbecco's modified Eagle's medium)/F12 media containing 10 % FBS (foetal bovine serum; Gibco BRL, Life Technologies). A single cell suspension was obtained by passing the digested cells through a 40 μm cell strainer. Following centrifugation, cells were resuspended in growth media at 2.5×10^7 cells/ml and spotted in 10 μl drops/well of a 24-well Corning tissue culture plate. The final cell density was therefore 2.5×10^5 cells/well. This high cell density is important for the complete progression to hypertrophic chondrocytes. After a 1 h incubation (at 37 °C) to allow for cell attachment, the micromasses were fed with growth media that consisted of 2:3 DMEM/F12, supplemented with 10 % FBS, 0.5 mM glutamine, 25 units/ml penicillin and 25 $\mu\text{g}/\text{ml}$ streptomycin, 0.25 mM ascorbic acid (Sigma) and 1 mM β -glycerophosphate (Sigma). Media were replenished daily, with p38 inhibitor supplementation from day 2. p38 MAP kinase inhibitors were used at 2 μM in DMSO unless indicated otherwise. DMSO concentration did not exceed 0.1 % of the well volume and therefore did not demonstrate adverse effects on chondrocyte differentiation. All experiments were performed independently at least three times.

Histological analysis of micromass sections

Micromass cultures were prepared, maintained and treated with p38 inhibitors as described earlier. At selected time points after isolation, micromasses were washed twice with cold PBS and then fixed in 4 % (w/v) paraformaldehyde at room temperature 22 °C for 2 h. Sections were dehydrated through graded alcohols and embedded in paraffin. Sections (5 μm) were cut before staining with haematoxylin and eosin or Alcian Blue according to standard methods. Images were recorded using a Leica DMRA2 microscope at $\times 40$ magnification.

Alkaline phosphatase activity

Alkaline phosphatase activity was visualized as described in [39]. Cells were washed twice with cold PBS before fixing for 2 h with 10 % (v/v) formalin in PBS. Following two washes with water (pH 8.0) and one 15 min incubation in water (pH 8.0), cells were stained with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.5 % *N,N*-dimethylformamide, 2 mM MgCl_2 and 0.6 mg/ml Red Violet LB salt (Sigma) in 0.1 M Tris/HCl (pH 8.3) in the dark at room temperature for 45 min, followed by observation using phase-contrast microscopy.

For quantitative assays, alkaline phosphatase activity from micromass cultures was measured at the indicated time as described in [40] with minor modifications. Briefly, micromasses were washed twice with cold PBS before scraping and harvesting the cells into 10 mM Tris/HCl (pH 8.3), containing 0.2 % Igepal and stored at -20 °C until the assay. Enzyme activity was determined in sonicated supernatants by measuring hydrolysis of the *p*-nitrophenyl phosphate substrate according to the manufacturer's instructions (Sigma). The reaction was terminated by the addition of 3 M NaOH. Using a standard curve, the amount of *p*-nitrophenyl substrate released was quantified by measuring the absorbance at 405 nm. The calculated units of activity were then expressed as a function of protein. Quantity of the protein within the supernatant was determined using the bicinchoninic acid method according to the manufacturer's instructions (Sigma). All data represent means $\pm$ S.E.M. from three different experiments.

Analyses of mineralization

Calcium deposition was visualized using von Kossa staining. After two washes with cold PBS, micromass cultures were fixed with 10 % formalin for 2 h at room temperature before staining for 30 min with 2.5 % (w/v) silver nitrate solution. The cells were then thoroughly washed with water and allowed to air-dry. All data represent means $\pm$ S.E.M. from three different experiments.

Calcium deposition was also quantified by Alizarin Red S staining as described in [41], with minor modifications. Cells were washed twice with cold PBS, fixed for 10 min in 70 % ethanol before staining with 0.5 % Alizarin Red S (Sigma) in water (pH 4) for 30 s. Following staining, the cells were washed three times with water and once with 70 % ethanol. Matrix mineralization was quantified by extracting the Alizarin Red S stain with 100 mM cetylpyridinium chloride (Sigma) at room temperature for 3–4 h. The absorbance of the extracted Alizarin Red S stain was measured at 570 nm.

Alcian Blue staining

Alcian Blue staining was performed as described in [42].

RNA isolation and RT (reverse transcriptase)–PCR

Total RNA was extracted from the micromass cultures at the indicated time, using the TRIzol[®] reagent RNA extraction method according to manufacturer's instructions (Gibco BRL, Life Technologies). cDNA was prepared from 1 μg of total RNA using the Superscript First-Strand Synthesis System for RT–PCR (Gibco BRL, Life Technologies) with p(dT)_{12–18} and random hexamer primers (Amersham Biosciences, Piscataway, NJ, U.S.A.). Amplification was performed in a 50 μl reaction mixture containing 1 μl of cDNA reaction and using ampliTaQ Gold polymerase (PerkinElmer, NJ, U.S.A.). The primer sequences used for each PCR are outlined below. The initial denaturation was done at 95 °C for 1.5 min, annealing temperatures ranged from 60 to

65 °C for 45–120 s, followed by extension at 72 °C. A final extension at 72 °C for 6 min concluded the reaction. PCR cycles varied from 20 to 37 cycles. PCR products were visualized on 1.5 % (w/v) agarose gels stained with ethidium bromide.

β -actin, collagen II, collagen X and MMP13 primer sequences were as described in [43,44]. p38 α forward AGGCCATGGTGC-ATGTGTGT, p38 α reverse AGTAGCTGGAGGAGGAGGAG, p38 β forward GGCTGCATCATGGCTGAACT, p38 β reverse TGAGAGGCGCTTCTTGAGGA, p38 γ forward TCTCAGCT-TCAAGCCTCCTA, p38 γ reverse TCCTAAGCTCTGCTCAC-TCT, p38 δ forward GTCTGTTGGTTGCATCATGG, p38 δ reverse GGATCTCTTGAGTTGCAGGT, MKK3 forward GCTA-CTGCCATTCTGTTTCCT, MKK3 reverse CCACAGTGATCG-AAGTCCAT, MKK4 forward TCTAGCGTAGGCTGAACTCT, MKK4 reverse TGAGCCGTGATACAAGTGAC, MKK6 forward GGAGTCTGCCACCTGTCATA, MKK6 reverse CAC-CAGACACAGGAGAGAT, MEF2C forward TCTGTCTG-GCTTCAACACTG and MEF2C reverse TGGTGGTACGGTCT-CTAGGA.

Real-time PCR

Real-time PCR was performed using 20 μ l final reaction volumes that contained the TaqMan one-step master mix kit (Applied Biosystems), gene-specific target primers and probes [FAM (6-carboxyfluorescein) dye layer] and endogenous reference primers and probes (VIC dye layer). Amplification was performed in an ABI Prism 7900 HT Sequence Detector (Perkin-Elmer) with 40 cycles and an annealing temperature of 60 °C. The FAM dye layer yields quantification of the target genes, whereas VIC yields simultaneous quantification of the 18 S rRNA as an internal control. All samples were amplified in five parallel reactions, and averages from these five amplifications are shown. Experiments were performed at least twice (from independent cell isolations) with similar results. All experiments contained controls without template. Primers and probes were designed using the software PrimerDesigner 2.0 (Applied Biosystems). Collagen II detection was performed using the following primer/probe set: forward primer 5'-GGCTCCCAACACCGC-TAAC, reverse primer 5'-GATGTTCTGGGAGCCCTCAGT and probe 6-FAM-5'-CAGATGACTTTCCTCCGTC-MGBNFQ. The following primer/probe set was used for Sox9 detection: forward primer 5'-CATCACCCGCTCGCAATAC, reverse primer 5'-CC-GGCTGCGTGACTGTAGTA and probe 6-FAM-5'-ACCATC-AGAACTCCGGCT-MGBNFQ. BSP (bone sialoprotein) expression was determined with the following primer/probe set: forward primer 5'-CGCTACTTTCTTTATAAGCATGCCTACT, reverse primer 5'-GCCTCCCTGGACTGGAAAC and probe 6-FAM-5'-ATCCTCCTCTGAAACG-MGBNFQ. Collagen X gene expression was detected with the following primer/probe set: forward primer 5'-ACGCCTACGATGTACACGTATGA, reverse primer 5'-ACTCCCTGAAGCCTGATCCA and probe 6-FAM-5'-AGTACAGCAAAGGCTAC-MGBNFQ. All data represent means $\pm$ S.D. from three different experiments.

Transient transfections and luciferase assays

Chondrocytes were released from micromass cultures on day 10 by digestion with collagenase P (3 mg/ml in DMEM containing 10 % FBS) for 1 h at 37 °C: 60 000 cells/well were plated of a 24-well plate and immediately transfected using FuGENE 6 (Roche) according to the manufacturer's instructions. The MEF2 reporter construct was kindly provided by Dr Mona Nemer (Clinical Research Institute of Montreal, Canada) [45], the dominant-negative p38 plasmid was a generous gift from Dr Roger Davis (University of Massachusetts Medical School)

[46] and the MEF2-engrailed fusion was kindly provided by Dr Ilona Skerjanc (University of Western Ontario). The collagen X promoter plasmid pGI colXH2500 has been described in [47]: 2 μ g of reporter vector was co-transfected with 1 μ g of empty expression vector (pcDNA3; Invitrogen) as control or the indicated expression vectors, and with 0.02 μ g of pRIMV (Promega) for standardization purposes. p38 inhibitors were included as indicated. Luciferase assays were performed as described in [19]. Data shown represent means $\pm$ S.D. from three independent experiments, performed in triplicate each.

RESULTS

Hypertrophic differentiation of mouse micromass cultures

Studies of mammalian cartilage development have been hampered by the limited availability of reproducible models for hypertrophic chondrocyte differentiation *in vitro*. We therefore investigated whether micromass cultures of mouse embryonic limb bud mesenchymal cells permit hypertrophic differentiation of chondrocytes. This system has been used extensively for studies of early chondroblast differentiation (chondrogenesis) as well as for hypertrophic differentiation of chicken chondrocytes [48,49]. Whereas one report described hypertrophic differentiation of mouse mesenchymal cells in these cultures based on morphological criteria [50], no biochemical or molecular characterization of these cultures has been provided. We therefore characterized differentiation of mouse mesenchymal cells in micromass cultures using biochemical and molecular markers. When cultured in the presence of ascorbate and different concentrations of β -glycerophosphate (1, 2.5 or 10 mM), micromass cultures initiate alkaline phosphatase activity by day 6 (Figure 1A). Appearance of alkaline phosphatase activity is accompanied by initiation of matrix mineralization, as indicated by von Kossa staining. Both alkaline phosphatase activity and mineralization increase throughout the culture period (Figure 1A), reaching a maximum around day 15 (see also Figures 4A and 5). In the absence of β -glycerophosphate, alkaline phosphatase activity and mineralization are strongly decreased; however, no differences were observed between different concentrations of β -glycerophosphate. All subsequent experiments were therefore performed in the presence of 1 mM β -glycerophosphate. These results suggest that mouse mesenchymal cells undergo hypertrophic chondrocyte differentiation in micromass culture in the presence of ascorbate and β -glycerophosphate.

At the molecular level, differentiating micromass cultures express collagen II throughout the culture as shown by RT-PCR analyses (Figure 1B). Expression of collagen X and MMP13, two markers of hypertrophic chondrocytes [51–53], is first induced around day 6, concurrent with the onset of alkaline phosphatase activity. Maximal expression of these genes is achieved by days 9–12. Real-time PCR analyses showed that collagen X and BSP, two markers of hypertrophic chondrocyte differentiation, are not expressed in early micromass cultures, but are induced strongly at later stages (results not shown). Finally, Alcian Blue-stained sections from day 15 micromass cultures show cartilage nodules containing numerous hypertrophic chondrocytes and surrounded by a perichondrium-like tissue (Figure 1C). These results demonstrate that mouse mesenchymal cells can successfully differentiate to hypertrophic chondrocytes *in vitro* in high-density micromass cultures.

Expression of p38 and MKK genes during micromass differentiation

Next, we examined the expression of the four p38 genes during micromass culture by RT-PCR analyses. p38 α , β , γ and δ

Figure 1 Hypertrophic differentiation of micromass cultures in ascorbate- and β -glycerophosphate-supplemented medium

(A) Micromasses were cultured for 6, 9 and 12 days in the presence of 0.25 mM ascorbate and various concentrations of β -glycerophosphate before staining for alkaline phosphatase activity and matrix mineralization (von Kossa staining). In the presence of β -glycerophosphate, the micromass cultures demonstrated increased alkaline phosphatase activity and von Kossa staining. (B) RT-PCR analyses of RNA from micromass cultures at selected times of differentiation demonstrate an acquisition of the hypertrophic phenotype as demonstrated by increased expression of hypertrophic chondrocyte-associated markers collagen X and MMP13. (C) Day 15 micromass cultures were embedded in paraffin, sectioned and stained with Alcian Blue. Cartilage nodules, containing large hypertrophic chondrocytes, are surrounded by perichondrium-like tissue.

are expressed throughout the culture period without significant changes in mRNA expression levels (Figure 2). Similarly, the upstream kinases MKK3 and MKK6 are expressed during all stages of chondrogenic differentiation. However, expression of MKK4 {which is an activator of JNK (c-Jun N-terminal kinase) MAP kinases, but appears to control p38 activities in some tissues as well; [54]} is rapidly down-regulated in micromass cultures as cells differentiate along the chondrogenic pathway. These results suggest potential biological roles for all four p38 genes during chondrocyte differentiation.

p38 activity is necessary for maximal alkaline phosphatase activity and mineralization

We next determine whether inhibition of p38 α/β activity by pharmacological inhibitors would affect hypertrophic chondro-

Figure 2 Expression of p38 MAP kinases and upstream kinases during micromass culture

Expression of p38 and MKK genes during micromass culture was investigated by RT-PCR. Expression of p38 α , p38 β , p38 γ , p38 δ , MKK3 and MKK6 mRNA levels appears unaltered during differentiation, whereas MKK4 expression is rapidly down-regulated in differentiating micromass cultures.

cyte differentiation. Unless otherwise noted, all experiments were performed with 2 μ M PD169316, a specific p38 inhibitor [55], and compared with cultures treated with 2 μ M SB202474, an inactive analogue [56]. Pharmacological p38 inhibitors have been shown to block chondrogenesis when present during early stages of micromass cultures [33]. Therefore we added inhibitors after 48 h of culture, after cartilage nodule formation had been initiated, to be able to analyse their effects on later stages of chondrocyte differentiation. Alcian Blue staining of micromass cultures on day 6 did not reveal any differences between cultures treated with DMSO or PD169316 (Figure 3A). These results suggest that early chondrogenesis is not affected under these conditions and that p38 inhibition does not induce chondrocyte de-differentiation, thereby confirming that all observed effects are due to specific interference with later stages of chondrocyte differentiation.

p38 inhibition does not appear to affect alkaline phosphatase activity as visualized by staining of micromasses (Figure 3B). However, quantitative analyses revealed delayed and reduced induction of alkaline phosphatase activity by 2 μ M PD169316 (Figure 4A). Micromass cultures treated with the control compound SB202474 show similar activity as cultures treated with DMSO. In contrast, cultures treated with 2 μ M PD169316 display markedly lower alkaline phosphatase activity starting on day 12. At 5 μ M concentration, effects of PD169316 are seen even earlier (day 6). Similarly, reduced alkaline phosphatase activity is observed in cultures treated with two different concentrations (2 and 5 μ M) of two additional p38 inhibitors, SB202190 and SB203580, on day 15 (Figure 4B). We also measured the concentration dependence of alkaline phosphatase inhibition by PD169316 on day 15. Marked inhibition of alkaline phosphatase activity was observed from 2 μ M and reaches a maximum at 10 μ M (Figure 4B).

Treatment with PD169316 results in a reduction in mineralization, as shown by von Kossa staining (Figure 3B).

Figure 3 p38 inhibition decreases mineralization

(A) Alcian Blue staining of day 6 micromass cultures that had been treated with DMSO or 2 μ M of the p38 inhibitor PD169316 from day 3 onwards reveals no differences in early chondrogenesis between the different treatments. (B) Staining of cultures for alkaline phosphatase activity reveals no strong differences on p38 inhibition with 2 μ M PD169316. In contrast, matrix mineralization (von Kossa staining) is suppressed by 2 μ M PD169316.

This is further demonstrated by quantitative analyses of Alizarin Red S staining (Figure 5). Whereas SB202474 treatment does not affect mineralization (compared with DMSO), treatment with 2 or 5 μ M PD169316 causes clear inhibition of mineralization starting on day 9. As in the case of alkaline phosphatase activity, the inhibitory effect of PD169316 is larger at the higher concentration. These experiments reveal a requirement for p38 signalling for normal alkaline phosphatase activity and mineralization during hypertrophic chondrocyte differentiation.

p38 inhibition results in decreased expression of chondrocyte marker genes and delays the onset of hypertrophic gene expression

We next analysed the effects of p38 inhibition on the expression of chondrocyte marker genes. Compared with SB202474, PD169316

Figure 4 p38 inhibitors decrease alkaline phosphatase activity

(A) Alkaline phosphatase activity was measured during differentiation of micromass cultures in the presence or absence of 2 μ M PD169316. PD169316 treatment resulted in a marked suppression of alkaline phosphatase activity when compared with micromasses differentiated in the presence of 2 μ M SB202474 or media supplemented with 0.1 % DMSO. (B) Effects of different concentrations of several p38 inhibitors on alkaline phosphatase activity were measured on day 15 of culture. All inhibitors resulted in decreased alkaline phosphatase activity in a dose-dependent manner. All data represent means $\pm$ S.E.M. from three independent experiments.

Figure 5 p38 inhibition leads to decreased mineralization

Mineralization of micromass cultures in the presence of DMSO, 2 μ M SB202474 or 2 or 5 μ M PD169316 was analysed by quantification of Alizarin Red staining and shown as a percentage of the value of SB202474-treated cells on day 15. Mineralization is delayed in the presence of PD169316. Data represent means $\pm$ S.E.M. from three independent experiments.

treatment causes a drastic reduction in the expression of collagen II, as shown by RT-PCR analyses (Figure 6). This down-regulation is confirmed by real-time PCR analyses of collagen II expression on day 6 of culture (Figure 7). Down-regulation of collagen II expression by PD169316 is accompanied by a decrease in mRNA levels of the chondrogenic master transcription factor Sox9, which directly activates the collagen II gene [57], as shown by real-time PCR analyses (Figure 7).

Figure 6 RT-PCR analyses of chondrocyte marker gene expression in response to PD169316

Expression of chondrocyte marker and p38 genes was analysed in micromass cultures incubated in the presence of PD169316 or the inactive analogue SB202474 (2 μ M each). Collagen II levels were decreased in PD169316-treated cells. Furthermore, maximal collagen X and MMP13 expression is delayed in the presence of PD169316. Expression of p38 α and p38 δ seems to be lowered at later stages by PD169316, whereas p38 β and p38 γ levels do not seem to be affected.

Figure 7 Real-time PCR analysis of chondrocyte gene expression

Collagen II, Sox9, collagen X and BSP mRNA levels in micromass cultures incubated with 2 μ M SB202474 (closed bars) or 2 μ M PD169316 (open bars) were analysed by real-time PCR and displayed as percentage of the value of SB202474-treated cells. Inhibition of p38 activity leads to lowering of collagen II and Sox9 transcript levels on day 6 as well as decreased levels of collagen X and BSP mRNA on day 15. All data represent means $\pm$ S.D. from three independent experiments.

PD169316 also induces a delay in the induction and a drastic decrease in maximal expression of the collagen X gene, the classical marker of hypertrophic chondrocytes (Figure 6). In agreement with this, quantitative analyses of collagen X expression on day 15 by real-time PCR analyses show strongly decreased levels of collagen X mRNA in PD169316-treated cultures (Figure 7). Similarly, expression of MMP13, another marker of hypertrophic chondrocytes, is slightly delayed and decreased under conditions of p38 inhibition. In addition, expression of BSP mRNA on day 15 is significantly decreased in the presence of 2 μ M PD169316 (Figure 7), whereas the control compound SB202474 does not affect BSP mRNA levels when compared

with untreated controls. Furthermore, expression of p38 α and p38 δ seems to be decreased at later stages in the presence of PD169316. In contrast, expression of p38 β and γ (Figure 6) and of MMK3 and MKK6 genes (results not shown) does not change in response to p38 inhibition, indicating that the observed effects reflect direct regulation of chondrocyte gene expression by p38 MAP kinases rather than a general down-regulation of gene expression. These results suggest a requirement for p38 signalling for normal gene expression in chondrocytes, in particular during hypertrophic differentiation.

Effects of p38 inhibition on micromass morphology

We next examined how p38 inhibition affects the morphology of micromass cultures. Cultures were incubated with 2 μ M SB202474 or PD169316 and embedded, sectioned and stained on days 4, 9 and 15. Histological analyses reveal that hypertrophic chondrocytes become apparent in condensations of day 9 cultures (Figure 8). The number and size of these hypertrophic chondrocytes increases further on day 15. Similar progression to hypertrophy is observed in control and p38-inhibited cultures; however, the number and size of hypertrophic cells appears to be larger in SB202474-treated cells. These results suggest that p38 inhibition does not only delay molecular markers of hypertrophy, but also the characteristic increase in cell volume.

MEF2C as a potential mediator of p38 signalling in chondrocytes

The transcription factor MEF2C is a substrate of p38 in monocytes and other cells [58,59]. Since the collagen X promoter contains a potential MEF2 binding site [60], we determined whether p38 signalling regulates MEF2C in chondrocytes as well. An MEF2 reporter plasmid was transfected into chondrocytes released from micromass cultures and p38 was inhibited by 2 μ M PD169316 or by overexpression of a dominant-negative form of p38. In both cases, p38 inhibition results in >40% reduction in MEF2-dependent transcription of a luciferase reporter gene (Figure 9A). Next we examined whether MEF2C regulates the activity of the collagen X promoter in hypertrophic chondrocytes using a fusion protein of MEF2C with an engrailed transcriptional repression domain. Overexpression of this dominant-negative form of MEF2C results in a 56% reduction in collagen X promoter activity, compared with a 66% reduction by dominant-negative p38 α . These results suggest that MEF2C activity in chondrocytes is regulated by p38 and that MEF2C activity is required for collagen X expression in hypertrophic chondrocytes (Figure 9B). Finally, we investigated MEF2C expression during chondrocyte differentiation by RT-PCR analyses. MEF2C is expressed throughout the differentiation of micromass cultures (Figure 9C).

DISCUSSION

Our results clearly show a delay of hypertrophic chondrocyte differentiation by established pharmacological inhibitors of p38 MAP kinases, as demonstrated by reduced and delayed induction of alkaline phosphatase activity, matrix mineralization and expression of the hypertrophic marker genes collagen X, MMP13 and BSP. Similar results were obtained with several different inhibitors at very low concentrations of inhibitors, and in comparison with a chemically similar control. Our results therefore suggest that these inhibitors delay chondrocyte differentiation through specific inhibition of p38 MAP kinases. These conclusions are supported by the fact that PD169316 inhibits the activity of the MEF2 reporter to a similar degree as a dominant-negative form of p38 α .

Figure 8 Histology of micromass cultures

Micromass cultures were incubated with 2 μ M SB202474 (**A–C**) or PD169316 (**D–F**), embedded on days 4 (**A, D**), 9 (**B, E**) and 15 (**C, F**), sectioned and stained with haematoxylin and eosin. Size and number of hypertrophic chondrocytes increases over time under both conditions, but appear greater in SB202474-treated cells.

Figure 9 MEF2C regulates collagen X transcription in chondrocytes

(**A**) Cells were released from micromass cultures by collagenase digestion and transfected with an MEF2 reporter plasmid and pRICMV. Cells were incubated with 2 μ M SB202474 or PD169316 for 48 h before harvesting. In parallel, cells were transfected with the MEF2 reporter, pRICMV and empty expression vector or an expression vector for dominant-negative p38 α (dn p38). Firefly luciferase activity was determined and standardized to *Renilla* luciferase activity. p38 inhibition by either pharmacological inhibitors or dn p38 results in >40% reduction in MEF2 activity. (**B**) Cells were released from micromass cultures by and transfected with pGICoX H2500, pRICMV and empty expression vector or expression vectors for a fusion of MEF2C with the engrailed transcriptional repression domain (MEF2C-en) or dn p38. Firefly luciferase activity was determined and standardized to *Renilla* luciferase activity. The MEF2C-engrailed fusion decreases collagen X promoter activity almost as much as dn p38. (**C**) MEF2C expression in micromass cultures was analysed by RT-PCR analysis. MEF2C is expressed throughout micromass differentiation.

Inhibition of p38 leads to a strong reduction in collagen II mRNA levels. Treatment with p38 inhibitors was only initiated after 48 h, when cartilage condensations have already been established. It is therefore likely that p38 inhibition at this stage does not interfere with chondrogenesis and does not induce de-differentiation of chondrocytes. This is also confirmed by Alcian Blue staining and histological sections, both of which clearly demonstrating chondrogenic differentiation of cells within the micromass cultures, irrespective of p38 inhibition. Furthermore, p38 inhibition represses collagen II expression throughout the culture period, rather than just delaying it. Therefore we suggest that p38 signalling is necessary for normal collagen II expression in differentiated chondrocytes, probably independent of effects on chondrogenesis. Similarly, on day 6, mRNA abundance of the chondrogenic master transcription factor Sox9, which directly activates the collagen II gene [57], is strongly decreased by PD169316 treatment. These results suggest that p38 signalling is also required for normal gene expression in differentiated chondrocytes that have not initiated hypertrophic differentiation. This effect could be of importance in articular chondrocytes, where normal gene expression is disrupted in diseases such as osteoarthritis. Our results also suggest that p38 signalling is required to maintain p38 α and p38 δ expressions at later stages of chondrogenic differentiation (possibly in a positive feedback loop), but further studies employing more quantitative methods will be required to confirm these observations. However, it is important to note that expression of many genes (such as p38 β and γ , MKK3 and MKK6) did not change significantly on p38 inhibition, suggesting that the observed down-regulation of gene expression does not reflect a general metabolic effect, but rather a specific modulation of chondrocyte-specific gene expression.

Quantitative analyses suggest that p38 inhibition strongly represses the expression of hypertrophic marker genes such as collagen X and BSP, in agreement with the strong reduction of alkaline phosphatase activity and mineralization. This is confirmed by our transient transfections of primary chondrocytes showing strong inhibition of the collagen X promoter by PD169316, suggesting that p38 effects are mediated, at least in part, by changes in gene transcription. Some of these effects of p38 signalling on hypertrophic gene expression appear to require MEF2C, a well-established substrate of p38 in other cell types [23,58,59,61]; however, it appears that, probably, additional transcription factors are involved. In this context, it is interesting to note that expression of MMP13 seems to be much less affected by p38 inhibition compared with the expressions of other hypertrophic markers such as collagen X or BSP. Hypertrophic differentiation appears to be regulated by several pathways and transcription factors; e.g. Cbfa1-dependent and -independent pathways have been described [62–64]. It is possible that some of these pathways (e.g. MEF2C-dependent pathways) are more sensitive to p38 inhibition, whereas others (e.g. pathways regulating MMP13 expression) might not require p38 activity to the same degree. Further studies into the substrates and mechanism of p38 signalling in chondrocytes will answer these questions.

Our co-transfection studies employing a dominant-negative form of p38 α suggest a role of this p38 gene in hypertrophic chondrocytes. However, since all four p38 proteins share most of their upstream activators and downstream substrates [35], it is likely that the dominant-negative p38 α interferes with the activities of all or most p38 kinases. Elucidation of the role of individual p38 genes in chondrocyte differentiation will therefore require specific inhibition of individual p38 genes, e.g. through gene-targeting technology or siRNA approaches. However, the broad expression patterns of all four genes in chondrocytes and

the overlap in substrate specificity suggest redundant functions of the different p38 kinases in cartilage development. This hypothesis is also supported by the analyses of p38 α -deficient mice that did not reveal any obvious cartilage phenotype [65,66].

The extracellular factors controlling p38 activity during hypertrophic differentiation are not known. Activation of p38 by FGFs [67], members of the TGF β family [34,36], retinoic acid [68], connective tissue growth factor/hypertrophic-chondrocyte-specific gene product 24 [69] and integrins [70] has been described in developing chondrocytes. In most cases, it is not clear how activation of p38 in response to these factors might contribute to hypertrophic differentiation. For example, analyses of mice deficient for FGF receptor 3 suggest that FGF signalling suppresses both proliferation and hypertrophic differentiation of chondrocytes [71], whereas our results clearly suggest a requirement of p38 signalling for normal hypertrophic differentiation. Recent results suggest activation of p38 by $\alpha 6 \beta 1$ integrins in chicken chondrocytes [70], which is accompanied by accelerated hypertrophic differentiation. These results suggest that integrins and their ligands might be important physiological regulators of p38 activity during chondrocyte differentiation.

Zhen et al. [38] have recently demonstrated that p38 signalling is necessary for normal collagen X expression in chicken chondrocytes and that p38 activity is inhibited by PTHrP. This appears to be in agreement with the well-documented inhibition of chondrocyte differentiation by PTHrP [14]. However, down-regulation of p38 activity by PTHrP appears to be mediated by the protein kinase C pathway, and not by the cAMP/protein kinase A pathway that has been shown to be responsible for the mitogenic and differentiation-inhibiting effects of PTHrP. In fact, *in vivo* studies recently demonstrated that activation of the phospholipase C pathway by the PTH/PTHrP receptor supports hypertrophic chondrocyte differentiation [72]. These conflicting results suggest complex regulation and roles of p38 kinases in the control of growth plate physiology.

Multiple substrates of p38 kinases have been described in other cell types, including transcription factors, kinases and heat shock proteins. Among these, only the transcription factors ATF-2 and cAMP response element binding protein have been investigated in the context of growth plate chondrocytes. However, *in vitro* and *in vivo* studies have shown that both of these factors are necessary for maximal proliferation and the expression of the mitogenic cyclin D1 and cyclin A genes in chondrocytes [18–21,73]. It is therefore questionable whether they are involved in the activities of p38 in hypertrophic differentiation. A binding site for MEF2 transcription factors (another target of p38) has been identified in the mouse collagen X gene [60]. Our results demonstrate that MEF2C is indeed regulated by p38 signalling in chondrocytes and required for maximal collagen X transcription, suggesting that it probably plays an important role in the chondrocyte response to p38 signalling. However, based on the large number of p38 substrates, it is probable that additional downstream pathways contribute to p38 effects in cartilage.

Currently available genetic models do not offer any insights into the roles of p38 signalling in skeletal development. p38 α -deficient mice have been created by two groups, but do not display any obvious skeletal phenotypes [65,66]. To date, no 'knockout' models have been reported for the other members of the p38 family. The activities of p38 proteins are controlled by upstream kinases, namely MKK3, MKK4 and MKK6. Among these, MKK3 and MKK6 are specific for p38, whereas MKK4 also activates JNKs. All of these genes have been interrupted by homologous recombination. Mice with targeted disruptions of the MKK3 [74] or MKK6 [75] genes display no developmental or growth abnormalities, whereas MKK4-deficient mice die around

embryonic day 12.5 with defects in liver organogenesis [54,76,77]. *In vivo* analyses of the roles of p38 pathways in cartilage development will therefore require the generation of 'knockout' models for the three remaining p38 kinases, simultaneous disruption of the MKK3 and MKK6 genes and chondrocyte-specific mutagenesis of the MKK4 gene.

In summary, we have demonstrated an important requirement for p38 kinase signalling in normal hypertrophic differentiation of chondrocytes. Future studies will address the identity of relevant upstream regulators and downstream substrates of p38 in chondrocytes, the specific functions of individual p38 proteins and the roles of p38 signalling in skeletal growth, development and pathology *in vivo*.

This work was supported by grants from the Canadian Institutes of Health Research to F.B. and T.M.U. and The Arthritis Society of Canada, the Canadian Arthritis Network, and the Canada Research Chair Program to F.B. We are grateful for technical assistance from Matthew Cowan.

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Received 12 June 2003/8 October 2003; accepted 31 October 2003

Published as BJ Immediate Publication 31 October 2003, DOI 10.1042/BJ20030874