Opposing Role of Mitogen-activated Protein Kinase Subtypes, Erk-1/2 and p38, in the Regulation of Chondrogenesis of Mesenchymes*

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The present studies were performed to determine subtype-specific roles of mitogen-activated protein kinase in chondrogenesis. Erk-1/2 activities, downstream of protein kinase C, decreased as chondrogenesis proceeded, whereas p38 activities, independent of protein kinase C, continuously increased during chondrogenesis. Inhibition of Erk-1/2 with PD98059 enhanced chondrogenesis up to 1.7-fold, whereas inhibition of p38 with SB203580 reduced it to about 30% of the control level. Inhibition of Erk-1/2 or p38 did not affect precartilage condensation. However, cartilage nodule formation was significantly blocked by the inhibition of p38, whereas Erk-1/2 inhibition did not affect it. Modulation of chondrogenesis by the inhibition of Erk-1/2 and p38 was accompanied by altered expression of adhesion molecules in an opposite way. Expression of N-cadherin was reduced as chondrogenesis proceeded. Inhibition of p38 caused sustained expression of N-cadherin, whereas Erk-1/2 inhibition accelerated the decrease of N-cadherin expression. Expression of integrin α5β1 and fibronectin were found to transiently increase during chondrogenesis. Inhibition of p38 caused continuous increase of expression of these molecules, whereas Erk-1/2 inhibition accelerated the decrease of expression of these molecules at a later period of chondrogenesis. Because temporal expression of these adhesion molecules regulates chondrogenesis, the above results indicate that Erk-1/2 and p38 conversely regulate chondrogenesis at post-precartilage condensation stages by modulating expression of adhesion molecules.

Formation of cartilage is initiated by differentiation of mesenchymes into chondrocytes. The differentiation process requires proliferation of chondrogenic competent cells that subsequently undergo precartilage condensation (1–3). Precartilage condensation is characterized by cells that are more densely packed in specific regions. This precartilage condensation is an important prerequisite for the initiation of chondrogenesis in mesenchymes in vivo. It becomes evident that several adhesion molecules including cadherins (4–7), integrins (8, 9), and extracellular matrix (ECM)

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§ The abbreviations used are: ECM, extracellular matrix; PKC, protein kinase C; MAP kinase, mitogen-activated protein kinase; PNA, peanut agglutinin; Erk, extracellular signal-regulated kinase.
Opposing Role of Erk and p38 in Chondrogenesis

Molecules including N-cadherin, fibronectin, and its receptor α5β1 integrin at post-precartilage condensation stage.

**Experimental Procedures**

**Micromass Culture of Mesenchymes—**Mesenchymes were derived from the distal tips of Hamburger-Hamilton stage 23/24 embryonic buda (27) of fertilized White Leghorn chicken eggs as described previously (18). The cells were suspended at a density of 2.0 × 10^5 cells/ml in Ham’s F-12 medium containing 10% fetal calf serum, and chondrogenesis was induced by adding the cells in 15-μl drops to culture dishes. The cells were incubated for 2 h at 37 °C to allow attachment and maintained in Ham’s F-12 medium containing 10% fetal calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin either in the absence or presence of various reagents as described in each experiment. Chondrogenesis was determined by examining the expression of type II collagen as described below and quantitated by staining sulfated cartilage matrix with Alcian blue. Briefly, micromass-cultured cells were stained with Alcian blue; Alcian blue bound to sulfated glycosaminoglycans was extracted with 4 M guanidine-HCl and quantitated by measuring absorbance at 600 nm.

**Immunocytochemical Detection of Type II Collagen Expression and Peanut Agglutinin (PNA) Binding—**Differentiating mesenchymes were fixed in 3% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature. The cells were washed and incubated for 1 h with anti-collagen type II monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) or 100 ng/ml of biotinylated PNA (Vector Laboratories Inc., Burlingame, CA). Type II collagen and PNA binding were visualized by developing with VECTASTAIN ABC and DAB substrate solution kits (Vector Laboratories Inc.) according to the procedure recommended by the manufacturer (18).

**Cell Proliferation Assay—**Proliferation of mesenchymes during micromass culture was determined by direct counting of viable cells as described previously (18). Briefly, mesenchymes were micromass-cultured (3 spots/3-mm dish) in the absence or presence of various reagents for the indicated time periods. Individual cells were suspended with 0.1% of trypsin and collagenase, and the number of viable cells was counted in triplicate using a hemocytometer.

**Cell Fractionation—**Cytosolic and particulate membrane fractions were prepared as described previously (18, 28). Briefly, cells micromass-cultured for indicated periods were scraped in buffer A containing 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM EGTA, and 2 mM EDTA supplemented with inhibitors of protease and phosphatase (18, 28). The cells were sonicated twice for 6 s and centrifuged at 100,000g for 1 h. The supernatant was designated as cytosolic fraction. The pellet was extracted with buffer B containing 20 mM Tris-HCl, pH 7.5, 1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and protease inhibitors. Following centrifugation at 15,000 × g for 15 min, the supernatant was saved as the particulate membrane fraction.

**Western Blot Analysis—**Total cell lysate was prepared from cells cultured for indicated periods were scraped in buffer A containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and inhibitors of proteases and phosphatases. The samples were precipitated with rabbit polyclonal anti-p38 antibody (Santa Cruz Biotechnology Inc.). Immunocomplexes were collected by binding to protein A-agarose beads (Pierce). After washing with lysis buffer, the beads were resuspended in 50 μl of kinase reaction buffer containing 25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM MgCl₂, 10 mM [γ-32P]ATP, and 2 μg of ATF-2 fusion protein as a substrate for p38 (New England Biolabs). Following incubation for 30 min at 30 °C, the reaction was stopped by the addition of 4× Laemmli’s sample buffer followed by boiling. Samples were resolved by electrophoresis, and then phosphorylation of ATF-2 protein was determined by autoradiography.

**RESULTS**

**MAP Kinase Activities during Chondrogenesis of Mesenchymes—**To determine subtype-specific functions of MAP kinases, we first examined their activities and changes during spontaneous chondrogenesis of mesenchymes initiated by micromass culture. Activation of Erk-1 and -2 was determined by Western blotting with antibody that recognized activated Erk-1/2. Because Erk-1 is a major Erk isofrom and the phosphorylation pattern of Erk-2 is very similar to that of Erk-1 in chick mesenchymes (18), we showed results of only Erk-1 in this study. Phosphorylation levels of Erk-1 were high at 1-day-old culture and decreased as chondrogenesis proceeded (Fig. 1A, upper panel). The addition of 5 mM PD98059, a specific inhibitor of MAP kinase kinase (30), to the culture significantly blocked Erk-1 activation. However, the addition of 10 μM SB203580, a specific inhibitor of p38 MAP kinase (31), to the culture did not affect the decrease in Erk-1/2 activities. SB203580 also did not affect PD98059-induced inhibition of Erk-1 phosphorylation.
Opposing Role of Erk and p38 in Chondrogenesis

Opposing roles of Erk-1/2 and p38 in the regulation of chondrogenesis of mesenchymes. A, expression of type II collagen was determined by immunocytochemistry in cells cultured for 4 days in the presence of vehicle alone as a control, 5 μM PD98059, or 10 μM SB203580. B and C, mesenchymes were micromass-cultured for the indicated time periods in the presence of vehicle alone as a control, 5 μM PD98059, or 10 μM SB203580 (B). Alternatively, cells were cultured for 4 days in the presence of various concentrations of PD98059 or SB203580 (C). Chondrogenesis was quantitated by staining sulfated proteoglycan with Alcian blue and measuring absorbance of bound Alcian blue extract at 600 nm. The data represent averages of six independent experiments with standard deviation.

Expression levels of Erk-1 remained constant in cells treated with PD98059 and/or SB203580 at 3-day culture (Fig. 1A, middle panel) or throughout the culture period (data not shown).

Activity of p38 was very low at 1-day culture times; however, it dramatically increased as chondrogenesis proceeded (Fig. 1B, upper panel). Treatment of cells with SB203580 significantly blocked p38 activity to about 10% of control level. Activity of p38 was not affected by inhibition of Erk-1/2 with PD98059. Also, the addition of PD98059 to the cells pretreated with SB203580 did not significantly affect SB203580-induced inhibition of p38 activity (Fig. 1B, upper panel). In contrast to the change in activities, expression of p38 did not change during chondrogenesis in cells treated with PD98059 and/or SB203580 (Fig. 1B, middle panel). Thus, Erk-1/2 and p38 activities are oppositely regulated during chondrogenesis, and it is not likely that Erk-1/2 and p38 directly cross-talk to regulate each other’s activities.

Opposing Roles of p38 and Erk-1/2 in Chondrogenesis—To determine whether the conversely regulated activities of Erk-1/2 and p38 have differential roles in chondrogenesis, chondrogenic differentiation was confirmed in control culture by examining cartilage-specific type II collagen expression (Fig. 2A). When Erk-1/2 was inhibited by PD98059, expression of type II collagen was significantly enhanced, to the extent of covering a whole area of individual spots (Fig. 2A). Alcian blue staining of sulfated proteoglycans revealed that chondrogenesis was enhanced up to 1.7-fold in a dose-dependent manner (Fig. 2, B and C). In contrast to the effects of Erk-1/2 inhibition, however, inhibition of p38 with SB203580 (10 μM) significantly blocked expression of type II collagen. Type II collagen expression was restricted to cartilage nodules whose numbers were significantly reduced compared with control culture (Fig. 2A). The degree of chondrogenesis in SB203580-treated cells was about 30% of the control, and the effect of SB203580 was dose-dependent as quantitated by Alcian blue staining (Fig. 2, B and C). The functional role of p38 in chondrogenesis was further supported by the observation that PD169316, another potent inhibitor of this kinase (32), inhibited chondrogenesis in a dose-dependent manner (data not shown). Taken together, the above results indicated that Erk-1/2 and p38 acted as negative and positive regulators of chondrogenesis, respectively, during in vitro micromass culture.

The inhibitory effect of SB203580 on chondrogenesis was abolished by the inhibition of Erk-1/2 with PD98059 at low concentrations of SB203580 (i.e. 2.5 and 5 μM). However, at high concentrations of SB203580 (i.e. 10 and 20 μM), Erk-1/2 inhibition did not completely promote recovery of chondrogenesis that was blocked by p38 inhibition (Fig. 3). The degree of chondrogenesis was 41 ± 4 and 29 ± 4% at 10 and 20 μM of SB203580, and inhibition of Erk-1/2 induced differentiation by 51 ± 4 and 35 ± 3%, respectively. Therefore, although inhibition of Erk-1/2 enhanced chondrogenic differentiation in the presence of p38 activity, Erk-1/2 inhibition appeared to be insufficient to induce chondrogenesis in the absence of p38 activity.

p38 and Erk-1/2 Do Not Regulate Proliferation of Chondrogenic Competent Cells—Because many extracellular molecules known to regulate chondrogenesis exert their effects at the stages of cell proliferation and/or precartilage condensation (5, 7, 33–36), we first examined whether the opposing roles of p38 and Erk-1/2 in the regulation of chondrogenesis were due to converse regulation of cell proliferation. As shown in Fig. 4, although chondrogenesis was significantly enhanced (Fig. 2), inhibition of Erk-1/2 did not affect proliferation of chondrogenic competent cells as determined by direct cell counting. Similarly, inhibition of p38 with SB203580 to block chondrogenesis up to about 30% of the control level (Fig. 2), also did not affect proliferation of the cells (Fig. 4). This indicated that negative and positive effects on chondrogenesis by the inhibition of p38 and Erk-1/2, respectively, were not brought about by modulat-
ing proliferation of chondrogenic competent cells.

*p38 Activity Is Required for the Progression of Precartilage Condensation to Cartilage Nodules*—We next examined the question of whether Erk-1/2 and p38 regulated chondrogenesis by modulating precartilage condensation or by affecting cellular events initiated by precartilage condensation. Precartilage condensation was verified by staining cells with PNA, a specific marker of precartilage condensation. PNA bound cell aggregates or precartilage condensation before the deposition of cartilage-specific ECM components, making it possible to distinguish between precartilage condensation and differentiated cartilage nodules (11, 13). On day 2 culture, numerous precartilage condensations or aggregates were present (Fig. 5), which were not differentiated to cartilage nodules as seen by the absence of Alcian blue staining (Fig. 5). The cell aggregates became large (Fig. 5) and differentiated to cartilage nodules on day 4 culture that were stained by Alcian blue (Fig. 5).

Inhibition of Erk-1/2 with PD98059 did not significantly affect precartilage condensation on 2-day-old culture. The size and number of cartilage nodules on 4-day-old culture were also not affected by the inhibition of Erk-1/2 (Fig. 5). However, Alcian blue staining was significantly enhanced when Erk-1/2 was inhibited with PD98059 (Fig. 5), suggesting that inhibition of Erk-1/2 enhanced expression of cartilage-specific molecules including type II collagen and sulfated glycosaminoglycans, but not by direct modulation of morphological changes such as precartilage cartilage condensation and cartilage nodule formation. Similar to the effects of Erk-1/2 inhibition, inhibition of p38 with SB203580 did not affect precartilage condensation on day 2 culture (Fig. 5). However, coalescence of cellular aggregates and differentiation to cartilage nodules on day 4 culture were significantly blocked in cells treated with SB203580 (Fig. 5). Therefore, p38 activity appeared to be required for the cellular events involved in progression of precartilage condensation to the cartilage nodule.

**Opposite Regulation of the Expression of Adhesion Molecules by Erk-1/2 and p38**—Precartilage condensation as well as its progression to cartilage nodules requires a regulated expression of cell adhesion molecules and ECM components (4, 8–11, 15). Therefore, the roles of the MAP kinase subtypes in the expression of cell adhesion molecules and ECM components were examined. Expression of N-cadherin was high in 1-day-old culture and was reduced as chondrogenesis proceeded (Fig. 6). However, the inhibition of p38 with SB203580 induced sustained expression of N-cadherin throughout the culture period, whereas the inhibition of Erk-1/2 accelerated the decrease of N-cadherin expression at the later periods of micromass culture (Fig. 6). This indicated that p38 and Erk-1/2 activities had opposing effects on N-cadherin expression during chondrogenesis.

Similar to converse regulation of N-cadherin expression, the expressions of fibronectin and its receptor integrin α5β1 were also conversely regulated by p38 and Erk-1/2. The expression of integrins α5 and β1 and fibronectin was increased transiently during chondrogenesis; their expression was detected in cells cultured for 1 day, increased as chondrogenesis proceeded, and decreased thereafter at the later stages of chondrogenic differentiation, i.e. the fifth day of culture (Fig. 6). Inhibition of Erk-1/2 or p38 did not significantly change expression patterns of these molecules at early periods of micromass culture, i.e. from 1 to 3-day-old cultures. However, the inhibition of Erk-1/2 accelerated the decrease of integrin α5 and β1 and fibronectin expression in the fourth and fifth day cultures, whereas the inhibition of p38 caused continuous increase of expression of integrins α5 and β1 throughout the culture period (Fig. 6). Because a reduction of N-cadherin, integrin α5β1, and fibronectin expression is necessary for chondrogenesis to progress after cell condensation (4, 8–10), the above results indicated that a balance of activities between p38 and Erk-1/2 appeared to regulate expression of these adhesion molecules at later periods of micromass culture.

**Erks-1/2 Are Downstream of PKC Signaling, Whereas p38 Is Independent of PKC**—Because our previous results indicated that PKC regulated chondrogenesis by acting upstream of Erk-1/2 (18), we next explored the possibility that p38 signaling was also linked to the PKC signaling pathway. Among the PKC isoforms such as α, ε, ζ, and λ/ι expressed in differentiating mesenchymes, expression of PKC α, and less dramatically so PKC ε, increased as chondrogenesis proceeded, whereas the expressions of PKC ζ and λ/ι did not vary significantly during chondrogenesis (Fig. 7A). Activation of PKC isoforms, as determined by examining translocation of cytosolic PKC to the particulate membrane fractions, was most evident in PKC α. The level of PKC ε also increased slightly, whereas levels of PKC ζ and λ/ι decreased slightly in the particulate membrane fraction during chondrogenesis. The amounts and expression patterns of the expressed PKC isoforms were essentially the same among cells cultured in the absence or presence of SB203580 or PD98059 (Fig. 7). Similarly, distribution of expressed PKC isoforms between cytosolic and particulate membrane fractions was not affected by the inhibition of Erk-1/2 or p38 (Fig. 7), indicating that the converse regulation of chondrogenesis by p38 and Erk-1/2 was exerted by modulating neither expression nor activation of PKC isoforms. Finally, we explored a possibility that p38 in addition to Erk-1/2 was a downstream target of PKC during chondrogenesis. Inhibition of PKC with Go6976 or down-regulation of PKC with prolonged treatment of cells with phorbol 12-myristate 13-acetate resulted in an increased phosphorylation of Erk-1 and inhibition of chondrogenesis (Fig. 8); however, neither inhibition of PKC with Go6976 nor down-regulation with phorbol 12-myristate 13-acetate affected p38 activity during micromass culture (Fig. 8). Therefore, the results indicated that, unlike Erk-1/2, p38 was not linked to the PKC signaling pathway.

**DISCUSSION**

**Converse Regulation of Erk-1/2 and p38 Activities and Their Opposing Roles in Chondrogenesis**—The MAP kinase subtypes Erk-1/2 and p38 are thought to play a key role in the signaling processes of many types of cellular differentiation. For example, it has been found that the activation of Erk-1/2 is required for the differentiation of fibroblasts to adipocytes (37), whereas inactivation of Erk-1/2 is required for C2C12 myoblasts to initiate myogenesis (38). Several recent studies also indicated
that p38 regulates cellular differentiation; for instance, the activation of p38 is required for erythroid differentiation (39), neuronal differentiation in PC12 cells (40), and adipogenesis of 3T3-L1 cells (41).

In this study, we show that activities of the MAP kinase subtypes, Erk-1/2 and p38, are conversely regulated during chondrogenic differentiation of chick limb bud mesenchymes induced by micromass culture (Fig. 1). We also demonstrate, by using specific inhibitors of the MAP kinase subtypes, that an increase of p38 activity is necessary for the progression of precartilage condensation to cartilage nodule (Fig. 5). A decrease in Erk-1/2 activities is also required for the progression of chondrogenesis, because inhibition of Erk-1/2 enhances chondrogenesis (Fig. 2) and activation of Erk-1/2 by the inhibition or down-regulation of PKC block chondrogenesis (18). On the other hand, phosphorylation of another major MAP kinase subtype, c-Jun N-terminal kinase, is not detected throughout chondrogenic differentiation (data not shown). The inhibitors used in this study, PD98059 and SB203580, are highly specific, and they have no inhibitory activity on all other kinases tested in vitro, including other MAP kinases at concentrations far greater than the concentration used in the present work (28, 29).

Our current results clearly indicated that both the decrease in Erk-1/2 and the increase in p38 activities during micromass culture are necessary for chondrogenic differentiation of mesenchymes. The stimulatory effect of Erk-1/2 inhibition on differentiation is most evident in the presence of p38 activity (i.e. in the absence or low concentration of SB203580), whereas inhibition of Erk-1/2 in the absence of p38 activity (i.e. at high concentration of SB203580) is not sufficient to induce chondrogenesis although it slightly increases differentiation (Fig. 3). Inhibition of p38 activity with SB203580, in the presence of Erk-1/2 activities, is sufficient to block chondrogenesis (Fig. 2). However, stimulation of Erk-1/2 by the inhibition or down-regulation of PKC, even in the presence of p38 activity, is sufficient to block chondrogenesis (Fig. 8). Therefore, oppositely regulated activities of both Erk-1/2 and p38 are essential for the induction of chondrogenic differentiation.

Currently, the signaling pathways leading to a decrease in Erk-1/2 activity and increase in p38 activity during chondrogenesis remain to be clearly characterized. However, Erk-1/2 activities appear to be regulated by PKC, because inhibition or down-regulation of PKC causes activation of Erk-1/2 (Fig. 8), and the decrease in Erk-1/2 activities is correlated with the increased expression and activation of PKC, especially PKC α and ε isoforms (Fig. 7). However, observations that inhibition or down-regulation of PKC does not affect p38 activity (Fig. 8)
cells treated with vehicle alone as a control, 20 nM phorbol 12-myristate 13-acetate, or 1 μM 13-acetate, or 1萌 enzymes were micromass-cultured for the indicated time periods in 5618

strongly correlated to the time course of cartilage nodule for-

ciation, and finally (iv) progression of precartilage condensation to cartilage nodules. Our observations that inhibition of p38 and Erk-1/2 activities regulated expression of adhesion molecules in an opposite way at later periods of culture, i.e. in day 4 and 5 cultures in which formation of cartilage nodules and expression of cartilage-specific molecules occurred (Fig. 5). These results strongly suggest that reduced expression of adhesion molecules is associated with formation and differentiation of cartilage nodules. Indeed, it has been shown that N-cadherin is expressed in prechondrogenic mesenchymes and during cell condensation but not in differentiated chondrocytes (4–7). In addition to N-cadherin-mediated cell-cell interaction, an interaction of mesenchymes with fibronectin via integrin α5β1 is necessary for cell condensation to occur, and reduced expression of fibronectin and its integrin α5β1 receptor following cell aggregation is associated with the progression of cartilage nodule formation (8, 9). Thus, the sustained expression of N-cadherin and the increased expression of integrin α5β1 and fibronectin at later stages of micromass culture upon inhibition of p38 is closely correlated with inhibition of chondrogenesis, whereas the accelerated decrease of expression of these molecules upon Erk-1/2 inhibition is correlated with enhancement of chondrogenesis. However, it remains to be more clearly elucidated whether the modulation of the expression of adhesion molecules by Erk-1/2 and p38 regulates cartilage nodule formation and expression of cartilage-specific molecules, and also whether modulation of chondrogenesis by the inhibition of Erk-1/2 and p38 causes altered expression of adhesion molecules.

Although our results clearly indicate that p38 and Erk-1/2 oppositely regulate expression of adhesion molecules, the molecular mechanism of their action is not clear. One of the possibilities is the regulation of Wnt signaling by MAP kinases. Stott et al. (16) has recently reported that Wnt, a putative ligand for frizzled receptor (42), is involved in chondrogenesis. They found that transfection of Wnt-7a to cells suppressed chondrogenic differentiation of mesenchymes. Wnt-7a did not inhibit precartilage condensation but blocked the progression of condensation to cartilage nodules with altered expression of adhesion molecules, sustaining expression of N-cadherin and wider distribution of integrins and fibronectin. Because the effects of p38 inhibition and Wnt-7a expression are very similar, it is of interest to determine whether Wnt signaling is conversely regulated by Erk-1/2 and p38.

While preparing our manuscript, Nakamura et al. (43) re-

and that expression and activation of PKC isoforms are not affected by the inhibition of p38 indicate that PKC is not involved in the regulation of p38 activity during chondrogenesis.

Regulatory Mechanism of Chondrogenesis by Erk-1/2 and p38—Many extracellular molecules known to regulate chondrogenesis exert their effects at the stages of cell proliferation and/or precartilage condensation (5, 7, 16, 18, 33–36). In this study, we investigated the role of p38 and Erk-1/2 in the various regulatory steps of chondrogenesis: (i) proliferation of chondrogenic competent cells, (ii) precartilage condensation that was determined by PNA staining, (iii) expression of cell adhesion molecules known to be involved in precartilage condensation, and finally (iv) progression of precartilage condensation to cartilage nodules. Our observations that inhibition of p38 and Erk-1/2 did not affect cell proliferation (Fig. 4) and precartilage condensation (Fig. 5) clearly indicated that the inhibitory and stimulatory effects on chondrogenesis by the inhibition of p38 and Erk-1/2, respectively, were not caused by modulation of cell proliferation and precartilage condensation.

In contrast to their roles in precartilage condensation, our present results demonstrated that p38 and Erk-1/2 differentially regulated the progression of precartilage condensation to cartilage nodules (Fig. 5). Inhibition of p38 activity blocked cartilage nodule formation demonstrating that the increase of p38 activity during micromass culture was required for the progression of precartilage condensation to cartilage nodules. Because cartilage nodules were formed on day 3 and 4 cultures as judged by the staining of PNA and Alcian blue (Fig. 5), the time course of p38 activation, which was very weak at one day and most active at 3 to 5-day-old cultures (Fig. 1B), was strongly correlated to the time course of cartilage nodule for-
ported that p38 contributed to chondrogenesis of ATDC5 cells, a clonal mouse chondrogenic cell line, induced by growth/differentiation factor-5. They showed that growth/differentiation factor-5 increased phosphorylation and activity of p38 dose dependently at early time periods, i.e. within an hour, and the inhibition of p38 activation with SB202190 suppressed expression of type II collagen and cartilage nodule formation that occurred at day 14. They also observed that growth/differentiation factor-5 induced phosphorylation of Erk-1/2 within 3 min after treatment, and inhibition of Erk-1/2 with PD98059 enhanced cellular differentiation. Although the culture systems and methods used to induce chondrogenic differentiation in Nakamura et al. (43) and ours are quite different, the functional role of MAP kinase subtypes is very similar, indicating a common regulatory mechanism of chondrogenesis by MAP kinases.

In summary, our present results emphasize the functional importance of two MAP kinase subtypes, Erk-1/2 and p38, in the regulation of chondrogenesis of limb bud mesenchymes during micromass culture. Activities of Erk-1/2 and p38 subtypes are oppositely varied during chondrogenesis, and the differential activities regulate chondrogenesis oppositely at post-precartilage condensation stages by modulating expression of adhesion molecules.

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