Integrin $\alpha_{6A}\beta_1$ and $\alpha_{6B}\beta_1$ Promote Different Stages of Chondrogenic Cell Differentiation*

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The differentiation of chondrocytes and of several other cell types is associated with a switch from the $\alpha_{6B}$ to the $\alpha_{6A}$ isoform of the laminin $\alpha\beta$ integrin receptor. To define whether this event plays a functional role in cell differentiation, we used an in vitro model system that allows chick chondrogenic cells to remain undifferentiated when cultured in monolayer and to differentiate into chondrocytes when grown in suspension culture. We report that: (i) upon over-expression of the human $\alpha_{6A}$ adherent chondrogenic cells differentiate to stage I chondrocytes (i.e., increased type II collagen, reduced type I collagen, fibronectin, $\alpha\beta$, and growth rate, loss of fibroblast morphology); (ii) the expression of type II collagen requires the activation of p38 MAP kinase; (iii) the over-expression of $\alpha_{6A}$ induces an incomplete differentiation to stage I chondrocytes, whereas no differentiation was observed in $\alpha_{6B}$ and mock-transfected control cells; (iv) a prevalence of the $\alpha_{6A}$ subunit is necessary to stabilize the differentiated phenotype when cells are transferred to suspension culture. Altogether, these results indicate a functional role for the $\alpha_{6B}$ to $\alpha_{6A}$ switch in chondrocyte differentiation; the former promotes chondrocyte differentiation, and the latter is necessary in stabilizing the differentiated phenotype.

Growth factors, cell-extracellular matrix (ECM), and cell-cell interactions are the primary determinants of lineage decisions and differentiation events in embryogenesis. These regulatory events involve different types of receptors and may lead to activation of several signaling pathways, such as those mediated by MAP kinases (1–3). Changes in the expression pattern of most of these receptors, including the integrins (heterodimeric $\alpha\beta$ receptors involved in cell-ECM and cell-cell interactions), are able to modulate several events associated with cell differentiation (3–20).

The onset of chondrogenesis in developing long bones is characterized by the reduction of intercellular spaces and establishment of extensive cell-cell contacts between mesenchymal chondrogenic cells, i.e., cell condensation (21). Several factors have been shown to play a role in this process, including cell-cell interactions (22–27), composition of the ECM (25, 28–30), changes in cell shape (31), and response to cytokines (32). Following cell condensation, chondrogenic cells that produce type I collagen, fibronectin (FN), and its integrin receptor $\alpha\beta$, differentiate to stage I chondrocytes that express type II collagen and eventually to stage II hypertrophic chondrocytes, characterized by type II and X collagen production (12).

Most of these events can be reproduced in vitro in a tissue culture model system that allows condensation and differentiation of chick embryo tibiae chondrogenic cells (12, 33–35). These cells, which adhere to tissue culture dishes and display a fibroblast-like phenotype (pre-chondrogenic cells), proliferate and secrete type I collagen and FN. When transferred to suspension culture, the cells rapidly aggregate and secrete type II collagen and other stage I chondrocyte-specific ECM molecules (12). In addition, differentiation is associated with (i) down-regulation of type I collagen, FN, and its receptor, $\alpha\beta$, integrin; (ii) switch from the $\alpha_{6B}$ to $\alpha_{6A}$ isoform of the integrin $\alpha\beta$ receptor for laminin (LN); (iii) reduced growth rate; and (iv) acquisition of a cobblestone morphology when re-plated in monolayer culture (12, 25). In ~3 weeks of culture, cell clusters are completely separated into single hypertrophic stage II chondrocytes that express type X collagen (12, 36).

A switch from $\alpha_{6B}$ to $\alpha_{6A}$ has been associated with the progression of cell differentiation of several cell types and tissues (13, 37–39). The two alternatively spliced isoforms of $\alpha_6$ differ in their cytoplasmic domain (40–43) and, upon binding to LN, induce different levels of phosphorylation of the signaling proteins paxillin and MAP kinases (44, 45). Furthermore, in cultured myoblasts, the ectopic expression of $\alpha_{6A}\beta_1$ (19, 20) modulates functions that are associated with differentiation and mitogenic responses, via MAP kinases.

The family of MAP kinases comprises several subtypes, including ERK-1 and -2 (extracellular signal-regulated kinases 1/2), JNK (c-Jun N-terminal kinase or stress-activated protein kinase), and p38. Each kinase subtype is activated by similar, but distinct, upstream kinases. Extracellular stimuli appear to activate either single or multiple MAP kinase pathways (46–48). In chondrocytes, inhibition of ERK1/2 and activation of p38 are associated with differentiation in vitro (49–51).

Here we have studied the role of the $\alpha_{6B}$ to $\alpha_{6A}$ switch, at the
onset of chondrocyte differentiation, by over-expressing α5A and α5B integrin subunits in chondrogenic cells derived from chick embryo tibiae. We have evaluated the effect on the expression of several differentiation markers (cell morphology, growth rate, collagen expression pattern) in cells grown under culture conditions that are either permissive or nonpermissive for chondrocyte differentiation, i.e. cells cultured in suspension or in monolayers.

Our results indicate a functional role for α5Bβ3 in the induction of chondrocyte differentiation and for α5Aβ3 in its progression. Furthermore, we suggest that these events depend on p38 MAP kinase activity.

**MATERIALS AND METHODS**

**Antibodies**—The following antibodies to integrin subunits were used: mouse monoclonal (mAb) BQ6 to human α6 (Dako, Glostrup, Denmark); mouse mAbs P1D6 to human α5 (Dako), mouse mAb V2E9 to avian β1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City), and rabbit polyclonal antibodies to avian α2, α3, and α5 raised against their C-terminal regions (a gift from Prof. G. Tarone, Torino, Italy). The following antibodies to matrix proteins were used: mouse mAb B3/D6 to avian fibronectin (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City), and rabbit polyclonal antibodies to chondroitin sulfate A type II collagen (52); rabbit polyclonal antibody AS126.46 to laminin-1 (a gift from Dr. R. Deutzmann, Regensburg, Germany). The following secondary antibodies were used: donkey anti-rabbit (Cy3TM), donkey anti-mouse (FITC), and donkey anti-mouse (FITC+Cy3) (Jackson Immunoresearch Laboratories, West Grove, PA; horsedarsh peroxidase conjugate (HRP) and swine anti-rabbit and rabbit anti-mouse immunoglobulins (Dako). For the MAP kinase assay the following antibodies were used: rabbit polyclonal to p38 MAP kinase (Cell Signaling Technology, New England Biolabs), rabbit polyclonal to pATF-2 (Thr-71) (Cell Signaling Technology, New England Biolabs), and mouse mAb AC-40 to actin (Sigma).

**Cell Culture**—Primary cultures of chondrocytes were isolated from chick embryo tibiae developed in virus-free eggs (Lohmann GMBH, AC-40 to actin (Sigma).

**Construction of α5A, α5B, and α5C cDNAs**—All constructs were assembled in the avian retroviral expression vector pSFCV-LE (54), a kind gift from B. Vennstrom (Department of Cell and Molecular Biology, Karolinska Institute, Stockholm). Human wild type α5 (3171 bp) and α5C (3207 bp) cDNAs cloned into the pRC-CMV vector (Invitrogen, Carlsbad, CA) were a kind gift from Dr. A. Sonnenberg (Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam). The full-length cDNAs were isolated by digestion with HindIII and subcloned into pSFCV-LE.

The human wild type α5 (3147 bp) cDNA, cloned in the pECE vector, was a kind gift from Dr. E. Rusloha (Cancer Research Center, The Burnham Institute, La Jolla, CA). The full-length cDNA was isolated by digestion with SalI and XbaI and, after Klenow treatment, inserted in-frame into the retroviral vector linearized with EcoRV.

**Functional Role of Integrin α5 Isoform Switch during Chondrogenesis**

**Transfection**—The empty pSFCV-LE vector or, alternatively, the vector containing the full-length human cDNA of either α5A, α5B, or α5C was transfected into avian chondrogenic cells using polybrene and MeSO. 24 h before transfection, 8 × 10^4–1 × 10^5 chondrogenic cells were seeded in culture dishes. After 24 h, cells were rinsed with PBS and resuspended in 3 ml of F-12 medium supplemented with 1% fetal calf serum, 1% chicken serum, and 30 μg of polybrene. Ten μg of circular plasmid DNA were admixed with the cells and incubated at 37 °C in 5% CO2 for 6 h with occasional agitation. Cells were then incubated at room temperature with fresh medium and 30% MeSO for 2 min, washed twice with PBS, and covered with complete medium (Coon’s modified F-12). Neomycin-resistant cells were isolated by use of a selection medium containing G418 (500 μg/ml, Invitrogen) for 1 week. When indicated, cells were transfected to suspension culture at day 10 of adherent culture to promote their differentiation. Using this method, we obtained a much higher transfection efficiency than with other protocols tested (not shown).

**Flow Cytometry**—For flow cytometric analyses, transfected cells were harvested with 2 ml EDTA in PBS, washed once in PBS, and counted. 1 × 10^6 cells were incubated with either a monoclonal antibody against human α6 (mAb-BQ6, diluted 1:20) or with a monoclonal antibody against human α5 (mAb-P1D6, diluted 1:20) for 20 min on ice. Cells were washed and incubated with donkey anti-rabbit FITC conjugated antibody (diluted 1:100) for 20 min on ice. Finally, samples were washed twice in PBS and analyzed in a FACScan using the CellQuest software (FACScalibur, BD Pharmingen). This analysis was performed on every new batch of transfected cells.

For the flow cytometric analysis of the endogenous levels of avian integrin subunits α2 or α3, transfected cells were permeabilized with PBS, 0.1% saponin for 10 min at room temperature. Cells were then incubated with antibodies to α2 (1:20) or α3 (1:5) diluted in PBS, 0.1% saponin and processed as described above. As a negative control, transfected cells were incubated with the secondary antibody alone. Each analysis was performed twice on two different batches of transfected cells.

**Immunofluorescence**—Immunofluorescence analyses were performed routinely on each new batch of transfected cells. At day 7 after transfection (end of the selection period), cells were seeded on 10-mm glass coverslips and grown adherent for 2 days. Coverslips were washed once with PBS and fixed for 10 min with 4% paraformaldehyde at room temperature, rinsed in PBS, incubated with 30 μm NH4Cl, and permeabilized with PBS, 0.1% saponin. Cells were subsequently incubated with primary antibodies to detect the presence of type I collagen (1:400), type II collagen (1:100), fibronectin (1:500), and TGF-β (1:1000), or when indicated, with antibodies to α2 (1:20) or α3 (1:5) diluted in PBS, 0.1% saponin and processed as described above. As a negative control, transfected cells were incubated with the secondary antibody alone. Each analysis was performed twice on two different batches of transfected cells.

**Evaluation of Apoptosis**—Cells were seeded on 10-mm glass coverslips, transfected, and grown in the presence or absence of 10 μM p38 inhibitor, SB203580, as described below. After 2 and 4 days, cells were fixed with 4% formaldehyde at 4 °C, washed several times with PBS, and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After several washes with PBS, apoptosis was determined using the TdT-mediated dUTP nick-end labeling (TUNEL) method according to the manufacturer’s instructions. Brieﬂy, cells were incubated at room temperature for 10 min with equilibration buffer followed by a 1-h incubation at 37 °C in a humidified chamber with TdT diluted in the reaction buffer. The TdT reaction was stopped with 2× SSC for 15 min at room temperature, and cells were washed with PBS and deionized water. Coverslips were mounted in Moviol, and apoptosis was assessed and quantified by nuclear staining observed with an epifluo-
rescence inverted microscope. Ten fields, obtained using the 40× lens, were counted for each sample.

**Type X Collagen and FN Metabolic Labeling**—Cells, cultured either in monolayer or in suspension, were starved for 2 h at 37°C in serum and methionine-free F-12 medium containing 100 μg/ml ascorbic acid. The cells were then cultured for 15 min in the presence of 0.4 μCi of [35S]methionine (Amersham Biosciences) for 2 h at 37°C. The culture medium was harvested and clarified by low speed centrifugation. To evaluate type X collagen secretion, aliquots containing 4 × 10^6 cpm of labeled medium were dialyzed against 0.5 N acetic acid for 24 h at 4°C, and proteins were digested with 100 μg of pepsin at 4°C for 12–15 h. The digested protein was lyophilized, dissolved in Laemmli reducing buffer, and separated by a 12.5% SDS-PAGE (57). The radiolabeled proteins were detected by autoradiography with Hyperfilm™ MP (Amersham Biosciences).

To analyze the levels of secreted fibronectin in dedifferentiated chondrocytes, aliquots of labeled medium containing 4 × 10^6 cpm were immunoprecipitated with the B3/D6 antibody in the presence of the FN inhibitor. Reactivity was evaluated using a chemiluminescence emission assay (Bio-Rad, Hercules, CA). Samples containing 50–100 μg of total protein were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and analyzed by immunoblotting. Membranes were blocked with 3% nonfat dry milk in TBS for 1 h at room temperature. HRP-conjugated swine anti-rabbit Ig (Dako) diluted at 1:2000 was used for detection. Staining. Membranes were blocked with 3% nonfat dry milk in TBS, 0.05% Tween, 3% nonfat dry milk for 1 h at room temperature. HRP-conjugated swine anti-rabbit Ig (Dako) diluted at 1:2000 was used for detection. Reactivity was evaluated using a chemiluminescence emission assay (ECL, Amersham Biosciences).

**p38 Kinase Assay**—The activity of p38 was determined using the Bradford dye colorimetric assay (Bio-Rad, Hercules, CA). Samples containing 50–100 μg of total protein were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and analyzed by immunoblotting. Membranes were blocked with 3% nonfat dry milk in TBS for 0.05% Tween for 1 h at room temperature and incubated with antibodies to p38 (1:1500) or type II (1:250) collagen diluted in TBS, 0.05% Tween, 3% nonfat dry milk for 1 h at room temperature. HRP-conjugated swine anti-rabbit Ig (Dako) diluted at 1:2000 was used for detection. Reactivity was evaluated using a chemiluminescence emission assay (ECL, Amersham Biosciences).

**Immunoblot**—Cells obtained after different times of culture under adherent or suspension conditions were pooled, washed with PBS, lysed for 1 h in ice-cold buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40) containing 2 mM PMSF and 2% aprotinin as proteinase inhibitors, and centrifuged. The protein concentration was determined using the Bradford dye colorimetric assay (Bio-Rad, Hercules, CA). Samples containing 50–100 μg of total protein were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and analyzed by immunoblotting. Membranes were blocked with 3% nonfat dry milk in TBS, 0.05% Tween for 1 h at room temperature and incubated with antibodies to p38 (1:1500) or type II (1:250) collagen diluted in TBS, 0.05% Tween, 3% nonfat dry milk for 1 h at room temperature. HRP-conjugated swine anti-rabbit Ig (Dako) diluted at 1:2000 was used for detection. Reactivity was evaluated using a chemiluminescence emission assay (ECL, Amersham Biosciences).

**RESULTS**

**Expression of α5, α6A, and α6B Integrin Subunits in Chondrogenic Cells**—We have previously reported that chondrocyte differentiation in vitro is associated with a down-regulation of the αβ1 fibronectin receptor and a switch from the α6A to the α6A isoform of the αβ1 laminin receptor (25). These results suggest that changes in the integrin expression pattern could be relevant for the induction and/or progression of chondrocyte differentiation. If this is the case, ectopic expression of either α6A or α6B should affect the differentiation pattern of cells cultured either in monolayer (permissive for differentiation) or in suspension (permisive for differentiation) (36).

Consequently, we transiently transfected (day 0) avian chondrogenic cells with full-length cDNAs coding for human α6A (pSFCV-LE/α6A) or α6B (pSFCV-LE/α6B) (40, 55) integrin subunits subcloned into the pSFCV-LE avian retroviral expression vector (54). As a control, we transfected α5 integrin subunit (pSFCV-LE/α5) (56) or the vector without an insert (pSFCV-LE). 24 h after transfection (day 1), cells were transferred into selective medium and cultured for 6 additional days (day 7). Successful expression of the transfected subunits was evaluated on day 7 by flow cytometry (Fig. 1).

The expression of the human α subunits could deplete the monomeric β1 pool available for endogenous integrins. To rule out this possibility, we surface-labeled transfected cells with biotin and analyzed the cell lysates by immunoprecipitation with an antibody to avian β1, followed by SDS-PAGE of precipitates, electrophoretic transfer to nitrocellulose, and detection of biotinylated integrins with streptavidin-horseradish peroxidase (Amersham Biosciences), diluted in TBS, 5% BSA (1:2000). Filters were developed using the ECL detection system.

**Densitometric Analysis**—Autoradiographic films from Western blot experiments were analyzed by calculating the total pixel value in the area of interest using Kodak Digital Science 1D image analysis software (Kodak, Rochester, New York).

**Conclusion**—Transfection of surface avian β1 expression, membranes were incubated with streptavidin-horseradish peroxidase (Amersham Biosciences), diluted in TBS, 5% BSA (1:2000). Filters were developed using the ECL detection system.
Transfection of $\alpha_6$ Reduces $\alpha_6\beta_1$ and Fibronectin, but Not Laminin, Expression—In chondrogenic cells, fibronectin and laminin-1 are the extracellular ligands for $\alpha_6\beta_1$ and $\alpha_6\beta_2$, respectively (25).

To assess whether transfection of ectopic $\alpha$ subunits affects the levels and distribution of FN and LN, immunofluorescence experiments were performed after 10 days of adherent culture, using a polyclonal antibody against the globular domains G3–G5 of laminin-1 or the B3/D6 monoclonal antibody against avian FN.

Staining for laminin was similar in all transfected chondrocytes, showing a weak punctate distribution around the nucleus, which is associated with a strong positive staining of focal contacts (Fig. 3A).

In contrast, we found that pSFCV-LE and pSFCV-LE/$\alpha_6$ cells displayed an extensive extracellular fibrillar network of FN underneath and between the cells (Fig. 3B), whereas pSFCV-LE/$\alpha_6A$ and pSFCV-LE/$\alpha_6B$ cells showed a faint staining with a few short fibrils (Fig. 3B).

To evaluate whether the reduced staining for FN was a consequence of a lower level of synthesis and/or secretion, we metabolically labeled transfected cells on day 10 and immunoprecipitated equal amounts of culture medium with an antibody to FN. The results (Fig. 3C) demonstrate a reduction in the levels of FN in all transfected cell populations in comparison with pSFCV-LE cells. However, although pSFCV-LE/$\alpha_6$ cells still secreted appreciable amounts of FN, almost no signal was detected in pSFCV-LE/$\alpha_6A$ and B. These results suggest that ectopic $\alpha_6$ inhibits FN synthesis and secretion.

Because reduced levels of FN during chondrogenesis are associated with a parallel down-regulation of the cognate $\alpha_6\beta_1$ receptor (25), the levels of endogenous $\alpha_6$ subunit were analyzed in the same cells. Western blot analysis using an antibody specific for the chick $\alpha_6$ subunit demonstrated a significant reduction in the expression of this subunit in cells expressing either of the two $\alpha_6$ isoforms but not in pSFCV-LE and pSFCV-LE/$\alpha_5$ cells (Fig. 3D), which instead gave a higher signal.

pSFCV-LE/$\alpha_6A$ and pSFCV-LE/$\alpha_6B$ Cells Display Different Levels of Differentiation under Nonpermissive Culture Conditions—During chondrogenic differentiation, the down-regulation of FN and $\alpha_6$ is associated with the loss of other markers of undifferentiated cells (i.e. fibroblast morphology, high proliferation rate, and type I collagen expression) and the expression of stage I chondrocyte markers (i.e. cobblestone morphology, low proliferation rate, and type II collagen expression) (12, 25).

To evaluate the functional role of $\alpha_6$ in these events, pSFCV-LE, pSFCV-LE/$\alpha_5$, pSFCV-LE/$\alpha_6A$, and pSFCV-LE/$\alpha_6B$ cells were cultured in monolayer. When observed by phase contrast microscopy on days 10 and 15, pSFCV-LE, pSFCV-LE/$\alpha_5$, and pSFCV-LE/$\alpha_6A$ cells displayed a normal fibroblast-like phenotype. In contrast, pSFCV-LE/$\alpha_6B$ cells exhibited a cobblestone-like morphology (Fig. 4A); this phenotype is typical of stage I differentiated chondrocytes, which are transferred to monolayer culture after differentiation in suspension (35). Furthermore, at day 15 the number of both pSFCV-LE/$\alpha_6A$ and pSFCV-LE/$\alpha_6B$ cells was significantly lower compared with pSFCV-LE and pSFCV-LE/$\alpha_5$ cells (Fig. 4A). This observation was confirmed by measuring the growth of the transfected cell populations. We plated an equal number of cells on day 7 and counted the cells on days 10, 15, and 17. At these three time points, 5-, 10-, and 20-fold fewer pSFCV-LE/$\alpha_6A$ and pSFCV-LE/$\alpha_6B$ cells were found in comparison with pSFCV-LE and pSFCV-LE/$\alpha_5$ cells (Fig. 4B).

Immunofluorescence experiments were conducted to analyze the pattern of type I and II collagen expression. pSFCV-LE,
pSFCV-LE/α5, pSFCV-LE/α6A, and pSFCV-LE/α6B cells from day 7 were plated onto coverslips for 2 additional days, fixed, and permeabilized (Fig. 4C). Control cells displayed an intracellular dotted and an extracellular fibrillar staining pattern for type I collagen and either faint (pSFCV-LE/H9251.6) or no staining (pSFCV-LE) for type II collagen (Fig. 4C). pSFCV-LE/H9251.6A and pSFCV-LE/H9251.6B cells instead exhibited strong intracellular staining for type II collagen. In addition, pSFCV-LE/H9251.6B cells but not pSFCV-LE/H9251.6A cells displayed intracellular staining for type I collagen (Fig. 4C).

To quantify these data, we performed Western blot and densitometric analysis of pSFCV-LE/α6A and pSFCV-LE/α6B cells, finding that they expressed 2.52 ± 0.33- and 6.09 ± 0.648-fold more type II collagen, respectively, compared with pSFCV-LE/α5 (Fig. 5, Adh., Col. II). No type II collagen was expressed by pSFCV-LE. On the contrary, type I collagen was produced by pSFCV-LE and pSFCV-LE/α5, and to a minor extent by pSFCV-LE/α6B, but it was undetectable in pSFCV-LE/α6A cells (Fig. 5, Adh., Col. I).

Altogether, these data suggest that over-expression of α6B induces full differentiation of chondrogenic cells to stage I chondrocytes under nonpermissive culture conditions. In con-

**FIG. 2.** Surface expression of endogenous avian β1, α2, and α6 integrin subunits in pSFCV-LE, pSFCV-LE/α5, pSFCV-LE/α6A, and pSFCV-LE/α6B adherent cells. A, cells cultured adherent for 10 days were surface-biotinylated, lysed, and immunoprecipitated with avian β1 antibody (V2E9). The precipitates were resolved by SDS-PAGE, blotted onto nitrocellulose membranes, and stained with streptavidin/HRP. The level of the surface expression of β1 was evaluated by densitometry. Top line, lane 1, pSFCV-LE; lane 2, pSFCV-LE/α5; lane 3, pSFCV-LE/α6A; lane 4, pSFCV-LE/α6B. Densitometric values are indicated by the numbers below each lane. This gel represents one of five independent experiments on different batches of transfected cells. B, on day 7 pSFCV-LE, pSFCV-LE/α5, pSFCV-LE/α6A, and pSFCV-LE/α6B cells were analyzed by flow cytometry using mAbs to avian α2 or α6 integrin subunits. In all panels, the gray lines show the expression of α2 and α6 subunits and the black lines the negative controls (see “Material and Methods”). The flow cytometry profiles show the results of one of two experiments performed on two different batches of transfected cells with similar results.
transfected cells synthesized type II collagen and down-regulated FN and $\alpha_5$. However, they were unable to modify other undifferentiated chondrogenic cell markers (i.e., type I collagen and cell morphology). Finally, control $\alpha_5$-transfected cells did not display any change when compared with pSFCV-LE, with the exception of a very low level of type II collagen expression.

Prevalence of $\alpha_6A$ and $\alpha_6B$ Is Necessary to Stabilize the Differentiated Phenotype in Chondrocytes Suspension Cultures—The expression of type X collagen was determined to evaluate whether ectopic expression of $\alpha_6A$ and $\alpha_6B$ is able to induce faster differentiation to stage II chondrocytes under nonpermissive culture conditions. Lysates of metabolically labeled cells were pepsin-digested and analyzed by SDS-PAGE. Under these culture conditions neither the $\alpha_6A$ nor the control transfected cells expressed type X collagen (Fig. 5, Adh., Col. X).

We next evaluated whether $\alpha_6A$ and $\alpha_6B$ subunits play any role in the progression from stage I to stage II chondrocytes in culture conditions permissive for differentiation (i.e., suspension cultures). Although in such cultures type X collagen is produced first after several days, pSFCV-LE/$\alpha_6A$ and pSFCV-LE/$\alpha_6B$ cells expressed type X collagen after only 24 h (Fig. 5, Susp., Col. X). This was due specifically to the over-expression of the $\alpha_6$ subunits, demonstrated by the absence of type X collagen after 24 h of suspension culture in pSFCV-LE and pSFCV-LE/$\alpha_6$ (Fig. 5, Susp., Col. X). Levels of type II collagen expression were also analyzed and, as anticipated, were found to be increased in pSFCV-LE/$\alpha_6A$, pSFCV-LE/$\alpha_6B$, and pSFCV-LE. In contrast, no type II collagen was expressed by pSFCV-LE/$\alpha_6B$ (Fig. 5, Susp., Col. II), and type I collagen was almost undetectable in all samples (Fig. 5, Susp., Col. I). These results suggest that a prevalence of $\alpha_6A$ versus $\alpha_6B$ is necessary to
**Fig. 4. Evaluation of chondrocyte differentiation markers in transfected cells cultured in monolayer.**

- **A.** Phase contrast microscopy of pSFCV-LE, pSFCV-LE/α₅, pSFCV-LE/α₅A, and pSFCV-LE/α₅B cells. For each new batch of transfected cells, an equal number of cells (5 x 10⁴) was seeded, grown in monolayer for 10 or 15 days, and photographed.

- **B.** Growth curve of the different transfected cells. The growth curve was determined at each new transfection. Equal numbers (5 x 10⁴) of cells were seeded in triplicate on day 7 after transfection (day 0 of the growth curve).

- **C.** Immunofluorescence staining for collagen types I and II in pSFCV-LE, pSFCV-LE/α₅, pSFCV-LE/α₅A, and pSFCV-LE/α₅B cells. The staining was performed on day 10 after transfection.

For more detailed information, refer to the full report or manuscript.
maintain the stage I-differentiated phenotype.

**p38 Kinase Activation Is Necessary for α6-induced Chondrocyte Differentiation**—p38 MAP kinase activation is associated with differentiation of chondrogenic cells (50, 51, 58, 59) and myoblasts (19, 20) in culture. The activity of p38 was determined in early phases of chondrocyte differentiation induced by α6-integrin transfection, with the aim of identifying the earliest events associated with differentiation to stage I chondrocytes. At early time points after chondrocyte transfection (days 2 and 4, before selection was completed), p38 activity was evaluated by measuring the levels of phosphorylated ATF-2, a major phospho-p38 kinase substrate. Equal amounts of total lysate proteins were immunoprecipitated with an antibody to p-p38. Immunoprecipitates were then incubated with ATP-2 fusion protein in the presence of ATP, thus allowing immunoprecipitated active p38 MAP kinase to phosphorylate ATF-2. Phosphorylation of ATF-2 at Thr-71 was detected by Western blotting using a phospho-ATF-2 (Thr-71) antibody. In addition, the total amount of p38 was also measured in each cell population by Western blot of total lysates. A densitometric analysis was performed, and the data for each sample were normalized to the actin and p38 contents. Values were further corrected for the number of cells expressing the ectopic α subunits as determined by immunofluorescence. The results indicate that p38 activity on day 2 is 2.56 ± 0.251 and 4.07 ± 0.266 times higher in pSFCV-LE/α6A and pSFCV-LE/α6B cells, respectively, in comparison with control cells (pSFCV-LE) (Table I). In the opposite direction, on day 2 the pSFCV-LE/α6 cells displayed 50% of the p38 activity detected in control cells (pSFCV-LE) (Table I, Fig. 6A). These results demonstrate that high levels of p38 activation are dependent on the expression of ectopic α6.

To address a possible role for p38 in the differentiation events associated with ectopic α6 expression, we first inhibited p38 activity using the specific inhibitor SB203580 (69). As expected, addition of 10 μM SB203580 to the culture medium of transfected cells had no effect on the expression of total p38, but it drastically reduced the level of p-p38 (Fig. 6B). In addition, the number of apoptotic cells was determined by analyzing the nuclear TUNEL staining using epifluorescence microscopy. No significant differences were found between the treated and nontreated cells (data not shown). Next, the amount of type II collagen synthesized on days 2 and 4 (Fig. 6C) in the presence or absence of SB203580 was determined. Western blot analyses of cell lysates from all of the transfected cell populations (pSFCV-LE, pSFCV-LE/α6, pSFCV-LE/α6A, and pSFCV-LE/α6B) showed that on day 4, in the absence of the p38 inhibitor, only pSFCV-LE/α6B synthesized type II collagen. The addition of SB203580 to the culture medium abolished such expression. It is notable that type II collagen is detected on day 10 in α6A-transfected cells only (day 10, Fig. 4). These results suggest that a functional correlation exists between p38 activation and expression of a stage I chondrocyte differentiation marker induced by α6A over-expression.

![Figure 5](image_url)

**Fig. 5.** Pattern of collagen expression in transfected cells growing either adherent or in suspension. pSFCV-LE, pSFCV-LE/α6, pSFCV-LE/α6A, and pSFCV-LE/α6B cells from day 10 of adherent culture were transferred to suspension culture for 24 h and then either lysed, separated by SDS-PAGE and blotted onto nitrocellulose (Col. I and Col. II), or labeled metabolically with [35S]methionine, peptide-digested, and separated by SDS-PAGE (Col. X) (see “Materials and Methods”). Western blots were developed with antibodies to either avian type I (Col. I) or type II (Col. II) collagens. Pepsin-resistant type X collagen bands, detected by autoradiography, were identified by their size (molecular mass, 59 kDa). Data are from one representative experiment of four from different batches of transfected cells. Adh., cells cultured adherently, day 10; Susp., cells cultured adherently for 10 days and then transferred in suspension culture for 24 h.

| Transfected subunits | Levels of p-ATF-2 per cell | Day 2 | Day 4 |
|----------------------|---------------------------|-------|-------|
| pSFCV-LE             | 251.0                      | 204.6 |
| pSFCV-LE/α6          | 127.0                      | 465.3 |
| pSFCV-LE/α6A         | 596.1                      | 588.8 |
| pSFCV-LE/α6B         | 1021.1                     | 444.1 |
DISCUSSION

A switch in expression from the \( \beta_6 \) to the \( \alpha_6 \beta_1 \) laminin receptor is associated with differentiation in several cell types (37–39). The two alternatively spliced isoforms of \( \alpha_6 \) differ in their cytoplasmic domains, which consist of 36 and 54 amino acids in \( \alpha_6A \) and \( \alpha_6B \) (40–43), respectively, and display similar binding affinities and ligand specificities. Moreover, upon phorbol ester treatment, only the \( \alpha_6A \) isoform is phosphorylated (41, 60). Differential interactions with cytoskeletal proteins have been suggested to explain the greater ability of \( \alpha_6A \) to induce pseudopodia formation and to promote migration in a macrophage cell line plated on a laminin-1 (19, 44, 61). However, little is known about the roles played by \( \alpha_6A \) and \( \alpha_6B \) in the modulation of cell differentiation. We have addressed this question using an in vitro model system that allows differentiation of chondrogenic cells transferred from nonpermissive (adherent) to permissive (suspension) culture conditions. The in vitro chondrocyte differentiation from prechondrogenic cells to stage I chondrocytes (33, 35) is associated with a switch from the \( \alpha_6B \) to the \( \alpha_6A \) (25). Our approach has been to over-express the two alternative isoforms, evaluating the effect on chondrocyte differentiation under both nonpermissive and permissive culture conditions.

\( \alpha_6B \) Expression Promotes Full Differentiation to Stage I Chondrocytes—Under nonpermissive culture conditions, ectopic expression of the \( \alpha_6B \) is sufficient to specifically promote chondrocyte differentiation to stage I, as indicated by activation of the expression of type II collagen, reduction of the growth rate, reduction of type I collagen, fibronectin and \( \alpha_5 \) expression, and changes in cell morphology from a fibroblast-like to a cobblestone shape.

The specificity of the \( \alpha_6B \) effects listed above is supported by the observation that transfection with human \( \alpha_6A \) or \( \alpha_5 \) subunits only prompts a low level and delayed expression of type II collagen. Furthermore, there is no loss of markers of the undifferentiated phenotype.

The full differentiation to stage I chondrocyte following \( \alpha_6B \) transfection is not due to a nonspecific imbalance in the expression of endogenous chick integrins. This is demonstrated by the unchanged levels of chick \( \alpha_2 \) and \( \alpha_3 \) after transfection of either human \( \alpha_6 \) or \( \alpha_5 \).

On the contrary, the specific \( \alpha_6 \)-dependent reduction of chick

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**Fig. 6.** Analysis of p38 MAP kinase activity and its role in type II collagen expression. Cells were cultured in the absence (A) or presence (B) of SB203580, a p38 inhibitor. For p38, equal amounts of cell extracts from pSFCV-LE (lane 1), pSFCV-LE/\( \alpha_6 \) (lane 2), pSFCV-LE/\( \alpha_6A \) (lane 3), and pSFCV-LE/\( \alpha_6B \) (lane 4) cells were analyzed on days 2 and 4. p38 activity was determined using an in vitro kinase assay and ATF-2 as a substrate. The levels of ATF-2 were determined by Western blot using an antibody to pATF-2. Equal amounts of total cell extracts from day 2 and 4 were analyzed by Western blot using an antibody to p38. For actin, equal amounts of total cell extracts from days 2 and 4 were analyzed by Western blot using an antibody to actin. For p38, cells, the total amount of p38/cell was determined. The results were evaluated by dividing the p38/actin ratio normalized to the number of cells expressing ectopic \( \alpha \) subunits as determined by immunofluorescence. This value ranged between 52 and 69% of the whole cell population. C, total extracts from pSFCV-LE (lane 1), pSFCV-LE/\( \alpha_6 \) (lane 2), pSFCV-LE/\( \alpha_6A \) (lane 3), and pSFCV-LE/\( \alpha_6B \) (lane 4) cells cultured in the absence (top) or presence (bottom) of SB203580 (10 \( \mu \)M). Cells were collected on days 2 and 4, and equal amounts of cell extracts were analyzed by Western blot with an antibody to type II collagen. Data are from one representative experiment of three, using different batches of transfected cells.
α5 may contribute to the differentiation process by removing the inhibitory activity exerted by α5β1 (62). The reduction in endogenous α5 is a direct and specific effect of α5 transfection and is not observed when cells are transfected with α5. In addition, it occurs despite the increased levels of endogenous β1, which provide sufficient protein to assemble chick α5β1 heterodimers.

α5A Prevalence Is Necessary to Fully Maintain the Stage I Differentiation State—Upon differentiation to stage I chondrocytes, the switch from expression of α5B to α5A leads to a prevalence of the latter on the cell surface. Experiments to impair this were performed by over-expressing α5B.

α5B-transfected chondrocytes, when differentiated in adherent cultures, are unable to maintain their phenotype once transferred in suspension, as shown by loss of expression of type II collagen. In contrast, α5A, α5A, and mock-transfected cells differentiate normally to stage I. These results suggest that the prevalence in α5A surface expression during chondrogenesis may contribute to sustaining the differentiated phenotype. In addition, upon transfection of α5B differentiation to stage II chondrocyte progresses normally in vitro. These observations suggest that other events may contribute to support the chondrocyte phenotype in vivo. Therefore, it is likely that transition to stage II chondrocytes implies several independent events, one of which may be the role of α5A in sustaining type II collagen expression.

Stage I to Stage II Transition Requires Suspension Culture and α5 to Occur—To assess whether α5B and/or α5A are involved in the progression from stage I to stage II chondrocyte differentiation, the expression of type X collagen was evaluated in transfected cells cultured either in monolayer or in suspension.

We found that type X collagen is exclusively expressed in suspension cultures but that it appears earlier (24 h) in both types of α5-transfected cells as compared with control cells where several days are required. These results further suggest that neither of the α5 subunits is sufficient to promote differentiation to stage II chondrocytes and that other conditions associated with culture in suspension are necessary. In addition, this further step of differentiation is likely to be independent from the expression of type II collagen, as shown by the behavior of α5-transfected cells.

Early Activation of the p38 MAP Kinase Pathway Is Functionally Associated with α5B-induced Type II Collagen Expression—p38 MAP kinase has been shown to modulate chondrogenic differentiation. Growth/differentiation factor-5 (GDF-5) induces p38 activation and chondrocyte differentiation in the mouse chondrogenic cell line ATDC5 (49). Chun and co-workers (50, 51, 58, 59), using a micromass culture system, have shown that chondrocyte differentiation associates with a protein Kinase C (PKC)-independent activation of p38 in chondrogenesis. In addition, treatment of micromass cultures with epidermal growth factor, a limb bud cartilage development modulator, decreases the rate of chondrogenesis by reducing p38 activity (51).

Furthermore, adhesion to substrates and actin cytoskeletal organization have been shown to be important in integrin growth factor receptor signaling via MAP kinases (2, 63–66). These pathways may be relevant to the induction of chondrocyte differentiation in anchorage-independent cultures (12) and following cytchalasin D treatment (31).

Here we show that inhibition of p38 activation by SB203580 inhibits induction of type II collagen expression by α5B. Therefore it appears that this signaling pathway is involved in chondrocyte differentiation. In addition, by determining the level and time of p38 activity we found a peak at day 2 in α5B transfected cells, which was about 2-fold higher than the levels measured on days 2 and 4 in α5A- and α5A-transfected cells. These results correlate with the early expression of type II collagen in α5B but not in α5A- and α5-transfected cells. Although these data do not allow us to conclude that p38 activation is directly dependent on α5B, they suggest that p38 activation is functionally related with type II collagen expression.

The presence of LN in chondrogenic area of the condensing mesenchyme suggests that the interaction of α5B receptor may be relevant in vivo, as well as in vitro. In addition, in vitro model systems more closely reproducing the limb bud chondrogenic area, i.e., micromass cultures, the inhibition of p38 activity has been proven to block type II collagen expression (50), further supporting this hypothesis.

The interpretation of the relevance of α5A role in vitro is more complex. The presence of LN in the ECM surrounding stage II chondrocytes in cartilage tissue (67), suggests that its interaction with α5B may play a role in chondrocyte differentiation in vivo, as we have highlighted in vitro. However, mice deficient in α5A do not display defective endochondral bone formation (68), suggesting that the potential role of α5B-LN interactions is not sufficient to promote the progression of chondrocyte differentiation and that other events may be relevant to this process. In particular, further studies are needed to resolve the role of other cell-ECM interactions and soluble factor effects.

In conclusion, our results demonstrate that ectopic expression of the α5B laminin receptor is sufficient to promote differentiation of chondrogenic cells even under nonpermissive culture conditions. Furthermore the expression of α5A contributes to stabilizing the differentiated phenotype and to sustaining the progression to further steps of chondrocyte differentiation in vitro.

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