Chondrocyte Aggregation in Suspension Culture Is GFOGER-GPP- and β1 Integrin-dependent

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Isolated chondrocytes form aggregates in suspension culture that maintain chondrocyte phenotype in a physiological pericellular environment. The molecular mechanisms involved in chondrocyte aggregation have not been previously identified. Using this novel suspension culture system, we performed mRNA and protein expression analysis along with immunohistochemistry for potential cell adhesion molecules and extracellular matrix integrin ligands. Inhibition of aggregation assays were performed using specific blocking agents. We found that: (i) direct cell-cell interactions were not involved in chondrocyte aggregation, (ii) chondrocytes in aggregates were surrounded by a matrix rich in collagen II and cartilage oligomeric protein (COMP), (iii) aggregation depends on a β1-integrin, which binds a triple helical GFOGER sequence found in collagens, (iv) integrin α10-subunit is the most highly expressed α-subunit among those tested, including α5, in aggregating chondrocytes. Taken together, this body of evidence suggests that the main molecular interaction involved in aggregation of phenotypically stable chondrocytes is the α10β1-collagen II interaction.

Cell-matrix and cell-cell interactions in cartilage are of crucial importance in mediating cartilage development (1), homeostasis (2), degradation (3), and cell survival (4). These interactions have been extensively studied in the context of limb bud mesenchymal cell condensation, an early stage of cartilage development that is believed to be mediated by cell-fibronectin interactions. In this case, antibodies against fibronectin interrupted cell condensation in limb bud mesenchymal micromass culture (5), and fibronectin depletion or inhibition of cell binding to fibronectin by RGD peptides, interfered with aggregation of prechondrogenic cells (6). After this initial condensation stage, cell-cell contacts are strengthened by direct cell-cell adhesion via the homotypic cell adhesion molecules, NCAM2 and N-cadherin (6, 7). Perturbation of NCAM and N-cadherin activity by blocking antibodies or transfection with mutated genes resulted in reduced or altered cell aggregation and chondrogenesis in vitro and in vivo. However, NCAMs and N-cadherins then disappear from the forming cartilage and are not expressed by differentiated chondrocytes in situ (8, 9). In healthy adult cartilage, there are no cell-cell contacts, but rather functional cell-matrix contacts, primarily integrin-mediated, between differentiated chondrocytes and components of the extracellular matrix (ECM).

Immunostaining of tissues as well as flow cytometry and Western blot analyses on freshly isolated chondrocytes have detected several integrins. In adult and fetal articular cartilage, integrin subunits α5, αV, and β1 are easily detected, whereas the expression of other subunits depends on the specific type of cells or tissue, or the technique used. For example, the expression of α1 and α2 varies between published studies, as does α3 (10–12). The α6-subunit was detected in fetal or new born cartilage (11, 13) but not in adult cartilage (10); a priori, chondrocytes can therefore potentially use integrins α1β1, α2β1, α3β1, α5β1, α6β1, and αVβ3 to bind ECM ligands.

In terms of ligand specificity, α3β1 integrin has been characterized as a receptor for laminins and failed to show a role in chondrocyte adhesion (11, 14). In contrast, α5β1 is the main chondrocyte receptor for fibronectin, as blocking antibodies almost completely abolished cell adhesion to this molecule (11, 15). α5β1 can also bind denatured collagens types VI and II (16, 17). α6β1 is one of the chondrocyte receptors for laminin (13) while αVβ3 is known to bind osteopontin, bone sialoprotein, and vitronectin (18, 19) found in bone or calcified cartilage (20, 21). Integrins α1β1 and α2β1 are collagen receptors (22), as is α10β1, which is thought to be a major collagen-binding integrin during cartilage development and in mature hyaline cartilage (12, 23). Integrin α11β1 also binds collagen but is expressed in the region just outside cartilage in cells around ribs, vertebrae and intervertebral disc, and in human embryonic tissues, showing a non-overlapping staining pattern with collagen type II (24). Finally, non-integrin collagen receptors are also expressed by chondrocytes in situ and in vitro, including DDR-2 and annexin V (25), which are cell surface molecules that can bind fibrillar collagens.

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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2 The abbreviations used are: NCAM, neural CAM; ECM, extracellular matrix; CAM, cell adhesion molecule; CHAD, chondroadherin; COMP, cartilage oligomeric matrix protein; DDR-2, discoidin domain receptor-2; EF1α, elongation factor 1α; FIC, freshly isolated chondrocytes; PBS, phosphate-buffered saline; QRT-PCR, quantitative real-time PCR; SEM, scanning electron microscopy; SFM, serum-free medium; TSP, thrombospondin; MAT, matrilins.
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The central role of cell-matrix interactions in controlling multiple processes in chondrocytes necessitates further investigation into the specific molecular interactions at play. Unfortunately monolayer culture dedifferentiates chondrocytes (26), while encapsulation in gels such as agarose impedes cell contacts and is cumbersome, rendering molecular analyses more difficult. An alternative to examine cell-cell and cell-matrix interactions for chondrocytes is suspension culture, where phenotypically stable chondrocytes are first dispersed in the culture medium and then proceed to form aggregates (27). This aggregation process likely occurs in a manner that reproduces in situ interactions, since phenotype is maintained in aggregates or pellet cultures that bear high similarity to the native cartilage environment (27–29). Given this similarity to the in situ chondrocyte environment, and the relatively fast dynamic process of chondrocyte aggregation seen in suspension cultures, we report here a series of suspension culture experiments that elucidate the specific molecular mechanisms involved in forming chondrocyte aggregates, taking into account the potential for both cell-cell and cell-matrix interactions.

Previous work in suspension culture has provided some insight into the aggregation process. For example anti-fibronectin serum or hyaluronidase failed to prevent aggregation (4, 30); hyaluronidase could even promote aggregation (4, 31). On the other hand, the presence of a blocking antibody against the β1 integrin subunit reduced cell condensation (4, 30), suggesting integrin involvement in aggregation of avian embryonic chondrocytes. These studies also showed that addition of collagen type I to chondrocytes in suspension increased aggregation, whereas collagenase inhibited it (4, 30). Although these previous studies suggest a collagen-integrin interaction is at play in the formation of aggregates of avian embryonic chondrocytes, the specific integrins and collagens involved, as well as the relevance of these findings for phenotypically stable mammalian chondrocytes, are not known. In this study we have therefore hypothesized that the aggregation of phenotypically stable mammalian chondrocytes in suspension culture is mediated by cell-matrix interactions rather than cell-cell interactions and involves a β1 integrin with known binding properties to collagen type II, namely integrins α1, α2, α10, or α11 β1. To test this hypothesis, expression analysis of candidate cell adhesion molecules and potential ECM ligands in suspension culture was performed. Inhibition of aggregation assays using specific blocking antibodies and peptides were performed to identify classes of molecules involved in chondrocyte aggregation.

**EXPERIMENTAL PROCEDURES**

**Culture Media—**SFM is a 1:1 (v/v) mix of calcium-free HAM’s F12 and calcium-free DMEM low glucose (both from US Biological) supplemented with 1 mM CaCl\(_2\), 0.4 mM proline, 1.5 mM glutamine, 22 mM sodium bicarbonate, non-essential amino acids 1X, penicillin-streptomycin 1X. Supplemented SFM is SFM with ITS+1 (Sigma-Aldrich), EGF, PDGF-BB, FGF-2 all 2 ng/ml (R&D Systems), 10\(^{-8}\) M dexamethasone, 5 × 10\(^{-5}\) M β-mercaptoethanol, and 30 μg/ml ascorbate, added fresh.

**Chondrocyte Isolation and Culture—**Chondrocytes were isolated from the femoropatellar groove of a 1–2-month-old calf. Briefly, cartilage was sequentially digested, first by protease Type XIV (Sigma-Aldrich Canada) at 56 units/ml and then by collagenase CLS2 (Worthington) at 752 units/ml and released cells were filtered, and washed with SFM. After isolation, chondrocytes were either cultured as static cell suspensions for 18 h or used to perform aggregation assays (see “Aggregation Assays”). For static suspension culture, cells were seeded at 0.4 × 10\(^6\) cells/ml in supplemented SFM in Petri dishes previously coated with 2% (w/v) agarose to prevent cell adhesion. Microscopic observation of cell aggregates was performed with an inverted microscope (Axiovert S100TV, Carl Zeiss) equipped with a 5× objective (NA 0.12), a QICAM Fast 1394 camera (QImaging) and Northern eclipse image acquisition software (Empix imaging). At different times after seeding (t = 0, 4 or 18 h) cell samples were taken and lysed in buffers for RNA or protein analyses (see below). At t = 18 h, cell aggregates were also fixed for immunostaining (see below).

**Immunoblotting—**Cell aggregates were extracted in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% w/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 10 mM Tris pH 7.2) with protease inhibitors (P8340, Sigma-Aldrich) and electrophoresed on 7.5% acrylamide/bisacylamide gel. Proteins were then transferred onto a polyvinyllidene fluoride membrane, and probed first with primary antibody (to NCAM, AB5032, to integrin β1, AB1952 both from Millipore, to N-cadherin, clone 389 from Zymed Laboratories Inc.) and then secondary antibody, coupled to horseradish peroxidase, which was detected by ECL (GE-Healthcare).

**mRNA Isolation and Quantitative Reverse Transcription PCR—**Total RNA was isolated with the RPN kit (Sigma-Aldrich). QRT-PCR was performed as previously described (27). Quantitative PCR occurred in the Rotorgene 6000 (Corbett Research) using SybrGreen to quantify cDNAs produced from various gene mRNA, using primers from BioCorp (see supplemental Table S1). Relative mRNA abundance was calculated following the Liu and Saint method (32). The housekeeping gene EF1α was used to calculate relative mRNA expression of target proteins. EF1α was tested and found to be an appropriate housekeeping gene for isolated chondrocytes cultured in suspension.

**Aggregation Assays—**600,000 cells/ml were inoculated into agarose-coated 48 well plates with supplemented SFM (control), supplemented SFM without CaCl\(_2\), or in the presence of the reagents listed in Table 1. The aggregation assays took place at 37 °C, for 4 h. Cell aggregation was monitored by microscopy. To quantify aggregation after 4 h of incubation, single cells were counted, and the percent aggregation was calculated as 100 × (1 – single cell concentration/initial cell concentration).

To verify the activity of blocking antibodies with bovine cells, cell adhesion assays were performed as positive controls. 96-well plates were coated with either collagen Type I at 100 μg/ml, following the manufacturer’s recommendations, or with fibronectin (both from Sigma) at 1 μg/ml in PBS, and then blocked with 10 mg/ml heat-denatured bovine serum albumin. Bovine chondrocytes, expanded in monolayer, were treated with trypsin (Invitrogen; 0.25% w/v in 1 mM EDTA), and incu-
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**TABLE 1**
Reagents used in aggregation assays

| Reactant           | Concentration | Supplier/comments             |
|--------------------|---------------|-------------------------------|
| Integrin β1        | 20 mg/ml      | Clone 4B4, Beckmann. Control: IgG1 |
| Integrin α1        | 20 mg/ml      | Clone FB12, Millipore. Control: IgG1 |
| Integrin α2        | 20 mg/ml      | Clone P1E6, Millipore. Control: IgG1 |
| Integrin α5β1      | 1/40          | Clone JBS5, Millipore. Control: IgG1 |

**Blocking antibodies**

| Reactant | Concentration | Supplier/comments |
|----------|---------------|-------------------|
| GRGDS    | 0.1–0.5 mg/ml | Negative control is SDGRG. Both from Sigma |
| GFOGER-GPP| 0.1–1 mg/ml  | Provided by Dr. Farndale. * Native triple helical collagen sequence recognized by integrins (61) with negative control non-triple helical GFOGER-GAP |

**Blocking peptides**

| Reactant | Concentration |
|----------|---------------|
| EDTA     | 5 mM          |

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**TABLE 2**
Antibodies used for immunostaining

| Staining           | Antibodies                      |
|--------------------|---------------------------------|
| Type II Collagen   | Clone II-IB3 (1/10), DSHB*      |
| Denatured Type II Collagen | Clone 3/4m. Provided by Dr. Poole* |
| Fibronectin        | Biogenesis 4470-3589             |
| Type I Collagen    | Clone Col 1 (1/100), Sigma      |
| COMP                | (1/1000) Provided by Dr. Zaucke* |
| Integrin α5        | AB 1928 (1/1000), Millipore.    |
| Integrin β1        | Clone 4B4 (1/200), Beckmann.    |

* DSHB, Developmental Studies Hybridoma Bank.
* From the Shriners Hospital, Montreal, QC.
* From the Institute from Biochemistry II, Medical Faculty, University of Cologne, Germany.

bated at 400,000 cells/ml in supplemented SFM with the different blocking agents (see Table 1) for 30 min, at room temperature. Then cell suspensions were transferred to coated wells and allowed to adhere during 30 min for fibronectin, or 1 h for collagen at 37 °C. Thereafter, wells were washed, adhered cells fixed with 5% (w/v) glutaraldehyde, and stained in 0.1% (w/v) in crystal violet. After additional washes the retained dye was solubilized with 100 μl of 10% (v/v) acetic acid, and the absorbance read at 570 nm.

**Immunostaining**—Chondrocyte aggregates after 18 h in suspension culture were fixed in 0.4% (w/v) paraformaldehyde in PBS, 10 min, and blocked in 10% (v/v) serum, in PBS. All subsequent steps were carried out in 1.5% (v/v) serum (complemented with 0.1% w/v saponin for integrin α5), in PBS, unless otherwise mentioned. Samples were stained for the antigens described in Table 2. All samples were counterstained for actin with Phalloidin-Alexa 488 (1/40, Molecular Probes), in 1.5% (v/v) serum, 0.1% (w/v) saponin, in PBS, and the nucleus stained with Hoechst 33258 (0.5 μg/ml, Molecular Probes) in PBS. Stained samples were mounted in 16.7% (w/v) Mowiol 4—88 (Fluka), 33.3% (v/v) glycerol, 0.75% (w/v) n-propyl gallate (Sigma-Aldrich Canada) in PBS. Confocal imaging was performed using an Achromat 40×/NA 1.2 water immersion objective mounted on an Axiosplan 2 microscope equipped with an LSM 510 META confocal laser scanning module, an Axiocam HRM camera and the LSM510 acquisition software (all from Zeiss).

**RESULTS**

**Chondrocytes Aggregate in Static Suspension over Agarose**—When cultured in static suspension, chondrocytes aggregate rapidly within the first hour, and these aggregates reach sizes of more than 1 mm after 18 h (Fig. 1). The rapidity of this aggregation process suggests that adhesion molecules mediating aggregation were present at early stages of the culture just after enzymatic isolation that is known to remove most cell surface proteins. Expression of candidate adhesion molecules during culture was assessed QRT-PCR and Western blot.
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Integrin Subunit β1 Is More Highly Expressed Than NCAM and N-Cadherin—QRT-PCR and Western blot analysis of freshly isolated chondrocytes or after 4 h or 18 h in suspension culture, revealed that NCAM and N-cadherin adhesion molecules were expressed at low levels relative to integrin subunit β1 (Fig. 2). mRNA levels for NCAM and N-cadherin remained low even after 18 h of culture (Fig. 2A), and none of the bands corresponding to the 131-kDa positive control for N-cadherin or the 3 bands at 131, 180, and 197 kDa of the NCAM positive control were detected by Western blot (Fig. 2B). NCAM are known to present 3 isoforms (120, 140, and 180 kDa, named 120-, 140-, or 180-NCAM) that can be differentially glycosylated (33).

Integrin subunit β1 was strongly expressed, and the protein level was found to increase with time, similar to the kinetics of aggregation (compare Fig. 2B to Fig. 1). The Western blot revealed bands at 135 and 120 kDa (Fig. 2B); the 120-kDa band was the intracellular precursor of the integrin subunit β1 and the 135 kDa its mature, transmembrane counterpart (34). In freshly isolated chondrocytes, the upper band, corresponding to the transmembrane protein was weakly detected, as expected since the enzymatic digestion used to isolate cells digested most cell surface proteins. After 4 h, the 135-kDa band became much more intense, indicating chondrocytes had synthesized new functional β1 integrins.

Chondrocyte Aggregation Requires Divalent Ions but Does Not Require Ca\(^{2+}\)—Previous work demonstrated the insensitivity of chondrocyte aggregation to calcium depletion (27) in long term suspension culture (10 days). This type of experiment was repeated here, but as a short aggregation assay where aggregation in complete medium (containing 1 mM Ca\(^{2+}\), 0.7 mM Mg\(^{2+}\), and 1.5 μM Zn\(^{2+}\) in a calcium-depleted medium or in a divalent ion-depleted medium (with EDTA) were compared (Fig. 2C). Here again the absence of calcium did not affect chondrocyte aggregation. On the contrary, chelation of divalent ions by EDTA inhibited cell-cell adhesion. These results suggest that chondrocyte aggregation in suspension culture is primarily mediated by one or multiple β1 containing integrins, known to be Mg\(^{2+}/\text{Mn}^{2+}\)-dependent (but less sensitive to Ca\(^{2+}\)).

Strong Expression of Collagen II Indicates Phenotype Maintenance and a Collagen-mediated Aggregation—In an effort to identify possible ligands for β1 integrin, and to examine to what extent aggregating chondrocytes synthesize a cartilage like pericellular matrix, several ECM molecules were analyzed. Among possible ligands, collagens and fibronectin could serve to link integrins on adjacent chondrocytes. The expression and presence of fibronectin and collagen types II, VI, and IX in aggregates was assessed by QRT-PCR and immunostaining. In addition, collagen type I, which could be synthesized by dedifferentiating chondrocytes, and denatured collagen type II that may arise from enzymatic treatment during cell isolation were examined. Collagen type II and fibronectin were positively immunostained (Fig. 3A), while collagen type I and denatured collagen type II were not detected (data not shown). Fibronectin was found only in small amounts and exclusively at the aggregate periphery (Fig. 3B), whereas collagen type II was strongly detected throughout the whole aggregate, appearing to fill the intercellular space. QRT-PCR confirmed these results since collagen type II was highly expressed by aggregating chondrocytes, while collagen types VI, and IX and fibronectin were expressed at much lower levels, and collagen type I was not detected (Fig. 3C).

Expression of Thrombospondins and Matrilins in Chondrocyte Aggregates Highlight TSP5/COMP as Potential Mediator of
Aggregate Formation—mRNA levels of thrombospondins (TSPs) and matrilins (MATs) were quantified by QRT-PCR, because these molecules have the ability to bind ECM components and cell membrane receptors (35–37). Among TSPs, TSP5/COMP was the most highly expressed followed by TSP1 with a 4-fold lower expression (Fig. 4). In comparison to TSPs, matrilins were weakly expressed with matrilin 2 showing the highest relative abundance (Fig. 4). These low levels of matrilins appear to exclude them from playing a central role in chondrocyte aggregation, whereas TSP1 and in particular TSP5 could certainly contribute to aggregate formation and organization. The significant presence of TSP5/COMP in aggregates was confirmed by immunostaining (Fig. 4C) where COMP was found between cells, throughout the aggregate, in regions where collagen type II was also detected.

Ultrastructural Analysis Shows Cell-Cell Contacts Are Absent and the Intercellular ECM Is Fibrillar Containing Both Collagen II and COMP—SEM was used to examine the ultrastructure of chondrocyte aggregates formed in suspension culture (Fig. 5). These analyses confirmed that chondrocytes in aggregates taken after 18 h of suspension culture were not in direct contact but were linked by a randomly oriented dense fibril network (Fig. 5A), with extensive cross-linking and fibril bonding (Fig. 5B). Fibrils appeared to arise from cells with some small fibrils merging to produce larger structures growing in diameter (Fig. 5B). In some locations fibrils were wider, creating a loose fiber network (Fig. 5, B and D), while other sites displayed a denser network with smaller diameter structures (Fig. 5C). Fibril diameter varied from 20 to 180 nm, as measured in environmental mode without gold coating. Immunogold staining revealed that the intercellular fiber network was positive for collagen type II and COMP confirming the presence of these molecules spanning the intercellular space in chondrocytes aggregates formed after 18 h (Fig. 5, C and D), and affirming their possible involvement in aggregate formation and organization.

Integrin Subunit α10 and Annexin V Are the Most Highly Expressed Collagen Receptors—mRNA levels for several integrins and collagen binding cell surface molecules were analyzed by QRT-PCR (Fig. 6A). Among integrin subunits, α10 was
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The Triple Helical GFOGER-GPP Peptide Significantly Inhibits Chondrocyte Aggregation, Whereas RGD Peptides Do Not—To further study the role of collagen II and TSP5/COMP, aggregation assays with peptides mimicking sequences present in these potential matrix molecule ligands, were performed (Fig. 7). RGD is a cell adhesion motif found in TSP5/COMP (and fibronectin and collagen I) and is recognized by several integrins (39). On the other hand, GFOGER is a triple helical motif present in collagens I, II, and XI and is recognized by collagen-specific integrins including α1-, α2-, and α1β1 (40). Our data found no inhibitory effect of RGD peptides on chondrocyte aggregation, while we observed triple-helical GFOGER to have a significant dose-dependent inhibition of aggregate formation (Fig. 7C). Cell adhesion on fibronectin or collagen type I-coated surfaces was also assayed in presence of RGD or GFOGER peptides, respectively, as a positive control. GFOGER at 500 μg/ml was found to inhibit chondrocyte adhesion on collagen type I by 57 ± 9% and RGD by 71 ± 6% on fibronectin-coated plates.

DISCUSSION

Chondrocyte Aggregation Is Driven by Integrin-mediated Cell-Matrix Interactions Rather Than Cell-Cell Interactions—N-CAM and N-cadherin are known to be expressed and involved in mesenchymal pre-chondrogenic cell condensation, but are no longer found in mature cartilage (8, 9). Although these molecules could be re-expressed after enzymatic isolation and contribute to chondrocyte aggregation, QRT-PCR, and Western blot analysis showed that this was not the case (Fig. 2A). In contrast, integrin β1 was strongly synthesized post-isolation, and its protein level correlated with the kinetics of aggregation (Fig. 2B). Moreover, the divalent ion-dependence of aggregation corroborates these latter results. Calcium was not required, affirming that calcium-dependent cadherins are not implicated in chondrocyte aggregation, but other divalent ions were needed (Fig. 2) as aggregation was strongly inhibited in presence of EDTA. NCAM and numerous other CAMs from the immunoglobulin-like CAM family are not divalent ion-dependent and thus are insensitive to EDTA, suggesting that CAMs are also not responsible for chondrocyte aggregation. However, integrins are Mg2+/Mn2+-dependent, and could also be inhibited by calcium in the mM range, or in the complete absence of calcium, depending on the ligand and the integrin type (41–43). Importantly, we previously measured ~14 μM calcium in the “calcium-free” SFM, likely from the added growth factors in this serum-free formulation (27). This residual concentration is sufficient to avoid integrin inhibition observed by others (41) in the complete absence of calcium. Therefore, these observations point to an integrin-mediated process and excluded all calcium-dependent cadherins, and other divalent ion-independent adhesion molecules such as N-CAMs, annexin V, and DDR-2.

Expression Profiling Suggests That Chondrocytes Aggregate Through Collagen II Binding to a Cellular Collagen Receptor—β1 integrins, in association with different α-subunits can bind collagens, fibroblastin (38), and other non-collagenous proteins

found to have the highest relative abundance at all time points. Annexin V showed even higher mRNA expression, with relative abundances of about twice those obtained for α10. Integrin subunits α1 and α2 displayed weaker expression while α11 was lower yet (not distinguishable in Fig. 6A but detected with a relative expression of 3.9 ± 2.7 × 10^-4). Finally DDR-2 showed a level of expression similar to α5, with a relative abundance of 0.024 ± 0.004 for DDR-2 and 0.027 ± 0.015 for α5. Thus significant expression of integrins α5β1 and α10β1 were found along with DDR-2 and Annexin V. Note that the latter two receptors (DDR-2 and Annexin V) are not likely to be involved in aggregation, considering the divalent ion-dependence of chondrocyte aggregation (Fig. 2C). Immunohistochemical staining confirmed high levels of α5 (Fig. 6B) and β1 (Fig. 6C) subunits.

Blocking Integrin β1 Inhibits Aggregation, Whereas Blocking α1, α2, and α5 Do Not—Aggregation assays with blocking antibodies against integrin β1, α1, α2, and α5β1 confirmed that a β1 integrin is implicated in aggregation since only the integrin β1 blocking antibody significantly reduced chondrocyte aggregation from 94 ± 3% in the control to 36 ± 5% with the integrin β1 blocking antibody (Fig. 7A). On the contrary, antibodies against α1, α2, and α5β1 had no effect on chondrocyte aggregation in suspension. As positive controls to ensure blocking functionality, all antibodies were tested for their capacity to inhibit bovine chondrocytes adhesion to collagen type I or to fibronectin in monolayer. Antibodies to β1, α1, α2, were found to efficiently block cell adhesion to collagen type I in monolayer with 89 ± 2, 90 ± 3 and 40 ± 14% inhibition, respectively (Fig. 7B). Similarly, antibodies to β1 and α5β1 inhibited adhesion to fibronectin in monolayer with 65 ± 12% and 72 ± 5% inhibition, respectively (Fig. 7B), in accordance with previous findings (11, 38).
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such as matrilins and TSPs (35, 37). As expected in this culture, collagen type I was not found, whereas collagen types II, VI and IX were expressed with collagen type II at a level more than 20 times higher than the others (Fig. 3). COMP was also found to be highly expressed and to be co-localized with collagen type II both at the light and electron microscopic level (Figs. 4C and 5D), in accordance with its role in promoting collagen fibrillogenesis (44). Although COMP can bind integrins α5β1 or αVβ1 (45) present at the chondrocyte surface, its proximity to collagen type II and lack of any blocking effect of anti-α5β1 and RGD peptides (Fig. 7A) suggest its role to be related to collagen fibrillogenesis rather than direct cell binding.

The Most Highly Expressed Collagen Receptor Compatible with the Divalent Ion Dependence of Chondrocyte Aggregation Is Integrin α10β1—In principle several matrix molecules analyzed in our study are compatible with the divalent ion dependence of chondrocyte aggregation, however only collagen II and COMP were highly expressed and detected in intercellular regions. Four different integrin-based collagen receptors are known, α1β1, α2β1, α10β1, and α11β1 (22), all of which can be expressed by chondrocytes or mesenchymal cells (10–12, 24), and therefore could be active in our aggregating freshly isolated chondrocytes. On the other hand, integrin α5β1 has the ability to bind COMP or denatured collagen type VI and type II in an RGD-dependent manner (16, 17, 45) and is the principal receptor for fibronectin, another abundant ECM molecule. Analysis of these five α-subunits by QRT-PCR revealed that α5 and α10 were indeed the most highly expressed (Fig. 6), where higher expression of α5 in comparison to α1 or α2 is corroborated by the literature (11, 25). The very weak expression of α11 was also expected, as α11 was previously detected in mesenchymal cells but not in cartilage (24).

Our data are the first to quantitatively compare α10 expression to other α-subunits in chondrocytes. We found α10 to be the most highly expressed of all α-subunits, suggesting that α10β1 is a main candidate integrin to bind collagen II in aggregating chondrocytes. We also analyzed other non-integrin collagen receptors known to be expressed in cartilage. In particular, DDR-2 and annexin V were found to be expressed by aggregating chondrocytes, with annexin V exhibiting the highest mRNA expression among all candidate collagen receptors tested. However, annexin V is primarily located intracellularly and adhesion of annexin V to collagen is not divalent ion-dependent (25, 46). Similarly no ion binding site has been described for DDR-2 (47). Thus the likelihood of these non-integrin collagen receptors driving chondrocyte aggregation appears to be minimal.

Function Blocking Antibodies and Peptides Further Identify Collagen II Binding to Integrin α10β1 as a Prime Mediator of Chondrocyte Aggregation—Although the above expression profiling and divalent ion-dependence of chondrocyte aggregation has pointed to particular collagens and their receptors as main molecular candidates in the chondrocyte aggregation process, function blocking experiments were required to further specify these interactions and their importance in chondrocyte aggregation. Antibodies to integrin β-subunits strongly inhibited chondrocyte condensation, but not antibodies to α1, α2, and α5β1 (Fig. 7). Unfortunately a blocking antibody against α11 was not available, but, as α11 mRNA expression was found to be very low, this molecule is unlikely involved in aggregation. We tested a blocking antibody against human integrin α10 (mAb 365 provided by Cartela, Lund, Sweden) but found this agent to be inactive with our bovine cells, even in immunocytochemistry, and thus was not useful in our study.

The lack of a role for α5β1 in the cell aggregation process was further confirmed by the lack of inhibition of aggregation by linear RGD peptides, the cell adhesion motif recognized by α5β1 (39) that is present in fibronectin, COMP, and denatured collagens types VI and II. In contrast, GFOGER-GPP peptides, a triple helical motif present in collagen types I, II, and XI (but not in collagen types III, V, VI, and IX) efficiently inhibited aggregation in a dose-dependent manner (Fig. 7). Because collagen type I was not detected in aggregating chondrocytes, while collagen type XI is buried inside collagen fibrils and therefore not accessible, collagen type II appears to be the only collagen implicated in aggregation that is blocked by GFOGER. The GFOGER motif is known to be recognized by integrins α1β1, α2β1, α11β1 (40), as well as by integrin α10β1.3

All of the above results, when taken together in the context of existing literature, lead to the following conclusions concerning the aggregation process of phenotypically stable freshly isolated mammalian articular chondrocytes: 1) α1β1, α2β1, α11β1-collagen interactions are not involved due to their weak expression, and the lack of effect of blocking antibodies to α1 and α2. 2) Ineffectiveness of RGD peptides or blocking antibodies to α5β1, also exclude an α5β1 interaction with fibronectin, denatured collagen type VI or II (16, 17) and with COMP/TSP5 (45) in the aggregation process. 3) The inhibition of aggregation obtained with the β1 blocking antibody and with the GFOGER-GPP peptide, indicates that aggregation is mediated by a collagen binding to a β1 integrin, most likely collagen type II binding to α10β1. Note that a lack of importance of DDR-2 and annexin V in aggregation is further supported here by the known non-GFOGER binding sites of annexin V, which rather bind to the N-telopeptide (48) and DDR-2 which binds most strongly to a very specific motif, GPRGQOGVGMFO in the D2 period of collagen type II, but not to GFOGER (49, 50). Other ECM components such as CHAD, or RGD-CAP, which were not studied here, are known to bind collagen and chondrocytes by integrins α2β1 or α1β1, respectively (51–53). Because these two integrins were excluded from being active in the aggregation process it appears that CHAD and RGD-CAP are also unlikely to be involved. Hence all of our results converge toward an α10β1-collagen type II interaction that drives the aggregation of freshly isolated phenotypically stable chondrocytes.

Chondrocyte Aggregates Simulate the in Situ Chondrocyte Environment—The integrin expression profile we found in nascent aggregates is similar to native cartilage where α1β1 and α2β1 were detected in smaller amounts than α5β1. Our study was the first to identify high expression levels of α10β1 that are compatible with its strong immunostaining in human cartilage (12), whereas α11β1 expression was not significant, also in agreement with its expression in mesenchymal cells in situ but

3 E. Lundgren-Akerlund and R. Farndale, personal communication.
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Chondrocytes condense rapidly in suspension culture, forming aggregates rich in extracellular collagen type II and COMP that are organized in a dense fibrillar network, similar to the in situ environment. Aggregate formation was not mediated by direct cell–cell contact but by cell-matrix interactions. Cell aggregation was strongly inhibited by both an antibody against integrin β1 and by the peptide GFOGER-GPP that mimics the triple helical integrin-binding motif of collagens, but not by RGD peptides or antibodies to α1, α2, and α5β1 integrins. Therefore it appears that aggregate formation is primarily mediated by a collagen interacting with a β1-containing integrin. Because collagen type II was detected in significant amounts in aggregates while collagen type I, VI, and IX were not expressed or minimally expressed, it appears that collagen II is the primary integrin binding ligand active in the aggregation of chondrocytes in suspension. Among integrin collagen receptors, α10 exhibited the highest mRNA expression, which combined with our blocking studies strongly suggests that α10β1 integrin mediates aggregation of chondrocytes in suspension. Therefore the most probable receptor-ligand pair responsible for aggregation of differentiated chondrocytes in suspension culture is α10β1-collagen type II.

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