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Differential Modulation of Integrin Expression in Chondrocytes during Expansion for Tissue Engineering

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Abstract. *Cartilage tissue engineering has an important role to play in the generation of graft material for reconstructive surgery. In cultured chondrocytes, the dedifferentiation of cells seems unavoidable for multiplication. Dedifferentiated cells produce matrix of less quality. Normal cartilage is composed of chondrocytes, which are embedded within an extracellular matrix (ECM). The ECM plays a key role in controlling cellular characteristics and contains the integrins as a large family of heterodimeric cell adhesion receptors involved in cell-cell and cell-matrix interactions. In this study, the characteristic changes of integrin expression and expression of matrix proteins during the course of dedifferentiation of chondrocytes in cell culture for 1, 6 and 21 days, analyzed at the mRNA level by microarray analysis and at the protein level by immunohistochemistry, are described. The components of the fibronectin receptor, integrin β1,α5, in conjunction with the ligand fibronectin, were up-regulated during dedifferentiation. Integrin β3 was expressed in the grey area. The components of the vitronectin-receptor, integrin α2b, αv, as well as integrin β5, were activated on day 21, but neither vitronectin nor osteopontin were expressed by the cells. With ongoing dedifferentiation, activation of the GPIIb/GPIIIa receptor was found. The integrins β2, β4, β6, β8 and α2, α4, α6, α7 and α11 were never expressed. ILK, CD47 and ICAP1, as components of the intracellular signalling cascade of several integrins, were activated with ongoing dedifferentiation. In conclusion, a candidate for signal transmission during dedifferentiation is the fibronectin receptor (integrin α5β1) in conjunction with its ligand fibronectin. Other receptors, e.g. for vitronectin and osteopontin (αVβ3) or laminin (α6β1) or their ligands, do not seem to be involved in signal transmission for dedifferentiation. In addition, the GPIIb/IIIa-receptor seems to assist the process of dedifferentiation. Intracellularly, ILK, ICAP1 and CD47 might assist the transduction of the integrin-dependent signals.*

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Tissue engineering represents a promising method for the construction of autologous chondrogenic grafts for reconstructive surgery. Implied in the definition of tissue engineering is the use of living cells and/or components of the extracellular matrix (ECM) in the development of implantable parts or devices that may lead to restoration or replacement of function (1). The promise of tissue engineering is perhaps most relevant to chondrogenic defects, because cartilage has little self-healing potential. Normal cartilage is composed of chondrocytes, which are embedded within an extracellular matrix that consists primarily of collagen type 2, aggrecan and hyaluronic acid. The unique expression and organization of macromolecules within the cartilage endows the tissue with exceptional strength, resilience and mechanical durability. The stability of the chondrocyte phenotype is determined by cell shape and cytoskeletal architecture. The ECM plays a key role in controlling these cellular characteristics. Culture conditions that allow chondrocytes to form a pericellular matrix promote the expression of cartilage-specific gene products, and thereby support the cartilage phenotype. The mechanism by which the ECM controls chondrocyte phenotypic expression is unclear. Specific studies that have attempted to define the regulatory role of the interaction between chondrocyte and the matrix are few. Beekman *et al.* found that, *via* an integrin-mediated mechanism, extracellular collagen down-regulated collagen synthesis in a negative-feedback manner (2). The integrin family of cell surface receptors appears to play a major role in the mediation of the cell-ECM interactions associated with structural and functional changes in surrounding tissues (3).

They are composed of noncovalently linked α and β subunits. Eighteen different αs and 8 βs can form dimers in 24 different combinations. The integrin β1 subunit is expressed on almost all cell types and it can be in complex with 12 different α subunits. Many of the functions of integrins are still unknown. So far, patterns of integrin expression have been shown to determine the adhesive properties of cells by modulating their interactions with specific ECM-proteins. In studies of the integrin-mediated attachment of chondrocytes to ECM-proteins, it was observed that the adhesiveness of chondrocytes increased when the cells were maintained in monolayer culture prior to use in attachment assays (4). These results suggest that chondrocyte-ECM adhesive interactions can be regulated by changes in integrin expression or function. Several recent studies have provided evidence that chondrocytes express integrins (5-10). Salter *et al.* used immunohistochemical staining in normal adult articular cartilage, and noted that integrin α5β1 was the most prominently expressed chondrocyte integrin (9). In contrast, the expressions of the integrin α1 and β3 subunits were weak and variable. A more recent study demonstrated that the chondrocyte expressions of α1β1, α5β1 and αvβ5 were accompanied by weak expressions of integrin α3β1 and αvβ3 (8). The integrin α2β (ITGA2B) gene encodes platelet glycoprotein IIb, the alpha subunit of the platelet membrane adhesive protein receptor complex GPIIb/IIIa. The beta subunit, GPIIIa, is encoded by the ITGB3 gene. The GPIIb/IIIa complex belongs to the integrin class of cell adhesion molecule receptors that share a common heterodimeric structure with alpha and beta subunits. The GPIIb/IIIa complex mediates platelet aggregation by acting as a receptor for fibrinogen. The complex also acts as a receptor for von Willebrand factor and fibronectin (11).

Integrin-mediated signalling seems also to be involved in differentiation and migration. Hannigan *et al.* isolated a gene that interacts with the cytoplasmic domain of beta-1 integrin. They found that this integrin-linked kinase (ILK) co-immunoprecipitated with beta-1 integrin from cell lysates, and that overexpression of ILK disrupted cell architecture and inhibited adhesion to integrin substrates, while inducing anchorage-independent growth in epithelial cells, suggesting that ILK regulates integrin-mediated signal transduction (12). In addition to ILK, integrin cytoplasmic domain-associated protein 1 (ICAP1) interacts with the cytoplasmic domain of beta-1 integrin. ICAP1 contains a protein kinase C phosphorylation site and multiple potential phosphorylation sites, including a calcium/calmodulin-dependent protein kinase II (13). CD47, or integrin-associated protein (IAP), is a membrane protein that is involved in the increase in intracellular calcium concentration that occurs upon cell adhesion to the ECM. IAP is also expressed on erythrocytes, which have no known integrins (14).

In this study, the characteristic changes of integrin expression and expression of matrix proteins during the course of dedifferentiation of chondrocytes in cell culture are described. Distinct patterns of expression were shown at the mRNA level by microarray analysis and at the protein level by immunohistochemistry.

Materials and Methods

Cell culture. Human septal cartilage of 32 patients was harvested during septoplasty. The cells were isolated using collagenase and pronase. Cell cultures were carried out in Falcon petri dishes at 37˚C in a 5% CO_2 fully humidified atmosphere, using Dulbecco's modified minimum essential medium (DMEM) (Fisher Scientific Co., Pittsburgh, PA, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics (Life Technologies, Inc. [Gibco BRL], Gaithersburg, MD, USA). The cells were cultured for 6 and 21 days without growth factors.

RNA extraction and microarray hybridization. Extraction of RNA was performed using the RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol and as published previously (15). The RNA concentration was estimated from the absorbance at 260 nm.

Approximately 1 μg total RNA was used in each microarray experiment and, for amplification and labelling of mRNA, the SMART technique (SMART Fluorescent Probe Amplification Kit; BD Clontech, Heidelberg, Germany) was applied according to the manufacturers' protocol. RNA samples from day 1 were labelled with Cy3 and day-6 or day-21 samples were labelled with Cy5 (Cy™3- and Cy™5-monoreactive dye; Amersham Pharmacia Biotech, Freiburg, Germany). Corresponding Cy3- and Cy5-labelled samples were mixed, vacuum dried and resuspended in 25 μl microarray hybridization buffer (MWG-Biotech, Ebersberg, Germany). Prior to hybridization, the samples were heat-denaturated at 95˚C for 5 minutes. The human 10K (MWG-Biotech) oligo microarray systems on glass slides were used for mRNA profiling. Hybridization of Cy3/Cy5-cDNA was performed using cover slips and a hybridization chamber for 16 hours at 42˚C in a water-bath. After stringent washing of the glass slides according to the manufacturer's specifications, the hybridization signals of the Cy3 and the Cy5 dyes were measured using a microarray laser scanner (GMS418; Affymetrix, MWG-Biotech).

Microarray data analysis and statistics. The ArrayVision (Imaging Research, Inc., St. Catharines, ON, Canada) software was used for evaluation and calculation of signal intensities from the raw data images in 16-bit tagged-image-file (TIF) format, as described previously (15). In brief, for evaluation of hybridization results, we defined a negative (<3,000), a grey area (3,000-4,999) and a positive range (≥5,000) of hybridization signal intensities. Signal-to-background (S/B) values were calculated by dividing the signal intensity for each spot with the background signal intensities of the hybridized glass slide. Computer-assisted evaluation of the raw data provides the mean signal intensity and the signal to background ratio for each individual gene spot. For statistical evaluation, the mean signal intensity and standard deviation (SD) was calculated for each spot from the values obtained in the 10 individual experiments. Functional grouping of genes was performed on the basis of the database supplied by the array manufacturer.

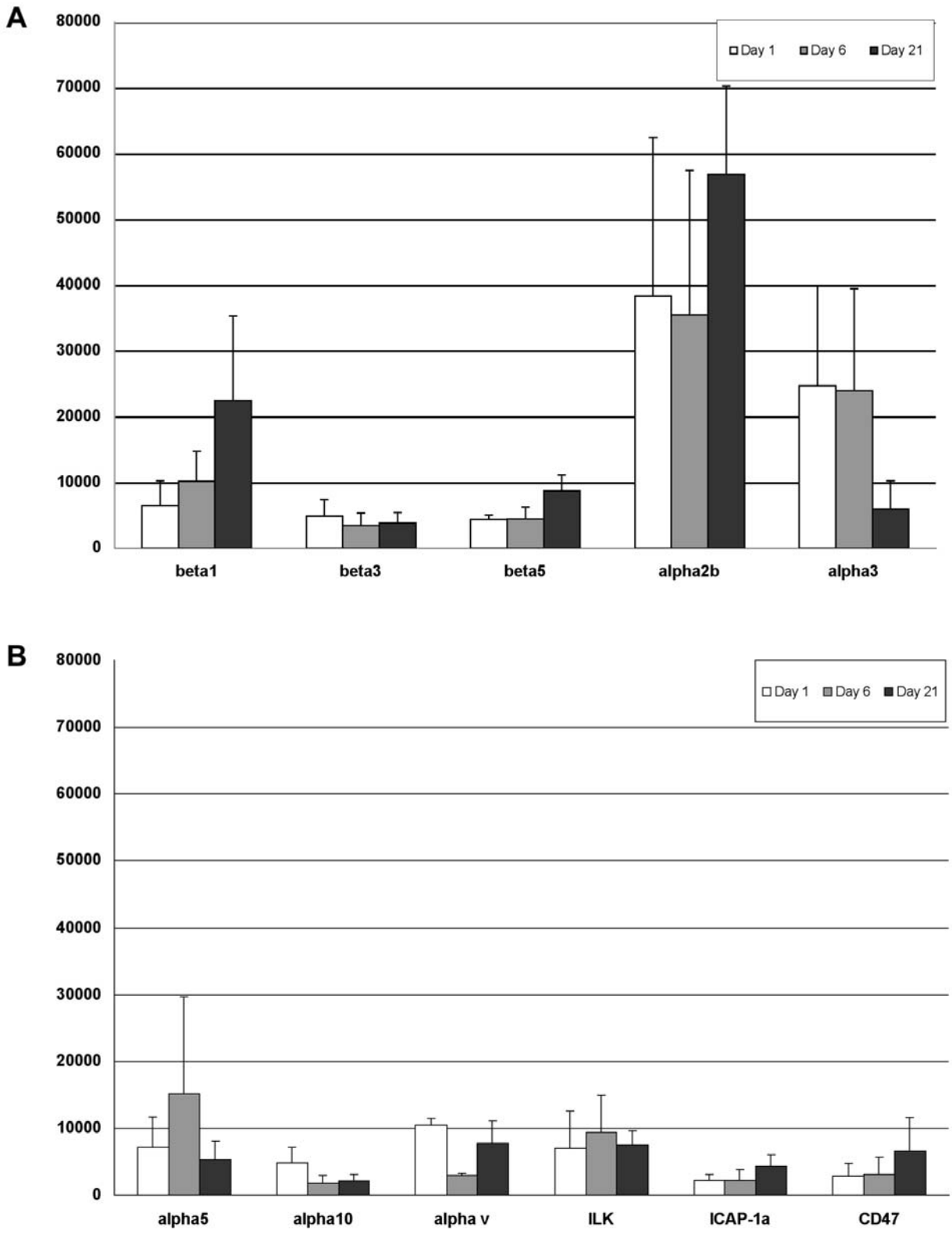

Figure 1. *Expression levels of genes for different integrins and ECM-proteins in chondrocytes measured by microarray hybridization analysis. Results are shown as mean signal intensity and standard deviation for the given genes in differentiated chondrocytes (white bars) on day 1, short-term cultured cells (grey bars) on day 6 and long-term cultured cells (black bars) on day 21.*

Table I. *Signal intensities of hybridization signals as measured using the microarray laser scanner and calculated by the ArrayVision software.*

Gene	Day 1	Day 6	Day 21	SD Day 1	SD Day 6	SD Day 21
beta1	6465	10128	22418	3749	4640	12814
beta3	4868	3481	3800	2506	1849	1600
beta5	4378	4463	8714	675	1738	2401
alpha2b	38256	35463	56775	24115	21947	13431
alpha3	24622	23926	5918	15239	15483	4294
alpha5	7150	15126	5311	4522	14590	2697
alpha10	4789	1717	2128	2302	1203	912
alpha v	10476	2903	7706	961	265	3346
ILK	7081	9330	7508	5523	5609	2127
ICAP-1a	2192	2245	4311	910	1618	1742
CD47	2835	3087	6582	1856	2641	5043

Immunohistochemistry. Immunohistochemistry for integrin β1, β3, β4, α2, α3, α5 and integrin-linked kinase (ILK) was performed by using a streptavidin-biotin complex procedure. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 minutes. Sections were washed with phosphate-buffered saline (PBS) and incubated with normal rabbit serum in PBS for 30 minutes at room temperature to block non-specific antibody reaction. The sections were then incubated overnight at 4˚C with the primary antibody (all from Santa Cruz Biotechnologies, Heidelberg, Germany). The slides were washed in several changes of PBS. The sections were then incubated with a peroxidase-conjugated secondary antibody (DAKO, Hamburg, Germany). After being washed twice in PBS, the sections were treated with a streptavidin-biotin-peroxidase complex and peroxidase reaction was performed using diaminobenzidine DAB (DAKO, Hamburg, Germany) as chromogen. The different antibodies were diluted to the desired concentrations in PBS. Controls were carried out by omitting the primary antibody. Light microscopy investigation was performed using a Zeiss Axiophot microscope.

Results

Microarray gene expression analysis. The expression level and the change in expression of 9,832 individual human genes in chondrocytes isolated from the septal cartilage was estimated on the basis of the hybridization signal intensity of each gene-specific probe represented by the microarray system. In the first series of microarray investigations, the gene expression in primary chrondrocytes (day 1) was compared to the gene expression after 5 and 20 days of cell culture (days 6 and 21) in five paired experiments.

Integrins. The microarray analysis included 36 genes for integrins and proteins of the ECM (Figure 1A, Table I). The gene for the fibronectin receptor (integrin β1) was expressed on day 1 and activated on days 6 and 21 (day 6/day 1 ratio 2.73, day 21/day 1 ratio 3.47). The gene for integrin β3 was expressed in the grey area with a day 6/day 1 ratio of 0.95 and a day 21/day 1 ratio of 0.78. The gene of integrin β5 was expressed on days 1 and 6 (day 6/day 1 ratio 1.07) and was then activated on day 21 (day 21/day 1 ratio 1.84). The genes for integrins β2, β4, β6, β7 and β8 were never expressed.

The gene for integrin α2b was expressed on days 1, 6 and 21 (day 6/day 1 ratio 0.92, day 21/day 1 ratio 1.49). The integrin α3-gene was activated on days 1 and 6 and then down-regulated on day 21 (day 6/day 1 ratio 1.04, day 21/day 1 ratio 0.24). Integrin α5 was expressed on day 1, activated on day 6 (day 6/day 1 ratio 2.11) and then down-regulated on day 21 (day 21/day 1 ratio 0.74). Integrin α10 was expressed on day 1, but then inactivated on days 6 and 21 (day 6/day 1 ratio 0.35, day 21/day 1 ratio 0.44). The gene for integrin αv was activated on days 1 and 21, but inactivated on day 6 (day 6/day 1 ratio 0.28, day 21/day 1 ratio 0.74). The genes for vitronectin and osteopontin were never expressed.

Integrin-associated proteins. The fibronectin gene was expressed on day 1 and then up-regulated on days 6 and 21 (day 6/day 1 ratio 1.83, day 21/day 1 ratio 4.06). The gene for the ILK was expressed on days 1, 6 and 21 with ratios of 1.32 and 1.06, respectively. Integrin αe, CD47 and ICAP-1 were not expressed on day 1, but were both activated on days 6 and 21 (day 6/day 1 ratios 1.03, 1.09 1.02 and, day 21/day 1 ratios 2.5, 2.32 and 1.97).

Immunohistochemistry. In order to confirm the mRNA data, the protein expression of different integrins and matrix-associated proteins was investigated by using immuno-histochemistry with specific monoclonal antibodies (Figure 2, Table II). As was found in the microarray analysis, integrin β1 was strongly expressed by differentiated primary chondrocytes (day 1) and then up-regulated on days 6 and 21. Integrin β3 was expressed on days 1, 6 and 21. Staining of the cells for integrin β4 revealed little expression on day 1, but no expression on days 6 and day 21. The mRNA results revealed a similar pattern of expression.

The monoclonal antibodies recognizing integrin α2 revealed protein expression on days 1 and 6 and up-regulation on day 21. In differentiated chondrocytes (day 1), a strong expression of integrin α3 was found. In contrast to the microarray analysis, it was followed by a rapid down-regulation of the protein expression in cultured cells already on day 6 and then on day 21. Integrin α5 was gradually up-regulated from day 1 to day 6, and then down-regulated.

Immunohistochemistry against ILK showed strong expression on days 1, 6 and 21. The mRNA results revealed a similar pattern of expression.

Discussion

In this study, we described the characteristic changes of integrin expression and expression of integrins and integrin-associated proteins during the course of dedifferentiation of chondrocytes in cell culture. We were able to show distinct patterns of expression at the mRNA level by microarray analysis and at the protein level by immunohistochemistry.

The physiologic response of chondrocytes to maintenance of the matrix and response to stimuli probably involves signalling from multiple sources including soluble cytokines, mechanical stimulation and signalling from the ECM. Cell-matrix interactions are important regulators of cellular functions, including matrix synthesis, proliferation and differentiation. This is well exemplified by the characteristically labile phenotype of chondrocytes that is lost in monolayer culture, but is stabilized in suspension under appropriate conditions.

We found that, with ongoing dedifferentiation, the expression of integrin β1 rose. The up-regulation could also be seen on the protein level with immunohistochemistry. Chondrocytes have previously been reported to express several integrins. Receptors have been detected for type 2 and type 6 collagen [α1β1, α2β1,α11β1; (9)]. The most abundant integrin detected on chondrocytes is the fibronectin receptor α5β1 (16). It has previously been described that chondrocytes down-regulate collagen type 2 during dedifferentiation on day 6, but then up-regulate on day 21 (17). The up-regulation of integrin β1 seems to correlate with these results, as the up-regulation would facilitate adhesion to collagen 2. Integrin β1 could be a marker for synthetic activity as the chondrocytes try to generate an extracellular matrix suitable for their survival. In this study, in conjunction with the up-regulation of integrin β1, we found an activation of integrin α5 on day 6 and a down-regulation on day 21. Similar results for integrin α5 and β1 were found using immunohistochemistry. Along with this activation of the fibronectin receptor, an up-regulation of fibronectin in the course of dedifferentiation could be observed. In conclusion, ongoing dedifferentiation seems to be correlated with synthetic activity of the cells. Remarkably, integrins have not only been previously found in the cell membrane in cartilage, but also in the ECM itself (16). The synthetic activity of the cells seems to be rather undirected, as they are trying to generate an environment suitable for their metabolic needs.

Other receptors have been described for vitronectin and osteopontin (αVβ3 and αvβ5) or laminin (α6β1) (18, 19). We found expression of integrin αv on day 1, down-regulation on day 6 and up-regulation on day 21. The gene for integrin β3 was expressed in the grey area and the gene for integrin β5 was up-regulated on day 21; similar results were attained by immunohistochemistry. Integrin β5 might play a role in vitronectin binding, as McLean and coworkers isolated cDNA clones coding for the beta-5 subunit of the novel integrin receptor involved in cell adhesion to the matrix protein vitronectin, which had been found in a human lung epithelial-derived cell line. This receptor had an alpha subunit that appeared to be identical to that in another vitronectin receptor, but the beta subunit was distinctive (20). In the present study, vitronectin or osteopontin expression was never observed, so these proteins do not seem to be involved in the dedifferentiation process.

Somewhat unexpected was the strong expression of integrin α2β. The integrin α2β (ITGA2B) gene encodes platelet glycoprotein IIb, the alpha subunit of the platelet membrane adhesive protein receptor complex GPIIb/IIIa. The beta subunit, GPIIIa, is encoded by the integrin β3 gene. The GPIIb/IIIa complex belongs to the integrin class of cell adhesion molecule receptors that share a common heterodimeric structure with alpha and beta subunits. The GPIIb/GPIIIa complex mediates platelet aggregation by acting as a receptor for fibrinogen. The complex also acts as a receptor for fibronectin (11). Integrin α2b expression seems to be more important in chondrocyte dedifferentiation than the β3 subunit.

Integrin α3 showed strong expression on days 1 and 6, but down-regulation on day 21, with ongoing dedifferentiation of the cells. Similar results were obtained on the protein level by immunohistochemistry. It has previously been shown that neuronal migration and layer formation might be regulated through modulation of α3β1 integrin-mediated neuronal adhesion and migration (21). Our experiments suggest that integrin α3-dependent signalling is abundant in differentiated chondrocytes, but is unnecessary in dedifferentiated chondrocytes.

In conclusion, certain candidate genes from the integrin family might be involved in transducing the signals mediating the dedifferentiation of the chondrocytes in monolayer cell culture. In a next step, we tried to find candidate genes that might transduce the signal to an intracellular level.

One of these candidates was ILK. Microarray analysis revealed that the gene for ILK was expressed on days 1, 6 and

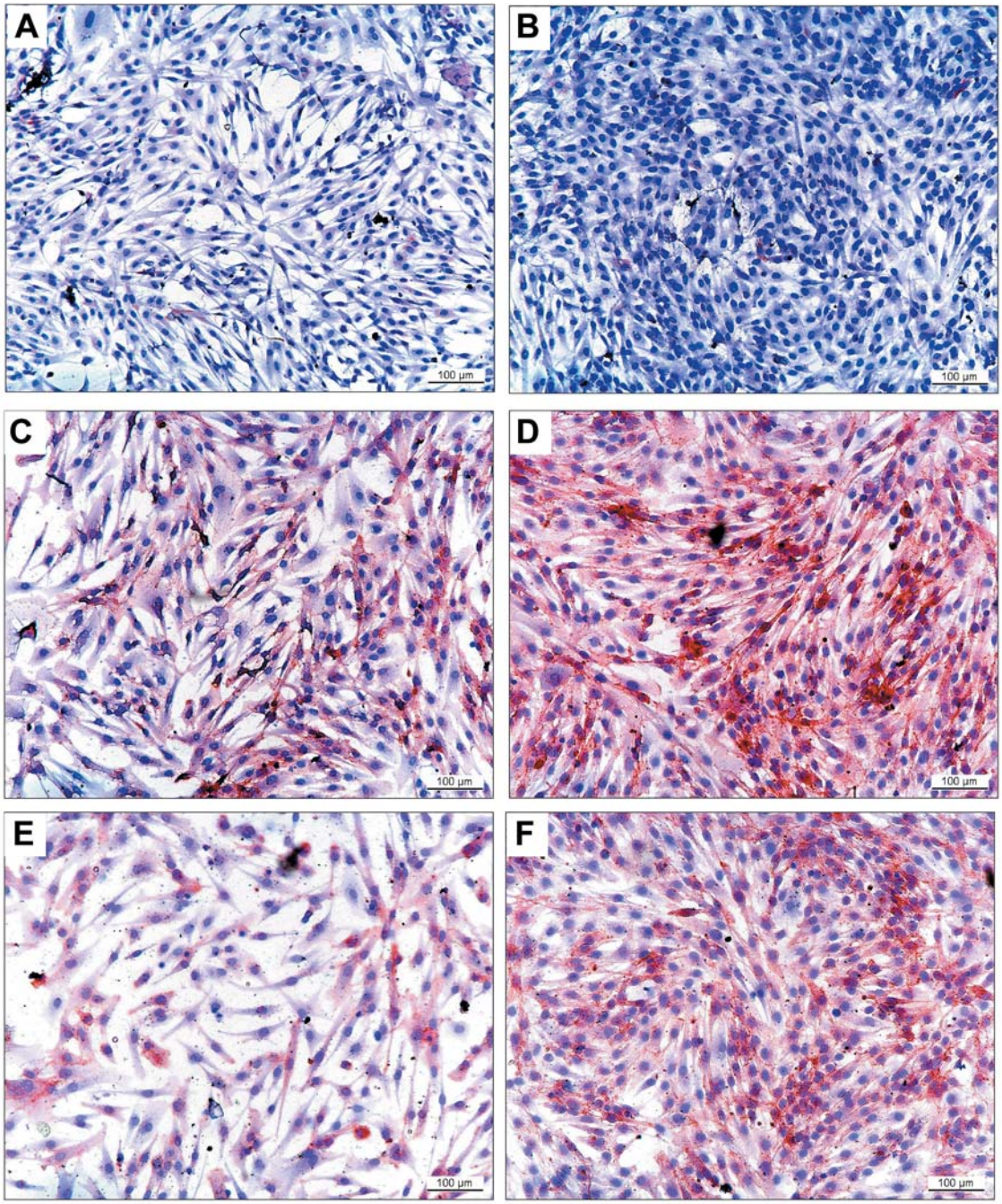

Figure 2. *Immunohistochemical staining against different integrins and ECM molecules after 6 days (left) and 21 days (right) of cell culture. A. Integrin β1 (day 6); B. Integrin β1 (day 21); C. Integrin α3 (day 6); D. Integrin α3 (day 21); E. Integrin-linked kinase (day 6); F. Integrin-linked kinase (day 21).*

21. Hannigan *et al.* isolated a gene that interacts with the cytoplasmic domain of beta-1 integrin. They found that ILK co-immunoprecipitated with beta-1 integrin from cell lysates, and that overexpression of ILK disrupted cell architecture and inhibited adhesion to integrin substrates, while inducing anchorage-independent growth in epithelial cells, suggesting that ILK regulates integrin-mediated signal transduction (12). The expression of this gene suggests that integrin-mediated signalling is involved in the process of dedifferentiation.

In concordance with ILK, ICAP1 and CD47 were up-regulated (13, 14). In conclusion, these three proteins involved in integrin-mediated processes during cell

Table II. *Immunohistochemical detection of integrins and integrin-associated proteins in primary (day 1) and cultured (day 6 and day 21) chrondrocytes.*

	Staining pattern*		
Antibody specificity	day 1	day 6	day21
Integrin β1	+	++	+++
Integrin β3	+	+	+
Integrin β4	±	-	-
Integrin α2	+	+	++
Integrin α3	+++	++	++
Integrin α5	+	++	+
Integrin-linked kinase	++	+	++

*amount of cells stained by the monoclonal antibodies is symbolized by ++++ (80-100%), +++ (60-70%), ++ - +++ (50-60%), ++ (40-50%), + - ++ (30-40%), + (20-30%), ± (<20%), and - for no staining.

adhesion, differentiation and migration were up-regulated during ongoing dedifferentiation in concordance with up-regulation of the key members of the integrin familiy, *e.g.* integrin β1. This substantiates the thesis of integrin-mediated signalling being involved in the process of dedifferentiation.

Conclusion

In the present study, the expression patterns of integrins and related ECM-proteins were analyzed. One of the candidates for signal transmission is the fibronectin receptor (integrin α5β1) in conjunction with its ligand fibronectin. Other receptors, *e.g.* for vitronectin and osteopontin (αVβ3) or laminin (α6β1) or their ligands, do not seem to be involved in signal transmission during dedifferentiation. Somewhat unexpected was the strong expression of the components of the GPIIb/IIIa- receptor, the latter seeming to be involved in the process of dedifferentiation. Intracellularly, ILK, ICAP1 and CD47 might be involved in transduction of the integrin-dependent signals.

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