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# Changes in the Expression of Annexin A5 Gene During In Vitro Chondrocyte Differentiation: Influence of Cell Attachment

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**Abstract** Several lines of evidence indicate that annexin A5, a membrane-associated protein with calcium-channel activity, plays a key role in cartilage calcification during endochondral ossification. As a major constituent of cartilage matrix vesicles, which are released from microvilli of hypertrophic chondrocytes, it is involved in calcium uptake necessary for the initial stages of cartilage calcification. Little is known, however, concerning transcriptional regulation of the annexin A5 gene during chondrocyte differentiation. Here, we report on changes in annexin A5 expression by measuring mRNA and protein levels during in vitro differentiation of chick sternal chondrocytes to the hypertrophic phenotype. Terminal differentiation of mature sternal chondrocytes was achieved in the presence of sodium ascorbate in high-density cultures growing either in monolayer or over agarose as cell aggregates. Differentiation of chondrocytes to hypertrophic cells was followed by morphological analysis and by the onset of type X collagen expression. High expression levels of annexin A5 mRNA were detected in chondrocytes freshly isolated from the sterna by enzymatic digestion and subsequently in cells growing in monolayer, but annexin A5 gene transcription was rapidly downregulated when cells were grown in suspension as aggregates over agarose. However, protein levels did not decrease probably due to its low turnover rate. In suspension culture, annexin A5 mRNA reappeared after 3 weeks concomitantly with segregation of the aggregates into single cells and onset of chondrocyte hypertrophy. The downregulation of annexin A5 expression in cells growing as matrix-rich aggregates was reverted when extracellular matrix components were removed and cells were reseeded onto tissue culture plastic, suggesting that cell spreading, formation of focal contacts and stress fibers stimulated annexin A5 expression in proliferating as well as in hypertrophic chondrocytes. J. Cell. Biochem. 84: 132–142, 2002. 

**Key words:** hypertrophic chondrocyte; chicken sterna; extracellular matrix; collagen

Annexin A5 has been originally isolated and characterized as a collagen-binding protein in chicken chondrocytes [Mollenhauer and von der Mark, 1983; Mollenhauer et al., 1984] and was later identified as a member of the annexin gene superfamily by its homology to annexin A5 from human origin [Fernández et al., 1988; Pfäffle et al., 1988]. At least 160 unique annexin proteins belonging to this family in more than 65 different species have been identified and have been grouped under the term "annexins" that refers to the ability of these proteins to interact in a calcium-dependent manner with biological membranes [for review, see Gerke and Moss, 1997]. A characteristic feature of all annexins is a protein core composed by 4 or 8 structurally homologous repeats, each of them containing the endonexin fold calcium binding motif [Swairjo and Seaton, 1994]. The N-terminal extensions of these proteins are however highly variable in length and sequence, indicating that they are probably responsible for the specific functions of each individual annexin and involved in the regulation of the membrane-associated functions

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[Smith and Moss, 1994; Swairjo and Seaton, 1994]. Many in vitro activities have been described for annexins, but conclusive data regarding their in vivo function are still missing [reviewed in Raynal and Pollard, 1994; Gerke and Moss, 1997]. Various lines of evidence indicate that annexins are involved in regulation of $Ca^{2+}$- and phospholipid-dependent protein kinase C activity, in cell growth and differentiation signaling, in cell-extracellular matrix interaction, as well as in tumor progression and malignancy.

Annexin A5 is one of the best-characterized members of this family of proteins. It is present predominantly in the cytoplasm, but has been also detected in the nucleus and on the outer surface of cells such as chondrocytes and fibroblasts [Pfäffle et al., 1988]. Although annexins lack signal peptide sequences, annexin A5 may be released from cells by membrane inversion and bind to the cell surface of apoptotic cells owing to its high affinity for phosphatidylserine [Martin et al., 1995; Verhoven et al., 1995].

Most remarkably, annexin A5 exerts a voltage dependent calcium channel activity when reconstituted into lipid vesicles [Rojas et al., 1990; Berendes et al., 1993]. Since annexin A5 binds specifically to native collagens II and X [Kirsch et al., 1994; Turnay et al., 1995; von der Mark and Mollenhauer, 1997], a model was developed proposing that these properties may be of particular relevance for the role of matrix vesicles in cartilage calcification. Matrix vesicles, which are released from hypertrophic chondrocytes in the cartilage growth plate, are rich in annexin A5 and alkaline phosphatase among other proteins [Anderson, 1995]. These vesicles act as nucleation cores for calcification, with annexin A5 as the major channel responsible for the calcium uptake. Binding of collagens II and X to annexin A5 not only contributes to the anchoring of matrix vesicles in the hypertrophic cartilage zone but also increases its calcium channel activity [Genge et al., 1989; Wu et al., 1991; Kirsch et al., 1994, 2000a,b,c].

Annexin A5 has been shown to be developmentally regulated. In the chick embryo it appears in development in sites of otic vesicle formation, in the anterior half of somites and in other chondrogenic regions, but its expression decreases before the onset of differentiation [Hofmann et al., 1992]. However, it reappears in the calcification zone of hypertrophic cartilage, both in hypertrophic chondrocytes and in osteoblasts as detected by in situ hybridization and by immunohistochemistry [Hofmann et al., 1992; Kirsch et al., 1997]. We have previously reported on the structure of the chicken annexin A5 gene and shown that the promoter region lacks the typical TATA-box but contains a GC-rich region, with multiple Sp1 sites typical of housekeeping genes which may possibly promote transcription from several start sites [Pfannmüller et al., 1993]. Little is still known, however, on the regulatory mechanisms of annexin gene expression and on its regulation by growth stimuli, such as the *c-fos* protooncogene [Braselmann et al., 1992], cell differentiation degree [Hofmann et al., 1992; Kirsch et al., 1997], or even in cartilage diseases such as osteoarthritis [Mollenhauer et al., 1999; Kirsch et al., 2000b]. For this reason, we have studied the expression of annexin A5 at the mRNA and protein levels in chicken chondrocytes during the in vitro induction of differentiation to the hypertrophic phenotype in order to establish a model system for studies on the regulation of the chicken annexin A5 gene expression.

## MATERIALS AND METHODS

### Chondrocyte Isolation

Sterna from 16-day-old chicken embryos were surgically excised and rinsed in cold phosphate buffered saline (PBS; 0.15 M NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$, pH 7.4). After removing the perichondria with a scalpel, the cephalic-third portions were dissected and discarded. Chondrocytes were obtained from the permanent cartilaginous caudal regions after digestion with 1 mg/ml trypsin (Boehringer Mannheim, Germany) in PBS for 1 h at 37°C followed by decantation, washing with cold PBS, and further digestion with 1 mg/ml collagenase P (Boehringer Mannheim) in complete culture medium (Ham's F12, containing 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum). Incubation was carried out under gentle shaking at 37°C until almost total disgregation of the tissue was observed. Dissociation of the cells was helped by repeated pipetting; after dilution with PBS, the suspension was filtered through a sterile nylon sieve (45 μm) and released chondrocytes were harvested by low-speed centrifugation and resuspended in complete medium containing

50 μg/ml sodium ascorbate (Sigma, St. Louis, MO).

### Chondrocyte Cell Culture

Cells were plated at a density of $2 \times 10^7$/10-cm diameter dish (high-density) and incubated at 37°C in a humidified atmosphere of 95% air/5% $CO_2$. After 4 days growing attached to plastic, chondrocytes were passaged by trypsin digestion (0.05%, w/v) in PBS containing 0.02% (w/v) EDTA. Part of the cells were reseeded on plastic and the rest were transferred to dishes coated with a thin layer of 1% (w/v) agarose in PBS, both types of culture at high-density. Culture medium was changed every second day, always containing fresh ascorbic acid at 50 μg/ml. Unattached cells were recovered by low-speed centrifugation and reseeded in the same dishes.

Segregation of chondrocyte aggregates formed after 1 week of culture on agarose was performed, after washing twice with PBS, by digestion with 1 mg/ml trypsin plus 1 mg/ml collagenase P for 30 min at 37°C. After addition of complete medium and centrifugation, a further digestion with 2 mg/ml collagenase P for 2 h at 37°C in complete medium was performed, followed by centrifugation, washing with PBS, and digestion for 30 min at 37°C with 1 mg/ml hyaluronidase (Boehringer Mannheim). After this treatment, no cell aggregates were visible and cells were sedimented, resuspended in complete medium containing 50 μg/ml ascorbate, and reseeded on plastic dishes at a density of $10^7$/10-cm diameter dish.

### RNA Extraction and Northern Blot Analysis

Total RNA was extracted from high-density adherent or nonadherent chondrocyte cultures by ultracentrifugation of guanidinium thiocyanate lysates through a CsCl cushion [Sambrook et al., 1989]. Northern blot analyses were carried out after electrophoresis of 10 μg total RNA in 1% (w/v) agarose gels containing formaldehyde and vacuum blotting onto Hybond N membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). cDNA probes were radiolabeled by random priming using the DNA labeling beads kit (Amersham Pharmacia Biotech) and [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech). Hybridization was carried out overnight at 42°C in the presence of 50% (v/v) formamide. Filters were washed twice in $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 5 min at room temperature, 3 times in $2 \times$ SSC containing 0.1% (w/v) SDS at 65°C for 15 min, and finally twice in $0.1 \times$ SSC containing 0.1% SDS for 2 min at room temperature. Filters were reused with different probes after washing the membranes with 0.1% SDS at 90°C until no radioactive probe was left on the filters (checked by autoradiography). Each hybridized filter was exposed for different lengths of time and only exposures in the proportional range of autoradiographic film darkening were considered for analysis. Autoradiograhs were scanned and the densitometric analysis of the gels was performed obtaining volumograms on a photodocumentation system from UVItec (Cambridge, UK) and by using the UVIBand V.97 software. The cDNA probe for chicken annexin A5 (pACII) was obtained in our laboratory [Turnay et al., 1995]; that for the chicken $\alpha$1 type X [SPLX; LuValle et al., 1988], and $\alpha$1 type II [pCS1; Young et al., 1984] collagen chains were kindly provided by Dr. P. LuValle (Department of Biochemistry and Molecular Biology, University of Calgary, Canada) and Dr. M.E. Sobel (National Cancer Institute, Bethesda, MD), respectively. Normalization was performed against hybridization signals using a chicken GAPDH probe (a gift from Dr. B. Trueb; M.E. Müller Institute, University of Bern, Bern, Switzerland). The cDNA probes were excised from the above mentioned plasmids by digestion with appropriate restriction enzymes and purified from agarose gels: pACII (BamHI-HindIII; ~2.2 kb), SPLX (EcoRI-HindIII; ~2,4 kb), pCS1 (PstI-PstI; ~0.7 kb), and pGAPDH (EcoRI-EcoRI; ~0.8 kb).

### Western Blot Analysis of Total Protein Extracts

Chondrocytes were collected under identical culture conditions to those described for the mRNA analyses. After washing twice with PBS, cells were homogenised in 10 mM Tris, pH 8.0, containing 140 mM NaCl, 2% (v/v) Triton X-100, 1 mM NEM, and 1 mM PMSF by repeated passaging of the cell suspension through a 25 gauge needle. Cell debris were removed by centrifugation at 13,000*g* for 15 min at 4°C and the supernatants were stored at −20°C until used. Protein concentration was determined using the detergent-compatible (DC) Protein Assay kit from Bio-Rad (Hercules, CA). PAGE–SDS was performed according to

Laemmli [1970] in 10% resolving gels and 20 μg total protein were loaded into each lane. Afterwards, the gels were either Coomassie blue stained or transferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech). After blocking in 5% (w/v) low-fat milk powder in PBS for 1 h at room temperature, the membranes were incubated for 1 h at room temperature with a 1:8,000 dilution of rabbit anti-chicken annexin A5 (obtained in our laboratory) in PBS containing 2% (w/v) low-fat milk powder, 0.05% (v/v) Tween-20, and 0.02% (w/v) sodium azide. After exhaustive washing with PBS/Tween-20, the blots were incubated for 1 h at room temperature with goat anti-rabbit IgG (Bio-Rad) conjugated with horseradish peroxidase (1:5,000 dilution), further washed with PBS/Tween-20, and finally with 50 mM Tris, pH 7.4, containing 0.5 M NaCl. The membranes were then immersed in ECL reagents (Amersham Pharmacia Biotech) and films (Hyperfilm ECL, Amersham Pharmacia Biotech) were exposed for different lengths of time in order to obtain exposures in the linear range of darkening. Densitometric analysis of the films was performed as described for the Northern blots. The membranes were always reprobed with a rat monoclonal antibody against constitutive 70 kDa heat shock protein (HSC 70; 1:5,000 dilution; StressGene, Victoria, Canada) after stripping the membranes by incubation in 62.5 mM Tris, pH 6.8, 2% (w/v) SDS, 0.1 M β-mercaptoethanol for 30 min at 50°C followed by reblocking in PBS containing 5% (w/v) milk powder. As secondary antibody, goat anti-rat IgG conjugated with horseradish peroxidase (Pierce, Rockford, IL) was used at a 1:20,000 dilution.

## RESULTS

### Morphology and Growth Characteristics of Chondrocytes

A homogeneous cell population of differentiated non-hypertrophic chondrocytes was prepared from the caudal region of 16 day embryonic chicken sternal cartilage and first grown in monolayer culture. Cells maintained a spherical shape for 12–24 h before spreading. After 4 days in high-density culture on tissue culture plastic, cells were passaged by trypsinization and split into non-adherent cultures growing over agarose and adherent cultures, both at high-density and always in the presence of complete medium containing 50 μg/ml sodium ascorbate.

After 2 days in monolayer culture, chondrocytes revealed a heterogeneous morphology showing spindle-shaped together with polygonal cells (Fig. 1A). At confluency, proliferation of these cells was very slow and declined to almost zero after 1 week in continuous culture; no further passages were performed for the rest of the culture period (4 weeks). The cell shape changed gradually; after 1 week in culture, almost all cells presented a polygonal morphology. After 2 weeks, approximately 40–50% of the cells assumed a swollen appearance (hypertrophy), with a 2 to 3-fold increase in cell diameter (>20 μm) (Fig. 1B), and tended to detach from the culture dish. After 3 weeks, the percentage of hypertrophic cells increased up to about 90% of the total cell population. Cells were almost completely detached at this stage and were maintained under these conditions for an additional week without symptoms of cell death.

On the other hand, cells grown on agarose remained round-shaped for some hours, but after 24 h almost the whole cell population was forming cell clumps that slowly grew in size (Fig. 1C). After 2 weeks on agarose, individual cells with a hypertrophic morphology were released from the cell aggregates, and after 4 weeks, most aggregates had segregated into single cells of hypertrophic morphology. When aggregates obtained after 1 week on agarose-coated dishes were enzymatically digested and cells transferred to plastic, they grew again as monolayer cultures showing a polygonal morphology (Fig. 1D).

### Expression of Type II and Type X Collagen mRNA During In Vitro Differentiation

Total RNA was extracted at different time intervals from cells growing either in monolayer or as aggregates on agarose-coated dishes, and the levels of annexin A5, α1(II), and α1(X) mRNA were analyzed by Northern blot using specific probes (Fig. 2). Signals obtained after densitometric analysis was normalized to GAPDH signals obtained after hybridization of the same blots (Fig. 3).

Collagen type X mRNA expression was zero in freshly obtained chondrocytes and also during the initial 4 days of monolayer culture prior to trypsinization and reseeding (Fig. 2, lanes a,b). Nine days after trypsinization and replating at

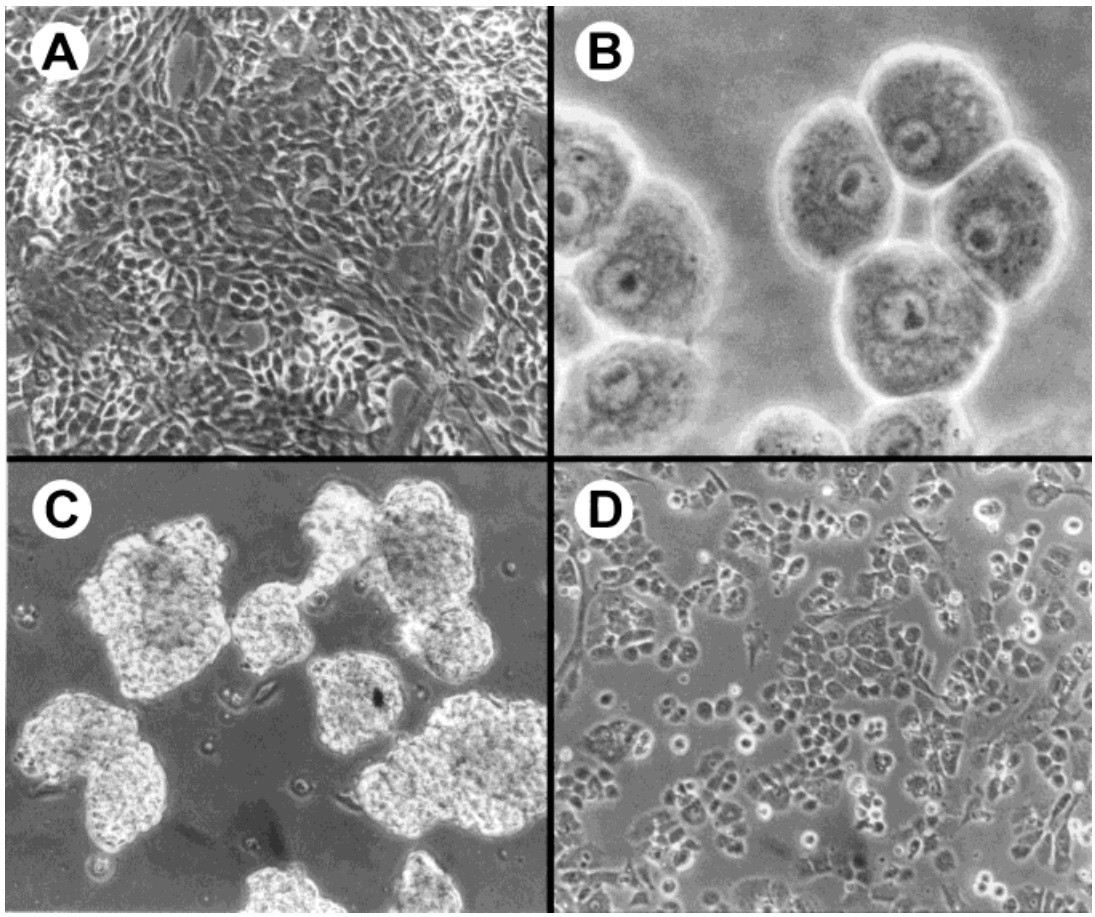


**Fig. 1.** Morphological analysis of cultured sternal chondrocytes. Freshly isolated chondrocytes derived from the caudal region of chicken embryo sterna were plated first on plastic, then trypsinized after 4 days incubation, and finally transferred to plastic or agarose-coated dishes. Micrographs show chondrocytes 4 days after reseeding on plastic (**A**, ×100) or on agarose (**C**, ×100). Morphologically hypertrophic chondrocytes appeared eventually in both types of culture maintained constantly in the presence of sodium ascorbate (**B**, ×400). Cell aggregates formed after incubation during 1 week on agarose-coated dishes were disrupted enzymatically, as described in Materials and Methods, and chondrocytes were transferred to plastic where they grew again as monolayer cultures (**D**, 48 h after disgregation, ×100).

high-density, type X collagen mRNA was detected, reaching highest expression levels after 4 weeks (Fig. 2, lanes g,h; Fig. 3). In contrast to type X collagen, type II collagen mRNA was strongly expressed in freshly isolated chondrocytes with almost identical levels after 4 days in monolayer culture. Nine days after reseeding on plastic, a 1.7-fold increase in collagen type II mRNA levels was observed, decreasing slightly after 4 weeks (Figs. 2 and 3).

The expression of both collagen types was also analyzed in chondrocytes grown as aggregates over agarose in the presence of sodium ascorbate. Collagen type X transcription was negligible 1 week after seeding (Fig. 2, lane c); at this stage, chondrocytes were forming aggregates but no single hypertrophic cells were observed. Cells showing hypertrophy appeared after 2 weeks in culture increasing their number thereafter. Two weeks after seeding α1(X) mRNA was first detected, with increasing levels until four weeks (Fig. 2, lanes d,e,f; Fig. 3). Final α1(X) mRNA levels were, however, always lower than those observed in cells growing and differentiating in monolayer (Fig. 2, lane h). The opposite occurred with expression of type II collagen. While transcriptional levels were relatively high in freshly obtained chondrocytes, expression of this collagen type decreased with time in attachment-independent culture (Figs. 2 and 3).

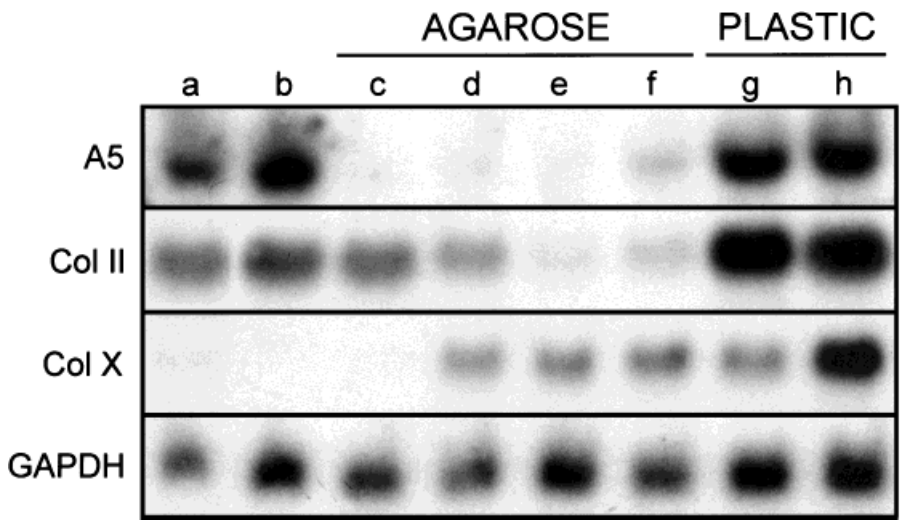


**Fig. 2.** Northern blot analysis of total RNA extracted from adherent and non-adherent chondrocyte cultures. Total RNA (10 μg) was prepared from freshly isolated chondrocytes (**lane a**), from cells grown 4 days in monolayer (**lane b**), and from cells growing as aggregates for 1, 2, 3, and 4 weeks on agarose-coated dishes (**lanes c**, **d**, **e**, and **f**, respectively) or in monolayer for 13 and 31 days (**lanes g** and **h**, respectively) and separated on formaldehyde-containing 1% agarose gels. Specific probes for chicken annexin A5, α1(II), and α1(X) collagen chains (Col II and Col X), and GAPDH were used for hybridization. Unique transcripts of ~1.7, ~5, ~2.4, and ~1.3 kb were detected for annexin A5, α1(II), α1(X), and GAPDH, respectively. Only the corresponding portions of the hybridized blots from a representative experiment are shown.

### Annexin A5 mRNA Levels During In Vitro Differentiation and Attachment-Dependent Transcriptional Regulation

Annexin A5 mRNA, which appeared in all the experiments as a unique transcript of around 1.6–1.7 kb, was strongly expressed in freshly isolated chondrocytes obtained by enzymatic digestion of the sterna (Figs. 2 and 3). High mRNA levels were maintained after 4 days growing in monolayer (Fig. 2, lanes a,b). When cells were allowed to differentiate in monolayer, annexin A5 mRNA levels gradually decreased; 9 days after reseeding on plastic, when type X collagen expression was first observed and morphologically hypertrophic cells were present, annexin A5 mRNA levels decreased by about 30% (Fig. 3). When cells were almost completely hypertrophic and collagen type X expression was maximal (4 weeks), annexin A5 mRNA levels did not increase but further declined (Fig. 3).

A different pattern of expression was detected when differentiation was induced in cells growing on agarose as aggregates. Attachment-independent growth of chicken chondrocytes induced an almost complete downregulation of annexin A5 mRNA (Figs. 2 and 3); after 1 week annexin A5 mRNA levels were not detectable under these experimental conditions. After 3 weeks, however, when terminal differentiation had taken place in the majority of chondrocytes, a moderate rise in annexin A5 mRNA was observed (Fig. 2, lane f; Fig. 3). However, mRNA levels were significantly lower than those observed in freshly isolated, attached chondrocytes.

In order to verify whether the downregulation of annexin A5 was dependent on the growth conditions, cells were first grown for 1 week on agarose forming aggregates until annexin A5 mRNA expression was almost completely suppressed (Fig. 4). Then, cell aggregates were dissociated by a combination of trypsin, bacterial collagenase, and hyaluronidase digestions, and reseeded on tissue culture plastic in complete medium with sodium ascorbate. Following replating into monolayer culture, cells rapidly attached and spread, assuming the polygonal morphology typical of differentiated chondrocytes (Fig. 1D). Under these attachment-dependent culture conditions, annexin A5 expression was rapidly reinitiated (Fig. 4, lanes g,h). Six days after replating, annexin A5 mRNA levels reached again to those of freshly isolated cells. At this time point, no hypertrophic cells and no type X collagen expression could be observed.

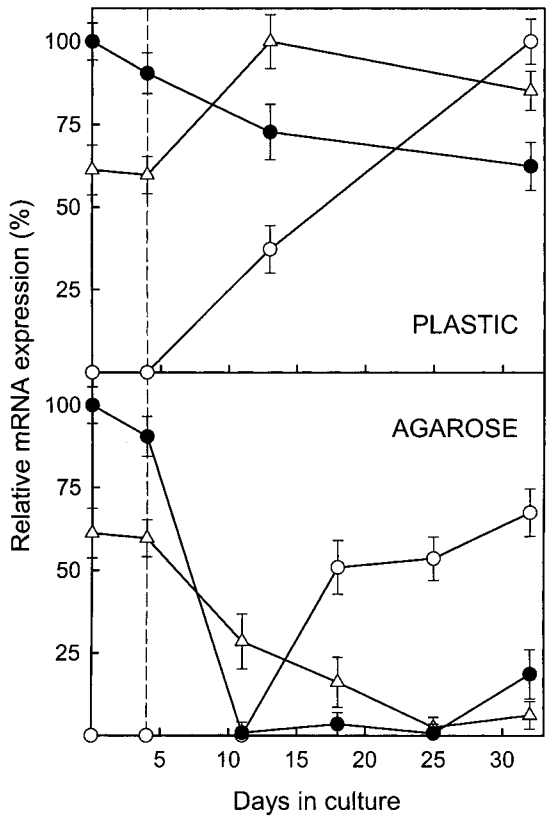


**Fig. 3.** Normalized levels of mRNA for annexin A5 and type II and type X collagens during in vitro chicken chondrocyte terminal differentiation. Blots from Figure 2 were analyzed by densitometry, and the results normalized for the GAPDH signal. Freshly isolated chondrocytes were first grown for 4 days in monolayer, trypsinized (vertical dashed-line), and transferred to tissue culture (plastic) or agarose-coated dishes. The changes in the mRNA levels of chicken annexin A5 (●), α1(II) (Δ), and α1(X) (○) were monitored with time in culture. Values are expressed as percentage of the highest normalized value reached for each mRNA and represent the mean (± SD) of five different independent experiments.

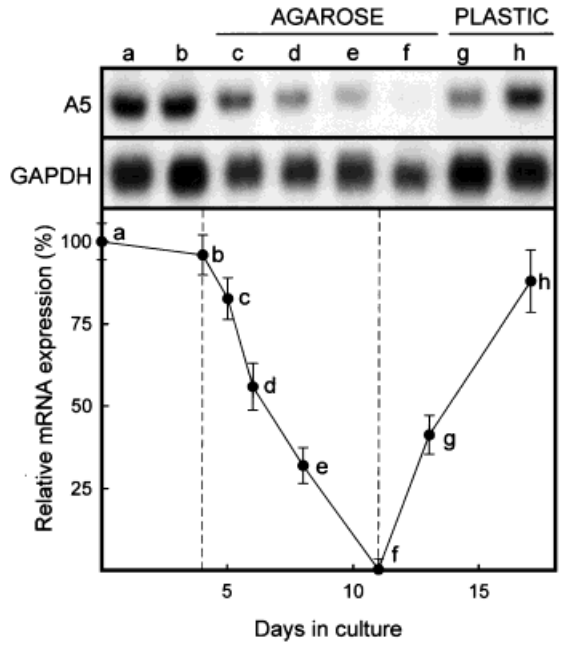


**Fig. 4.** Loss of chicken annexin A5 mRNA expression in suspension culture and reactivation in monolayer conditions. Total RNA (10 μg) from chondrocyte cultures was analyzed by Northern blot as described in Figure 1. Freshly isolated chondrocytes (**lane a**) were grown on monolayer and, after 4 days (**lane b**), were trypsinized and transferred to agarose-coated dishes and incubated under normal culture conditions for 1, 2, 4, and 7 days (**lanes c**, **d**, **e**, and **f**, respectively). Chondrocyte aggregates were then dissociated and cells were seeded on tissue culture dishes for a further 2 or 6 days (**lanes g** and **h**, respectively). The upper part shows the hybridization of a representative membrane using the chicken annexin A5 and GAPDH probes; the lower part shows the results of the normalized densitometric analysis. Data represent the mean (± SD) of five different independent experiments. The first vertical dashed-line indicates the transfer of chondrocytes to agarose-coated dishes, and the second, the time point of dissociation of cell aggregates and replating as monolayer culture.

### Annexin A5 Protein Levels During In Vitro Chondrocyte Culture and Differentiation

Annexin A5 protein content was determined in the different conditions described for the analysis of its mRNA levels. In all cases, the analysis was performed by Western blot of total cell or cell-aggregate associated proteins using specific antibodies against chicken annexin A5. Even though the same amount of protein was loaded into each lane, reprobing of the membranes was always performed using monoclonal antibodies that recognize constitutive chicken HSC 70 in order to normalize the obtained data. In this way, possible artifacts arising from the accumulation of extracellular matrix proteins in the cell aggregates growing on agarose was excluded.

Figure 5 shows the Western blot analysis corresponding to the in vitro induction of chondrocyte differentiation on agar and on monolayer culture under the same experimental conditions as the Northern blot analyses described in Figures 2 and 3. Even though mRNA levels at Day 4 on monolayer are nearly identical to those in freshly obtained chondrocytes, a 25% decrease on the cell-associated annexin A5 protein content was detected, probably due to a partial segregation of annexin A5 to the culture medium. When cell aggregates

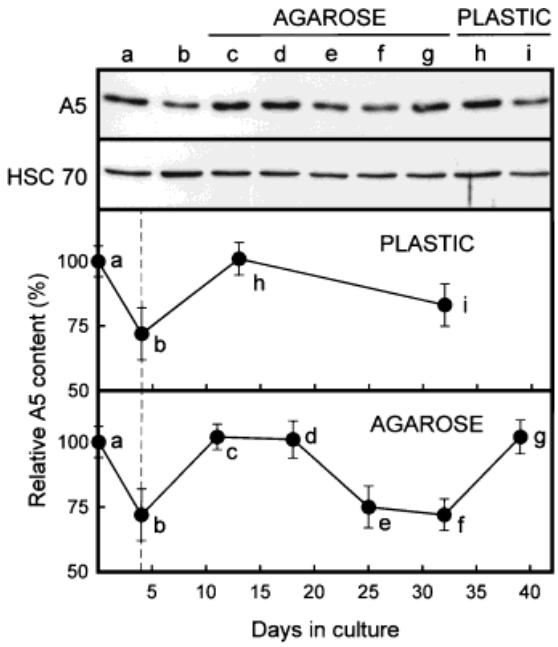


**Fig. 5.** Western blot analysis of annexin A5 protein levels during in vitro chicken chondrocyte differentiation. Cell homogenates from freshly isolated chondrocytes (**lane a**), and from cells growing as aggregates for 1, 2, 3, 4, and 5 weeks on agarose coated dishes (**lanes c, d, e, f,** and **g,** respectively) or in monolayer for 13 and 31 days (**lanes h** and **i**, respectively) were obtained as described in Methods. The same amount of protein (20 μg) was loaded into each lane and PAGE–SDS on 10% polyacrylamide resolving gels was carried out followed by transfer onto nitrocellulose membranes. Immunodetection of annexin A5 and of constitutive HSC 70 was performed as described using specific antibodies. Representative blots are presented in the upper part of the figure. Annexin A5 protein levels were normalized to the HSC 70 signals obtained on the same membrane after reprobing. Data are the mean (± SD) of three independent experiments.

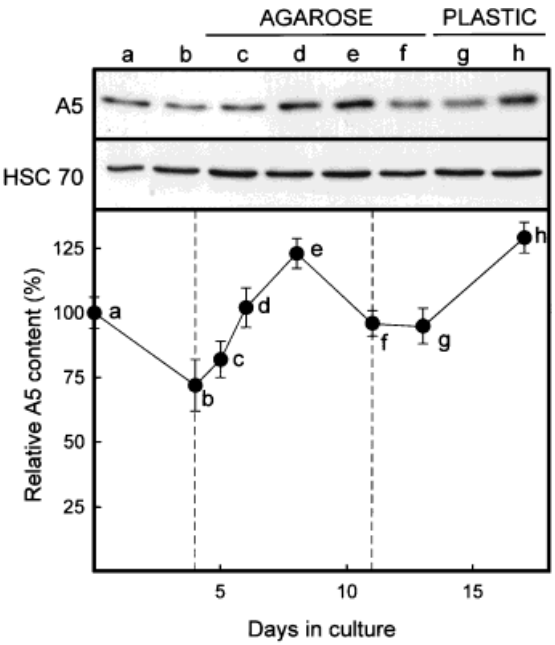


**Fig. 6.** Variation of annexin A5 protein levels in chicken chondrocytes in suspension culture and in monolayer conditions. Annexin A5 protein levels were analyzed by Western blot as described in Figure 5. Culture conditions and lanes correspond to those described in Figure 4: freshly isolated chondrocytes (**a**); cells growing 4 days on monolayer (**b**); cells transferred to agarose-coated dishes and grown in suspension culture for 1 (**c**), 2 (**d**), 4 (**e**), and 7 days (**f**); enzymatically dissociated chondrocyte aggregates grown in monolayer for 2 (**g**) or 6 (**h**) additional days. The upper part shows the signals obtained after immunodetection of annexin A5 or HSC 70 of one representative experiment; the lower part shows the results of the normalized densitometric analysis. The first vertical dashed-line indicates the transfer of chondrocytes to agarose-coated dishes, and the second, the time point of dissociation of cell aggregates and replating as monolayer culture. Data represent the mean (± SD) of three independent experiments.

begin to form on agar, a recovery of the protein levels detected in freshly obtained chondrocytes is observed after 1 week even though at this time mRNA levels are negligible. After 3 weeks on agar, a decrease in protein levels appears as a consequence of the complete blocking of annexin A5 transcription detected at 1 week on attachment independent conditions. Only after 5 weeks under these experimental conditions, an increase in annexin A5 is observed probably as a consequence of the slight increase in transcription detected at 4 weeks. When cells are kept in monolayer culture after trypsinization, a recovery in annexin A5 protein levels is detected at 13 days decreasing slightly thereafter.

Figure 6 shows annexin A5 protein levels during the first week of attachment independent culture of chicken sternal chondrocytes followed by enzymatic dissociation of the aggregates and further reseeding on monolayer culture (equivalent to the experiment described on Fig. 4 for the mRNA levels). A gradual increase in annexin A5 protein content up to Day 4 is observed after seeding chondrocytes on agar, decreasing afterwards. Thus, a significant delay between protein synthesis and mRNA transcription must exist for annexin A5. After enzymatic digestion of chondrocyte aggregates and reseeding of cells in plastic, protein content rises again also showing a delay when compared to mRNA levels.

## DISCUSSION

In this study we show that expression of annexin A5, a major constituent of chondrocyte membranes, is regulated in vitro at two

different levels. First, at developmental level, annexin A5 expression is downregulated with differentiation from proliferating chondrocytes to hypertrophic cells, a process running over 1–4 weeks in chicken chondrocyte cultures both in monolayer or in suspension cultures. Only in the last phase of hypertrophic differentiation in agarose cultures annexin A5 expression was reinitiated possibly also as a result of cellular heterogeneity developing in the chondrocyte aggregates. Second, at environmental level, annexin A5 expression is highly and immediately upregulated after release of chondrocytes from its extracellular matrix and following attachment and spreading in monolayer culture. This upregulation is reversible, since after transfer from monolayer to suspension culture annexin A5 expression declines to null level.

In the chick embryo, annexin A5 is expressed transiently in various phases and sites of chondrogenesis; it is focally expressed in otic vesicles and in the anterior part of somites in developing limb cartilage, but decreased in differential cartilage [Hofmann et al., 1992]. In situ hybridization data indicate that annexin A5 is again expressed with onset of maturation of chondrocytes in the epiphyseal growth plate [Kirsch et al., 1997], while recent data on annexin A5 expression in percoll-gradient separated growth plate chondrocytes indicate downregulation of annexin A5 expression in terminal differentiated hypertrophic chondrocytes (Schmidl and von der Mark, unpublished observation) which is in line with the findings reported here. Downregulation of annexin A5 mRNA levels in hypertrophic chondrocytes is, however, not in conflict with the presence of significant amounts of annexin A5 protein in the membranes of hypertrophic chondrocytes [Pfäffle et al., 1988, Hofmann et al., 1992, Kirsch et al., 1997] and in matrix vesicles [Genge et al., 1989; Wu et al., 1991], since annexin A5 is a rather stable protein. In fact, we have detected that the protein remains present even though the transcription of its gene may be shut off. Thus, although annexin A5 mRNA levels may be low in hypertrophic chondrocytes, we have observed that the protein persists and thus, may play a major role as calcium channel in hypertrophic chondrocytes and in matrix vesicle membranes. By regulating $Ca^{2+}$ influx into chondrocyte membranes, it may be responsible for the elevated intracellular $Ca^{2+}$ level in hypertrophic chondrocytes [Kirsch et al., 1992] and for the induction of type X collagen by high $Ca^{2+}$ levels [Bonen and Schmid, 1991]. As constituent of matrix vesicles, it promotes calcium influx, regulated by interaction with matrix vesicle-associated type II and type X collagens [Kirsch et al., 1994, 2000a,b,c; von der Mark and Mollenhauer, 1997].

In suspension culture on agarose chicken chondrocytes form aggregates and rebuild a cartilage-like extracellular matrix containing collagen II and aggrecan within a few weeks in the presence of ascorbate [Castagnola et al., 1987, 1988; Tacchetti et al., 1987]. Under these culture conditions, which allow chondrocytes to reassume a spheroidal cell shape and a chondrocyte-typical actin skeleton which consists mostly of a cortex-like cytoskeleton but no stress fibers [Marchisio et al., 1984; Benya et al., 1988], annexin A5 mRNA declines to zero levels within 1 week, even though protein levels are somehow maintained. Onset of hypertrophic differentiation, as indicated by the appearance of $\alpha$1(X) mRNA, commences at the 2nd week [see also Castagnola et al., 1988], while low levels of annexin A5 mRNA reappeared only after 3–4 weeks.

Enzymatic dissociation of the chondrocyte aggregates formed in suspension and replating of the isolated cells onto tissue culture plastic, however, rapidly stimulated expression of annexin A5 mRNA to levels comparable to those seen in chondrocytes freshly liberated from cartilage tissue. Annexin A5 protein levels increased with a delay after the increase of mRNA. This indicates that annexin A5 expression is upregulated first by the loss of the extracellular cartilage matrix, and second, as a result of cell spreading and formation of focal contacts and actin stress fibers. Enforcement of chondrocyte spreading has been shown to initiate also the upregulation of type II collagen and fibronectin [Dessau et al., 1978] and finally induces dramatic changes in the gene expression pattern leading to "dedifferentiation" or modulation of the chondrocyte phenotype to fibroblast-like cells. This is associated with the onset of type I, III, and V collagen expression and the loss of aggrecan and type II collagen expression [Mayne et al., 1976; Benya et al., 1978; for review see Cancedda et al., 1995].

The stimulation of annexin A5 gene expression by cell spreading explains why annexin A5

levels remained at high level in monolayer culture and declined only moderately with time in culture, in contrast to the rapid decrease of annexin A5 after transfer of cells to suspension culture. Protein levels do not decrease, but rather increased after transfer of chondrocytes to suspension culture; this is probably due to the delay in the translation process and to the high stability of this protein. A decrease in protein content is only observed after 2 weeks on agarose. The high levels of adhesion-related annexin A5 mRNA expression may have obscured the moderate increase seen after 3 weeks in suspension culture. Anyway, this increase is reflected in an increase in the protein content after 5 weeks. This indicates that developmentally controlled regulatory signals responsible for downregulation of annexin A5 during chondrocyte differentiation or upregulation in late stages of hypertrophy are by far overrun by strong regulatory signals elicited by cell spreading and focal contact formation.

Whether integrins or other receptors are responsible for these modulation events in chondrocytes in general, and in the upregulation of annexin A5 expression, in particular, remains to be elucidated. It is likely that the activation of the integrin-associated focal adhesion kinase $pp^{125}$FAK [Hanks and Polte, 1997; Cary et al., 1999] triggers onset of annexin A5 gene expression, but further studies will be required to elucidate the specific signaling pathway leading to onset of annexin A5 gene expression following chondrocyte spreading in monolayer culture.

So far little is known on the transcriptional regulation of the annexin A5 gene. Mouse and rat annexin A5 genes present two promoter regions; different transcripts and alternatively spliced mRNA species have been detected [Imai and Kohsaka, 1995; Rodríguez-García et al., 1999]. However, we have only detected a unique transcript for chicken annexin A5; moreover, the analysis of its gene revealed only one promoter and the absence of TATA boxes and other cis-regulatory elements [Pfannmüller et al., 1993; Fernández et al., 1994], suggesting that the annexin A5 gene might be more related to that of a housekeeping gene. Here we show for the first time that at least two regulatory mechanisms exist which control annexin A5 expression in a developmental and an environmentally regulated manner.

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