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Inhibition of Terminal Differentiation and Matrix Calcification in Cultured Avian Growth Plate Chondrocytes by Rous Sarcoma Virus Transformation

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Abstract Endochondral bone formation involves the progression of epiphyseal growth plate chondrocytes through a sequence of developmental stages which include proliferation, differentiation, hypertrophy, and matrix calcification. To study this highly coordinated process, we infected growth plate chondrocytes with Rous sarcoma virus (RSV) and studied the effects of RSV transformation on cell proliferation, differentiation, matrix synthesis, and mineralization. The RSV-transformed chondrocytes exhibited a distinct bipolar, fibroblast-like morphology, while the mock-infected chondrocytes had a typical polygonal morphology. The RSV-transformed chondrocytes actively synthesized extracellular matrix proteins consisting mainly of type I collagen and fibronectin. RSV-transformed cells produced much less type X collagen than was produced by mock-transformed cells. There also was a significant reduction of proteoglycan levels secreted in both the cell-matrix layer and culture media from RSV-transformed chondrocytes. RSV-transformed chondrocytes expressed two- to- threefold more matrix metalloproteinase, while expressing only one-half to one-third of the alkaline phosphatase activity of mock infected cells. Finally, RSV-transformed chondrocytes failed to calcify the extracellular matrix, while mock-transformed cells deposited high levels of calcium and phosphate into their extracellular matrix. These results collectively indicate that RSV transformation disrupts the preprogrammed differentiation pattern of growth plate chondrocytes and inhibit chondrocyte terminal differentiation and mineralization. They also suggest that the expression of extracellular matrix proteins, type II and type X collagens, and the cartilage proteoglycans are important for chondrocyte terminal differentiation and matrix calcification. J. Cell. Biochem. 69:453–462, 1998.

Key words: Rous sarcoma virus; chondrocytes; matrix calcification

During vertebrate skeletal development, the epiphyseal growth plate is the primary site of longitudinal bone growth and de novo mineralization [Hunziker, 1994]. During this process, a tightly regulated developmental cascade causes the growth plate chondrocytes to proliferate, differentiate, become hypertrophic, and, through the release of matrix vesicles, initiate the calcification of the extracellular matrix [Wuthier, 1982]. The expression of alkaline phosphatase is a marker for chondrocyte differentiation. Localized differences in the distribution of extracellular matrix proteins is another indication of the developmental stage of the chondrocytes. For example, type X collagen is a typically found in the hypertrophic cartilage region of the growth plate [Petit et al., 1992]. Chondrocytes also abundantly express type II collagen and proteoglycans. The importance of these extracellular matrix proteins in skeletal development is illustrated by genetic studies showing that mutations in types II and X collagens lead to a variety of chondrodysplasias [Olsen, 1995].

Our laboratory has established an in vitro culture model that mimics the terminal differentiation and calcification process of growth plate chondrocytes [Wu et al., 1989, 1995]. In

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this culture system, the normal developmental progression of chondrocytes is achieved by culturing cells in serum-containing DATP-5 medium that contains physiological levels of amino acids and electrolytes, supplemented with ascorbic acid [Ishikawa and Wuthier, 1992]. Extensive biochemical and morphological studies have indicated that growth plate chondrocytes cultured in DATP-5 medium express alkaline phosphatase (ALP) activity, synthesize a proteoglycan-rich matrix containing type II and type X collagens, and produce abundant extracellular calcium phosphate mineral deposits [Wu et al., 1995, 1997a].

Rous sarcoma virus (RSV) infection of embryonic chondrocytes results in a tumorigenic transformation of cells, including profound changes in cell morphology and extracellular matrix (ECM) synthesis. These changes include a switch from type II to type I in collagen synthesis by RSV-transformed chondrocytes [Gionti et al., 1983; Allebach et al., 1985] and the down-regulation of proteoglycan synthesis [Muto et al., 1977; Pacifici et al., 1977]. While these studies have illustrated phenotypic changes in the expression of ECM, the effects of RSV transformation on chondrocyte terminal differentiation and calcification have not been studied. It is of interest, therefore, to study the effect of RSV transformation on growth plate chondrocytes in terms of their biological endpoint (i.e., matrix calcification). This study illustrates that alteration of the normal chondrocyte developmental pathway by RSV transformation prevents terminal differentiation and mineralization of the ECM.

MATERIALS AND METHODS

Isolation of GP Chondrocytes

Epiphyseal growth plate chondrocytes were obtained from the tibiae of 6- to 8-week-old broiler chickens as described by Wu et al. [1995]. Briefly, small blocks of cartilage tissue were collected in a synthetic cartilage lymph (SCL) and digested in 1% trypsin solution (prepared in SCL) for 15–20 min at 37°C. After trypsin digestion, the trypsin solution was removed and 10 ml of SCL and 0.5 ml of 0.78% collagenase solution were added to the tissue slices. After 30 min of collagenase/trypsin digestion at 34°C, the liquid was removed from the slices and replaced with 0.5 ml of 0.78% collagenase solution plus 15 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The collagenase-DMEM solution was removed from the cartilage digest after 15 hours of incubation at 34°C. The chondrocytes were released from the partially digested residual tissues by vortexing 3 times (1 min each time) in fresh medium. Isolated cells were demineralized in iso-osmotic citrate buffer (8.0 mM citric acid in 80.5 mM sodium citrate, pH 6.0) for 10 min at 37°C, washed with fresh medium, resuspended in DMEM containing 10% FBS, and counted using a hemocytometer. Cell viability was determined by trypan blue exclusion. Cells were plated at 3–4 $\times$ 10^5 cells per 35-mm dish in 2 ml of medium. The cells were initially cultured in DMEM with 10% FBS and 1% penicillin/streptomycin solution. From day 6 onward, cells were cultured in DATP-5 medium [Ishikawa and Wuthier, 1992]. The culture medium was changed every 2–3 days. Ascorbate was provided at a concentration of 50 mg/ml starting from day 3.

Transformation of Chondrocytes Using Rous Sarcoma Virus

RSV A (ATCC VR-724) was purchased from American Type Culture Collection (ATCC) (Rockville, MD). Three days after initiating chondrocyte cultures, the cells were washed with serum-free DMEM three times prior to infection of the cultures with RSV stocks at a multiplicity of infection of two focus-forming units per cell. After 4 h incubation in serum-free media, FBS was added to the final concentration of 10% and the cultures were continued as described as above. RSV-transformed chondrocytes appear spindle-shaped, and this hallmark was used to monitor the process of RSV transformation [Okayama et al., 1977; Gionti et al., 1983]. To ensure complete transformation of all cells in treated dishes, both normal control cells and RSV-treated cells were passaged once on day 7, using EDTA-0.05% trypsin. The passaged cells were plated at 0.2 million per dish in DMEM-10%FBS and then cultured as described previously above.

Proline Incorporation Analysis

Three days after plating the cells, the culture media was replaced with an equal volume of DMEM/10% FBS containing 1 mCi/ml ^{3}H-proline. After 16 h, the labeled media was removed and proteins precipitated with 10% tri-

chloroacetic acid (TCA). After washing three times with cold 10% TCA, the protein pellets were resuspended in 0.5 ml phosphate-buffered saline (PBS), and 0.1-ml aliquots were counted for radioactivity. To analyze the incorporation of ^{3}H-proline into the cell-matrix layer, the cell-matrix layer remaining in the 35-mm culture dishes after removal of the labeled media was washed 3 times with ice-cold PBS and lysed in 0.5 ml of TMT (10 mM Tris, pH 7.5; 0.5 mM $MgCl_2$; 0.05% Triton X-100). Aliquots of 0.1 ml were added to 5 ml of scintillation cocktail and radioactivity was counted using a Wallac 1409 counter.

Cell Harvesting and Analyses

At selected time points, the cell-matrix layers were harvested by removing the medium, washing twice with 1 ml of TMS (50 mM Tris, pH 7.5; 1.5 mM $MgCl_2$; 10% sucrose) buffer, and scraping into 4 ml of TMS buffer. After centrifugation at 2,000 rpm for 25 min in a clinical centrifuge, the cell pellets were frozen under 1 ml of TMT buffer. Aliquots of the culture media were also frozen at −80°C. When ready for analyses, the pellets were thawed and resuspended by sonication. Samples were analyzed for protein levels by the method of Lowry et al. [1951] using bovine serum albumin (BSA) as the standard, and for alkaline phosphatase activity using p-nitrophenylphosphate as a substrate, as described in Nie et al. [1995]. To analyze the levels of proteoglycan in the cell monolayer and the medium, aliquots (20 ml for TMT sonicate, or 10 ml for medium) were mixed with 250 ml of dimethylmethylene blue reagent and the absorbency difference at 595 and 520 nm was measured [Chandrasekhar et al., 1987] and converted to proteoglycan concentrations using chondroitin sulfate (Sigma, St. Louis, MO) as a standard. Cellular DNA levels were determined using bis-benzimidazole (Hoechst 33258) with aliquots of the TMT cell sonicate; the DNA standard curve was constructed using the same level of TMT vehicle [Rago et al., 1990]. After the biochemical assays, the TMT sonicate was re-pelleted by centrifugation (2,000 rpm, 30 min), the supernatant was removed, and the pellet was digested overnight at room temperature in 1 ml of 0.1 N HCl. Calcium and phosphate levels in the acidic extract were measured spectrophotometrically as previously described [Nie et al., 1995].

Gel and Immunoblot Analyses of Proteins

Medium or cell-matrix layer proteins were analyzed by 8% acrylamide, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis [Laemmli, 1970]. Immunoblot analysis of type X collagen was carried out after SDS-PAGE by the method of Towbin and Gordon [1984]. Proteins were transferred from the gels onto nitrocellulose membranes, and probed with anti-chicken type X collagen monoclonal antibody (a gift from Dr. G. Balian, University of Virginia).

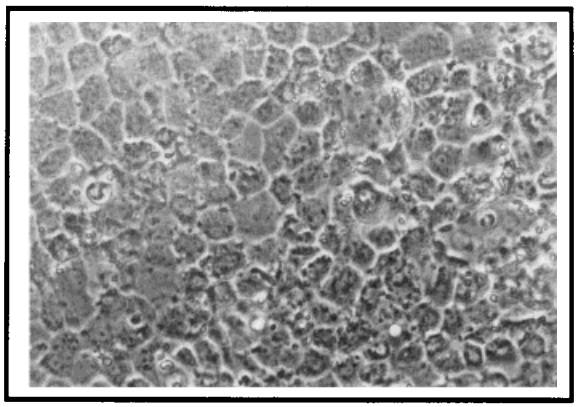
Zymogram Analysis of MMP-2 Expression

Proteolytic activity in the culture medium was assayed by a modification of the method of Kleiner and Stetler-Stevenson [1994]. In brief, 8% acrylamide gels containing 0.2% gelatin were used. After electrophoresis, gels were washed twice with 2.5% Triton X-100 for 30 min with gentle shaking and incubated in 1% Triton X-100, 50 mM Tris, 5 mM $CaCl_2$, 0.02% sodium azide, pH 8.0, at 37°C for 18 h. Gels were stained with Coomassie Brilliant Blue R-250. The presence of protease activity was revealed as negative bands against a blue background.

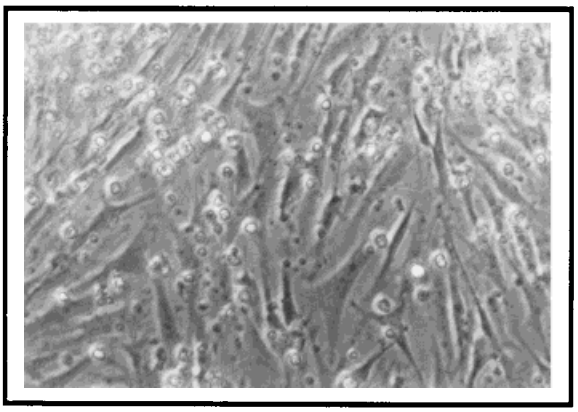
RESULTS

RSV Transformation of GP Chondrocytes and Its Effect on Cell Growth

Epiphyseal growth plate chondrocytes, upon infection by RSV, changed morphology from a polygonal to a bipolar shape (Fig. 1). The change of morphology was similar to that observed in RSV-transformed chicken embryo chondrocytes [Okayama et al., 1977; Gionti et al., 1983; Pacifici et al., 1977]. Mock-transformed and RSV-transformed chondrocytes were passaged once after release with trypsin-EDTA to achieve complete transformation. Passaging facilitated complete transformation of the RSV-exposed cells but did not alter the polygonal morphology of the normal mock-transformed cells. Transformation by RSV stimulated chondrocyte growth at early stages; however, at later stages, mock-transformed chondrocytes grew better than the RSV-transformed cells as indicated by their higher protein and DNA levels (Fig. 2A,B). Upon achieving confluency, both chondrocyte phenotypes continued to grow forming multi-layered nodules.



A Non-Transformed



B RSV-Transformed

Fig. 1. Normal (mock-transformed) and RSV-transformed growth plate chondrocyte morphology 3 days after passaging. **A:** Normal polygonal chondrocytes ×100. **B:** RSV-transformed GP chondrocytes. ×100.

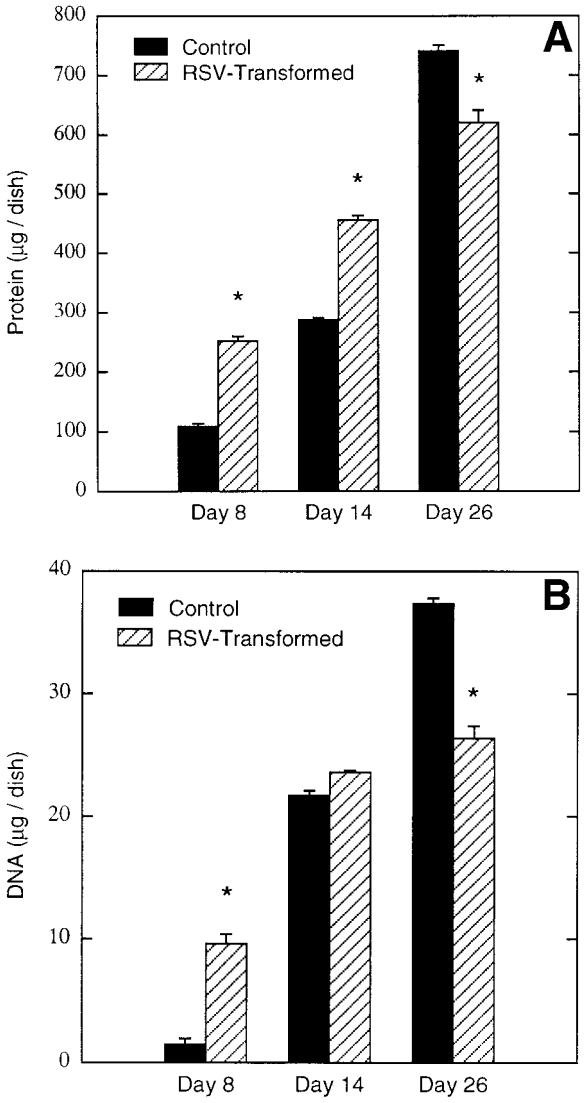


Fig. 2. Effect of RSV transformation on cell protein and DNA content. Mock-transformed (control) and RSV-transformed chondrocytes were seeded into 35-mm petri dishes at 0.2 million cells per dish. At the indicated time intervals, cells were harvested and analyzed. **A:** Cell-matrix total protein; **B:** DNA content. Asterisks (*), values significantly different from that of control; $P < 0.01$.

Effect of RSV Transformation on Proteoglycan Levels

RSV transformation of growth plate chondrocytes dramatically reduced the levels of proteoglycan in both the cell-matrix layer and the culture media. The levels of proteoglycan in mock-transformed chondrocyte cell-matrix layers increased steadily (Fig. 3A), whereas in RSV-transformed cells the increase was minimal. Transformed cells showed higher levels of proteoglycan in cell layers at early stages of the cultures (day 8). RSV-transformed chondrocytes secreted about one-eighth to one-tenth the normal level of proteoglycans into the media, especially at later, postconfluent stages (day 14 and later) (Fig. 3B). Thus, RSV transformation had a strong inhibitory effect on proteoglycan synthesis in confluent chondrocyte cultures.

Effect of RSV Transformation on Matrix Composition

Since we observed that RSV-transformed chondrocytes actively synthesized and formed

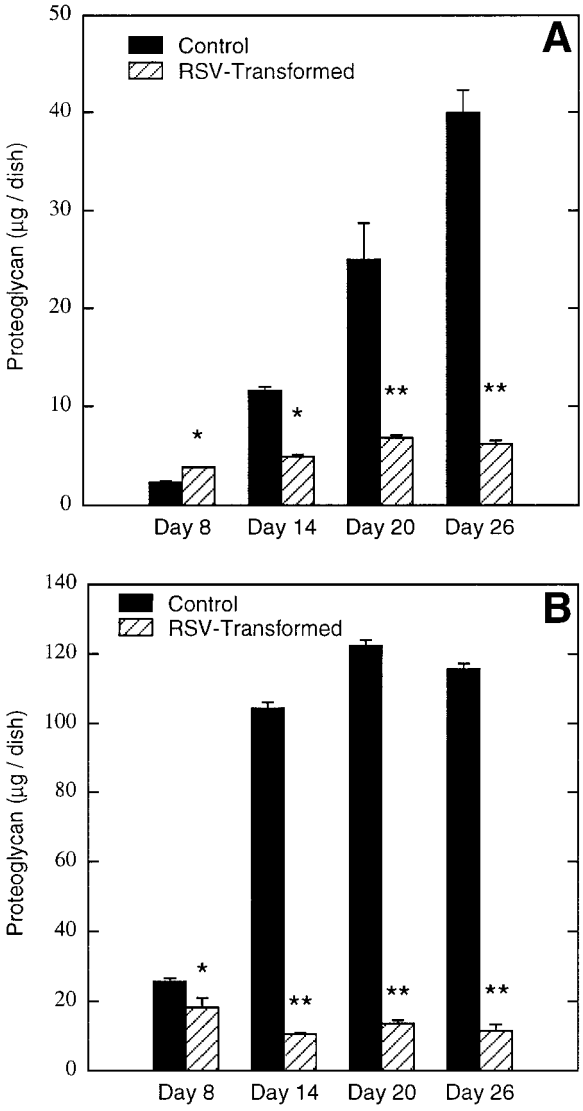


Fig. 3. Effect of RSV transformation on proteoglycan synthesis. Normal mock-infected and RSV-transformed chondrocytes were seeded into 35-mm petri dishes at 0.2 million cells per dish. At the indicated time intervals, cell and culture media were harvested and the proteoglycan levels were analyzed as described in Materials and Methods. **A:** Cell-matrix layer proteoglycan. **B:** Media proteoglycan. Asterisks (*), values significantly different from the control; $P < 0.01$. Double asterisks (**), values significantly different from that of control; $P < 0.001$.

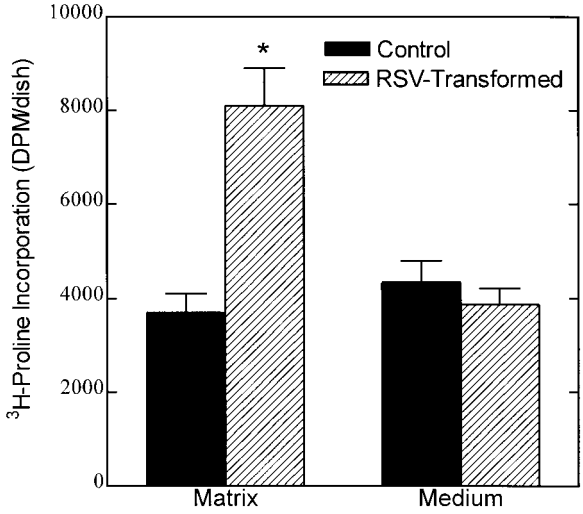


Fig. 4. Effect of RSV transformation on collagen synthesis. Incorporation of ^{3}H-proline into cell-matrix and secreted media proteins during early culture (day 4). Asterisks (*), values significantly different from the control; $P < 0.01$.

extracellular matrix proteins at early, preconfluent stages, we assayed the incorporation of proline into these fractions as an indication of collagen synthesis. RSV-transformed cells incorporated ^{3}H-proline into the cell-matrix at approximately twice the rate of mock-transformed cells (Fig. 4). However, both types of cells secreted similar amounts of proline-labeled macromolecules into the media. Analysis of cell and matrix proteins by SDS-PAGE revealed that RSV-transformed cells synthesized larger amounts of fibronectin and type I collagen, whereas in mock-transformed chondrocytes, type II and type X collagens were predominant (Fig. 5A). To further confirm the inhibition of type X collagen synthesis in RSV-transformed chondrocytes, we performed immunoblot analyses of type X levels in cell monolayers and media using a monoclonal antibody. As shown in Figure 5C, there was minimal level of type X collagen associated with monolayers in RSV-transformed cells when compared to that of mock-transformed chondrocytes. Type X collagen in the culture media from RSV-transformed chondrocytes was undetectable (Fig. 5B).

Effect of RSV Transformation on Enzyme Activities

In order to examine the effect of RSV transformation on matrix metalloprotease (MMP) expression, gelatin zymography was performed. These chondrocyte cultures showed a predominant lucent band of gelatinase activity at 72 kDa in the culture media (Fig. 6). This enzyme was previously identified as MMP-2 (Wu et al., 1997a). RSV transformation caused a marked increase in the levels of latent MMP-2 (Fig. 6). In contrast, while alkaline phosphatase activity steadily increased throughout the culture period in both mock-transformed and RSV-transformed cells (Fig. 7), the levels in RSV-transformed chondrocytes were significantly

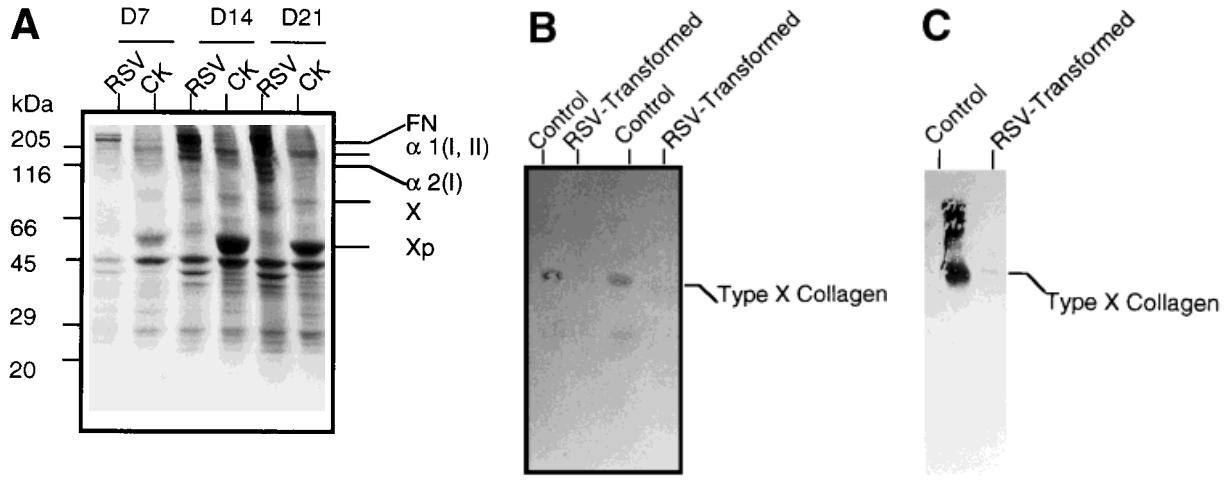


Fig. 5. Effect of RSV transformation on the expression of extracellular matrix proteins. **A:** SDS-PAGE analysis of cell-matrix total protein. Cell-matrix layers were harvested at the indicated time and total proteins were separated on 8% SDS-PAGE gel and stained with Coomaise blue R-250. Note the increased synthesis of fibronectin and type I collagen a 2-chain and the reduction of type X collagen synthesis in RSV-transformed chondrocytes. **B:** Immunoblot analysis of type X collagen in culture media; the left two lanes were culture media harvested at day 14, while the right two lanes were culture media of day 21. Note the absence of type X collagen in the culture media from RSV-transformed chondrocytes. **C:** Immunoblot analysis of type X collagen in cell-matrix layers harvested at day 21. Note the absence of type X collagen in cell-matrix layer of RSV-transformed chondrocytes. The presence of type X collagen at higher molecular weight is likely due to the covalent cross-linkage between type X collagen and other matrix proteins.

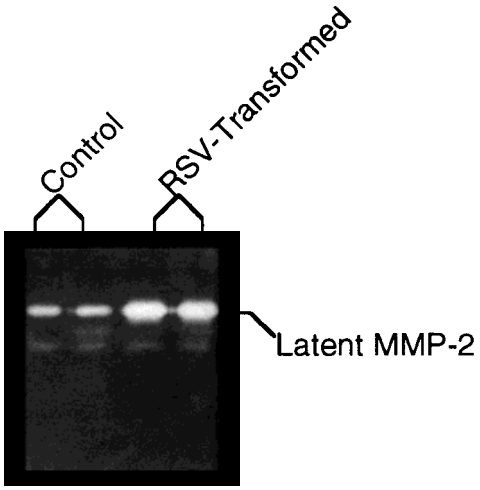


Fig. 6. Zymographic analysis of gelatinase expression in chondrocyte culture medium. Both normal and RSV-transformed chondrocytes predominantly express a 72-kDa gelatinase and RSV transformation enhanced the expression of this gelatinase.

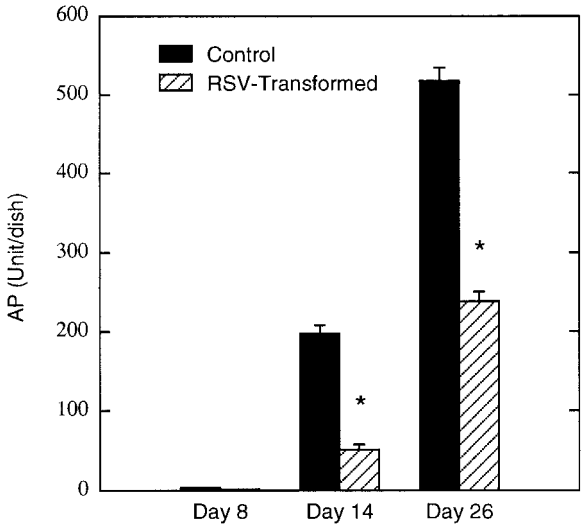


Fig. 7. Effect of RSV transformation on alkaline phosphatase expression. There was a greater than twofold reduction of ALP activity in RSV-transformed chondrocytes. Asterisks (*), values significantly different from that of control; $P < 0.01$.

lower than those in mock-transformed cells at all time periods.

Effect of RSV Transformation on Calcification

To determine the effect of RSV transformation on mineral formation, we analyzed calcium and phosphate levels in cell monolayers. Mock-transformed chondrocytes steadily accumulated calcium indicative of calcium phosphate mineral formation, while RSV-transformed cells did not (Fig. 8). Patterns in phosphate accumulation by the matrix paralleled those of calcium (data not shown).

DISCUSSION

Our study indicates that RSV transformation caused profound changes in epiphyseal growth

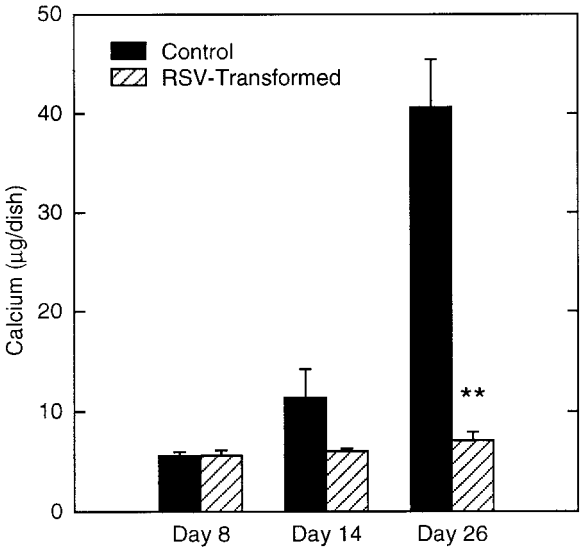


Fig. 8. Inhibition of mineral deposition in GP chondrocyte cultures by RSV transformation. At the indicated time intervals, cell-matrix layers were harvested and the levels of calcium analyzed. Double asterisks (**), values significantly different from that of control; $P < 0.001$.

plate chondrocytes in terms of their morphology, extracellular matrix (ECM) profiles and mineralization. First, RSV transformation caused chondrocytes to change from polygonal morphology to spindle shaped. Second, type I collagen and fibronectin synthesis were enhanced in RSV-transformed chondrocytes. Third, synthesis of proteoglycans was significantly reduced in RSV-transformed chondrocytes. These results are consistent with several previous studies using embryonic chondrocytes which have shown that RSV transformation causes a shift in the expression of collagen from type II to type I [Gionti et al., 1983; Allebach et al., 1985]. It has also been shown that RSV transformation causes a marked decrease in proteoglycan synthesis [Muto et al., 1977; Pacifici et al., 1977; Yoshimura et al., 1981].

In the present study, we have further observed that the synthesis of type X collagen, a molecule characteristically synthesized only by hypertrophic chondrocytes [Schmid and Linsenmayer, 1985; Poole and Pidoux, 1989; Schmid et al., 1991], was also markedly reduced in RSV-transformed chondrocytes. The failure of RSV-transformed chondrocytes to produce type X collagen suggests that they were unable to progress through their normal differentiation pathway to the hypertrophic phenotype. In addition to these changes in ECM composition, the rate of ECM synthesis, as indicated by proline incorporation, was increased in RSV-transformed cells, especially at the early stages of culture. We also found that ALP activity, another marker for chondrocyte differentiation, was reduced more than two-fold in RSV-transformed cells, and the cultures failed to mineralize the ECM. By contrast, mock-transformed cells produced large amounts of extracellular mineral in the later stages of culture. These results collectively demonstrate that RSV transformation inhibits the normal differentiation and mineralization process undergone by growth plate (GP) chondrocytes.

The failure of RSV-transformed chondrocytes to undergo extracellular mineralization may be due to either the altered properties of their matrices or their inability to form functional matrix vesicles. The matrices produced by normal growth plate chondrocytes are thought to play a role in supporting the nucleation of apatite precursors and crystal growth, however, the initiation of mineral formation in cartilage is mediated by matrix vesicles [Anderson, 1969; Bonucci, 1970; Wuthier, 1982].

Type X collagen is a short, non-fibril-forming collagen exclusively found in hypertrophic cartilage [Schmid and Linsenmayer, 1985]. The co-incident appearance of type X collagen with cell hypertrophy in growth plate tissues has led to the suggestion that it is essential to matrix mineralization which begins in this region of the growth plate [Leboy et al., 1988; Schmid et al., 1989]. Mice harboring a dominant negative mutation in type X collagen gene developed skeletal deformities ranging from compression of hypertrophic growth plate cartilage to a decrease in newly formed bone [Jacenko et al., 1993]. Fourier transform infrared microscopic analysis of the calcified cartilage of normal and type X deficient transgenic mice indicated that the mineral from transgenic mice exhibited less crystallinity and higher acidic phosphate content than the corresponding mineral from normal tissues [Paschalis et al., 1996], suggesting a regulatory role for type X collagen in the growth of normal minerals. In vitro mineralization studies have shown that, type X collagen, alone or with type II collagen, regulates matrix vesicle-mediated mineralization by interacting with the calcium channel protein annexin V [Kirsch and Wuthier, 1994; Kirsch et al., 1994]. Thus, the virtual absence of type X collagen in the ECM of RSV-transformed chondrocytes may

compromise the initiation of extracellular mineralization by impairing matrix vesicle function.

A number of studies indicate that RSV transformation of embryonic chondrocytes cause a switch in collagen synthesis: type II collagen synthesis was inhibited, while synthesis of type I collagen was initiated [Allebach et al., 1985; Gionti et al., 1983; Okayama et al., 1977]. The present study showed that there was strong type I collagen expression in RSV-transformed GP chondrocytes. Although the expression of type I collagen has been shown in hypertrophic chondrocytes in concert with cartilage matrix calcification and with transition from carilage to bone [Kirsch et al., 1997; Mundlos et al., 1990], the premature switch from type II collagen to type I in RSV-transformed chondrocytes may compromise mineralization ECM. The inability of RSV-transformed chondrocytes to mineralize, in spite of the rich matrices they produced, suggest that type I collagen cannot substitute for type II or type X collagen in initiating or facilitating endochondral mineralization.

We observed a marked reduction of proteoglycan synthesis in RSV-transformed growth plate chondrocytes. It has been shown that proteoglycans inhibit hydroxyapatite crystal growth in vitro and thus may inhibit tissue mineralization [Eanes et al., 1992; Chen et al., 1984]. If proteoglycans function primarily as crystal growth inhibitors in growth plate cartilage we should have observed an increase in mineral deposition with RSV transformation. Proteoglycan has also been shown to be important for chondrocyte differentiation. Inhibition of proteoglycan biosynthesis in a mineralizing chondrocyte culture has been shown to interfere with chondrocyte differentiation and in this way to decrease calcification [Hunter and Weinert, 1996]. Similarly, we have found that treatment of DATP5 chondrocyte cultures with 4-methyl umbelliferyl-α-D-xyloside, a specific inhibitor of proteoglycan biosynthesis, caused a significant, dose-dependent decrease in mineral deposition [Y. Ishikawa, unpublished observations]. Our findings suggest that synthesis of cartilage proteoglycans is perhaps critical for further cell differentiation.

RSV-transformed chondrocytes also expressed more matrix metalloproteinase 2 (MMP-2) as shown by zymographic analysis. In GP chondrocyte cultures, the expression of this enzyme is also up-regulated by retinoic acid [Wu et al., 1997; Nie et al., 1998]. Interestingly, retinoic acid treatment of GP chondrocyte cultures, like RSV-transformation, caused chondrocytes to change their morphology from polygonal to spindle-shaped. Similar to RSV-transformation, retinoic acid significantly decreased, if not abolished, type X and type II collagen expression while initiating or stimulating type I collagen and fibronectin expression in GP chondrocyte cultures [Wu et al., 1997a]. Retinoic acid treatment also inhibited proteoglycan synthesis in GP chondrocyte cultures. These close similarities suggest that retinoic acid treatment and RSV-transformation must share some common or convergent regulatory mechanism in the control of ECM protein expression. But despite these similarities, retinoic acid enhances culture mineralization [Iwamoto et al., 1994; Wu et al., 1997a], while RSV-transformed cells fail to mineralize. Thus, it is evident that more than ECM composition alone directs the mineralization process.

There is ample evidence that the mineralization of the growth plate ECM is directly related to the progressive developmental changes undergone by the associated chondrocytes [Hunziker, 1994]. Recent studies have demonstrated that the terminal stage of the chondrocyte differentiation pathway involves programmed cell death [Gibson et al., 1995, 1997; Hatori et al., 1995]. An apoptotic fate for growth plate chondrocytes can account for many of the commonly observed features of endochondral mineralization including changes in chondrocyte redox state [Shapiro et al., 1982], depletion of cellular ATP [Matsumoto et al., 1988], loss of mitochondrial calcium [Brighton and Hunt, 1976], an increase in cytosolic calcium [Wu et al., 1997], and blebbing of the plasma membrane in the biogenesis of matrix vesicles [Anderson, 1969]. Very recently, it has been suggested that the failure of the growth plate to undergo mineralization in avian dyschondroplasia is because of the lack of apoptotic cells in this disease condition, perhaps due to an imbalance in cytokine and growth factors [Ohyama et al., 1997]. In the present study, the failure of RSV-transformed cultures to mineralize may have been due to a developmental detour away from the hypertrophic phenotype to a fibroblastic one. This side-tracking of the maturational sequence then prevents the formation of matrix vesicles that occurs during terminal differentia-

tion and thereby prevents matrix calcification. The determination of whether the failure in mineralization is primarily the result of RSV-induced changes in cell-mediated processes, or rather is a consequence of alterations in extracellular matrix composition, will require further study.

ACKNOWLEDGMENTS

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