

# Chondrocyte Survival and Differentiation In Situ Are Integrin Mediated

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**ABSTRACT** Chondrocytes in specific areas of the chick sternum have different developmental fates. Cephalic chondrocytes become hypertrophic and secrete type X collagen into the extracellular matrix prior to bone deposition. Middle and caudal chondrocytes remain cartilaginous throughout development and continue to secrete collagen types II, IX, and XI. The interaction of integrin receptors with extracellular matrix molecules has been shown to affect cytoskeleton organization, proliferation, differentiation, and gene expression in other cell types. We hypothesized that chondrocyte survival and differentiation including the deposition into interstitial matrix of type X collagen may be integrin receptor mediated. To test this hypothesis, a serum-free organ culture sternal model that recapitulates normal development and maintains the three-dimensional relationships of the tissue was developed. We examined chondrocyte differentiation by five parameters: type X collagen deposition into interstitial matrix, sternal growth, actin distribution, cell shape, and cell diameter changes. Additional sterna were analyzed for apoptosis using a fragmented DNA assay. Sterna were organ cultured with blocking antibodies specific for integrin subunits ( $\alpha 2$ ,  $\alpha 3$ , or  $\beta 1$ ). In the presence of anti- $\beta 1$  integrin (25  $\mu\text{g/ml}$ , clone W1B10), type X collagen deposition into interstitial matrix and sternal growth were significantly inhibited. In addition, all chondrocytes were significantly smaller, the actin was disrupted, and there was a significant increase in apoptosis throughout the specimens. Addition of anti- $\alpha 2$  (10  $\mu\text{g/ml}$ , clone P1E6) or anti- $\alpha 3$  (10  $\mu\text{g/ml}$ , clone P1B5) integrin partially inhibited type X collagen deposition into interstitial matrix; however, sternal growth and cell size were significantly decreased. These data are the first obtained from intact tissue and demonstrate that the interaction of chondrocytes with extracellular matrix is required for chondrocyte survival and differentiation. *Dev. Dyn.* 1997;210:249-263. © 1997 Wiley-Liss, Inc.

## INTRODUCTION

The regulation of chondrocyte differentiation from the proliferative stage to the hypertrophic stage has been studied in many laboratories in situ and with

tissue culture models (Leboy et al., 1989; Eavey et al., 1988; Quarto et al., 1992). Proliferating chondrocytes synthesize and secrete a complex extracellular matrix (ECM) including collagen types II, IX, and XI, proteoglycans, and other matrix molecules. Terminally differentiated, hypertrophic chondrocytes express type X collagen (LuValle et al., 1992). The hypertrophic stage of chondrogenesis is characterized by an increase in individual chondrocyte size and volume, pericellular matrix accommodation, and a concomitant decrease in collagen types II, IX, and XI (Schmid and Linsenmayer, 1985; Linsenmayer et al., 1991; Schmid et al., 1991). The hypertrophic cells are also characterized by secretory vesicles that contain annexin V and alkaline phosphatase activity (Kirsch et al., 1997). It has been hypothesized that the accumulation of type X collagen mRNA is the result, and not the cause, of hypertrophy (Linsenmayer, 1991; Reichenberger et al., 1992). The theory that hypertrophy precedes type X collagen expression has been termed the "hypertrophic program" (Linsenmayer, 1991; Reichenberger et al., 1992). Studies using in ovo embryonic chick sterna have demonstrated that changes in chondrocyte size precede the deposition into interstitial matrix of type X collagen (Hirsch et al., 1996). However, the mechanisms for chondrocyte terminal differentiation have not been determined.

Chondrocyte-matrix interactions, via  $\beta 1$ ,  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  integrin subunits have been demonstrated in freshly isolated human fetal chondrocytes (Durr et al., 1993) and cultured chick prehypertrophic chondrocytes (Enomoto et al., 1993). The  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ , and  $\alpha \nu\beta 3$  integrin receptors have been identified as collagen binding receptors, although some of these receptors may also bind other ligands. For example,  $\alpha 2\beta 1$  binds laminin in addition to collagen, and the  $\alpha \nu\beta 3$  also binds vitronectin and laminin (Zutter and Santoro, 1990; Albelda and Buck, 1990; Hynes, 1992).

Attachment assays have demonstrated that freshly isolated (Durr et al., 1993) and cultured chondrocytes

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(Enomoto et al., 1993) bind to types I and II collagen via  $\beta 1$  integrin (Durr et al., 1993; Enomoto et al., 1993; Loeser, 1993) and  $\alpha 2$  integrin subunits (Durr et al., 1993), although the combination of anti- $\alpha 1$  and anti- $\alpha 2$  acted synergistically to decrease binding to types I and II collagen further (Durr et al., 1993). Chondrocyte binding to collagen substrates was also found to be  $Mg^{++}$  dependent (Durr et al., 1993; Enomoto et al., 1993) but  $Ca^{++}$  and RGD sequence independent (Durr et al., 1993).

The localization of integrin subunits on cartilage sections with immunofluorescence has been problematic. The  $\alpha 2$  integrin subunit was not detected in human fetal cartilage (clone P1E6) although the same antibody blocked attachment to types I and II collagen by 40–50% (Durr et al., 1993). The same antibody did not block articular chondrocyte attachment to type II collagen (Loeser et al., 1995) or reduce collagen gel contraction by dermal fibroblasts at 40  $\mu g/ml$  (Langholz et al., 1995). However, we used the same antibody (clone P1E6) to detect  $\alpha 2$  on chick chondrocytes *in situ* using confocal microscopy (Hirsch et al., 1996). In addition, chick chondrocytes form cell matrix attachment complexes (CMAX) that include integrin molecules ( $\beta 1$ ,  $\alpha 2$  and  $\alpha 3$ ) and cytoskeletal proteins (F-actin, vinculin, focal adhesion kinase,  $\alpha$ -actinin, zyxin, and paxillin) (Wu et al., 1995; Hirsch et al., 1996). This morphological observation led to the hypothesis that changes in chondrocyte cell size and gene expression may be regulated by integrin receptor signaling (Hirsch and Svoboda, 1993, 1994, 1996; Hirsch et al., 1994, 1996). Inhibition of cell-matrix interactions may prevent subsequent terminal differentiation as measured by the synthesis and deposition into interstitial matrix of type X collagen.

To test this hypothesis we developed a serum-free organ culture system that duplicated normal development in the chicken sternum. We used this model to evaluate possible functional properties of specific proteins involved in cell-matrix attachment complexes. Whole embryonic chicken sterna (day 14) were organ cultured (OCS) with antibodies directed against the  $\beta 1$ , (Hirsch and Svoboda, 1996)  $\alpha 2$ , or  $\alpha 3$  integrin subunits. The  $\beta 1$ ,  $\alpha 2$ , and  $\alpha 3$  integrin subunit antibodies were chosen for the blocking assay as  $\alpha 2\beta 1$  and  $\alpha 3\beta 1$  have been previously demonstrated to function as collagen receptors (Hynes, 1992). In addition, the same (anti- $\alpha 2$  or - $\alpha 3$ ) or similar antibodies demonstrated effects on chondrocyte attachment to collagen substrates (Durr et al., 1993; Enomoto et al., 1993; Loeser, 1993), cell differentiation (Menko and Boettiger, 1987; Gamble et al., 1993), and collagen or collagenase gene expression (Langholz et al., 1995). Type X collagen secretion and deposition into the pericellular interstitial matrix in the sternal medial cephalic region were used as a terminal differentiation marker. In addition, we assessed overall sternal growth, individual cell diameter, and filamentous actin (F-actin) distribution. Finally, the role of chondrocyte matrix attachment in survival of

all cells in the developing sterna was examined using a standard apoptosis assay.

After establishing a dose response curve, sterna were incubated with optimal doses of blocking antibodies for 8 days. We will demonstrate and discuss the following results: (1) All three integrin blocking antibodies stunted overall sternal length; (2) The decreased sternal length was partially caused by smaller cells throughout the tissue and decreased type X collagen deposition into interstitial matrix in the hypertrophic zone; (3) The actin cytoskeleton was disrupted in chondrocytes treated with anti-integrin antibodies and the cells had fewer filopodial extensions reaching to the surrounding matrix; (4) There was increased apoptosis in anti- $\beta 1$  treated sterna that also contributed to decreased growth.

## MATERIALS AND METHODS

### Cartilage Preparation

White Leghorn chicken eggs were obtained from SPAFAS (Norwich, CT) at embryonic day 13 or 14. Some eggs were incubated at 39°C to obtain chicks at later developmental stages. Whole sterna were removed and dissected free of all tissue and perichondrial membranes in Ham's F12 medium (GIBCO-BRL Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 100  $\mu g/ml$  penicillin/100 U/ml streptomycin (Sigma, St. Louis, MO), 1% non-essential amino acids (GIBCO-BRL Laboratories, Grand Island Biological Co.), and 1% antibiotic/antimycotic (GIBCO-BRL Laboratories, Grand Island Biological Co.). The whole sternum was either immediately fixed for transmission electron microscopy or immunohistochemistry or it was used for organ culture studies (see Cartilage Organ Culture). Anatomically, the chick sternum has a central spine (keel) that is perpendicular to two lateral plates. Spines and lateral plates of fixed sterna were separated with a sterile scalpel, maintaining cephalic-caudal and medial-lateral orientations. The complete lateral plate or keel was kept as a single specimen. In this study, three regions were examined in each specimen: the hypertrophic cells in the cephalic region, the prehypertrophic chondrocytes in the middle region, and the proliferative chondrocytes in the caudal region (Hirsch et al., 1996).

### Cartilage Organ Culture

Day 14 sterna were dissected as described above and cultured at 37°C with 95% air/5%  $CO_2$  in a humid environment for 8 days in sterile tissue culture chambers (Nunc, Inc., Naperville, IL). The media composed of Ham's F12 medium as described above supplemented with  $10^{-11}$  M dexamethasone (Sigma), 60 ng/ml insulin (Sigma),  $10^{-11}$  M triiodothyronine (T3, Collaborative Research, Bedford, MA), and 100  $\mu g/ml$  ascorbic acid (Bohme et al., 1992; Burch and Lebovitz, 1982a,b; Dozin et al., 1992; Eavey et al., 1988; Leboy et al., 1992; Quarto et al., 1992), and was termed "DITA" ( $n = 31$ ). A dose-response curve was generated (Table 1, see Fig. 2) using various concentrations of blocking antibodies.

**TABLE 1. Average Sternal Length of Organ Culture Sterna Grown in the Presence of Anti-Integrin Subunit Antibody<sup>a</sup>**

Sterna culturing conditions	No. of sterna	Length (mm)
Control media, DITA	31	26.15 ± 0.37
30 µg/ml anti-Mouse IgG Control	11	24.75 ± 0.49
Clone W1B10		
1 µg/ml anti-β1	2	25.00 ± 0.00
2 µg/ml anti-β1	1	20.00
10 µg/ml anti-β1	2	16.5 ± 0.50
20 µg/ml anti-β1	1	16.00
25 µg/ml anti-β1 <sup>b</sup>	13	16.00 ± 0.42
50 µg/ml anti-β1 <sup>b</sup>	2	16.00 ± 1.00
100 µg/ml anti-β1 <sup>b</sup>	2	15.75 ± 0.75
200 µg/ml anti-β1 <sup>b</sup>	1	14.50
Clone P1E6		
5 µg/ml anti-α2	2	17.00 ± 0.00
10 µg/ml anti-α2	8	18.13 ± 0.77
25 µg/ml anti-α2	2	15.5 ± 0.50
Clone P1B5		
5 µg/ml anti-α3	1	19.00
10 µg/ml anti-α3	8	18.00 ± 0.65
25 µg/ml anti-α3	2	14.5 ± 0.50

<sup>a</sup>Values presented as average sternal length (mm) ± standard error (unless N = 1).

<sup>b</sup>No type X collagen secreted into the ECM.

The blocking antibodies used were chicken specific (anti-β1; clone W1B10, Sigma) and anti-human integrin subunits (anti-α2 (clone P1E6), -α3 (clone P1B5, Becton Dickinson, San Jose, CA). Cell-matrix interactions were inhibited by culturing sterna with one of the following: 25 µg/ml anti-chick β1 integrin (n = 13), 10 µg/ml anti-human α2 integrin (n = 8), or 10 µg/ml anti-human α3 integrin (n = 8), and compared to control sterna cultured with 30 µg/ml anti-mouse IgG (n = 11). All experiments were repeated multiple times, with the culture medium changed daily. Subsequent to culturing, the cartilage was immediately fixed for either transmission electron microscopy, organelle or actin staining, immunohistochemistry, or in situ hybridization. Each lateral plate was examined as a total specimen so that orientation was maintained. One lateral plate from each cultured sternum was immunolabeled with type X collagen and phalloidin, whereas other lateral plates were fixed for electron microscopy. The media was frozen from each sternum daily for biochemical analysis.

### Transmission Electron Microscopy

Pieces of cartilage (with known orientation markers) from organ cultured sterna were fixed in ½ strength Karnovsky's (Hirsch and Svoboda, 1993) and postfixed in 1% osmium tetroxide (Electron Microscopy Science, Fort Washington, PA) and 1.5% potassium ferrocyanide (Fisher Scientific, Pittsburgh, PA) (Farnum and Wilsman, 1987). The tissue was stained en bloc in fresh 1% uranyl acetate (Fisher Scientific) for 30 min, dehydrated and embedded in Poly 812 epon (Electron Microscopy Science) and araldite (Tousimis Research Co., Rockville, MD). Thin sections were examined on a JEOL (Peabody, MA) 100CX transmission electron mi-

croscope. Images were photographed on Kodak (Rochester, NY) electron microscope sheet film 4489.

### Filamentous Actin Labeling

F-actin was visualized with FITC or TRITC tagged phalloidin (Molecular Probes, Eugene, OR) as described previously (Hirsch et al., 1996). For double-labeled studies, indirect immunohistochemistry was performed on whole lateral plates of sterna as described below, prior to incubation in TRITC-phalloidin. The stained sternal lateral plates were mounted in slowfade mounting media (Molecular Probes, Eugene, OR) on glass slides with nail polish spacers, coverslipped, and viewed on the confocal laser scanning microscope (CLSM).

### Chondrocyte Diameter Determination

Sterna were stained with phalloidin and cells were measured as described previously (Hirsch et al., 1996). Briefly, a grid overlay on the CLSM computer monitor was used to select areas and measure chondrocytes along their long and short axes at the surface, 10, 20, 30, and 40 µm into the cartilage. At least 40 chondrocytes from each sternal region (cephalic, middle, and caudal) in at least three different sterna were used to determine the average chondrocyte size.

An overall comparison of chondrocyte long axis lengths was conducted with a three-way analysis of variance with depth, age, and region as "between" subject variables. More specific comparisons were then obtained by two-way analyses of variance at each age level with depth and region as "between" subject variables as described previously (Hirsch et al., 1996). *P* values were set at 0.05.

### Indirect Immunohistochemistry

Isolated organ cultured sterna were incubated at 37°C for 1.5 to 3 hr in 0.1% testicular hyaluronidase (280 U/mg; Sigma) to unmask the epitope (Schmid and Linsenmayer, 1985; Hirsch et al., 1996). The tissue was fixed and immunostained for type X collagen as previously described (Hirsch et al., 1996). Briefly, freshly prepared 4% paraformaldehyde/PBS (pH 7.4) was used for fixation (15 min). Nonspecific staining was blocked with 10% normal goat serum (NGS) (GIBCO Laboratories, Grand Island Biological Co.). The type X collagen antibody (AC9; Schmid and Linsenmayer, 1985) was a generous gift from Dr. Thomas Linsenmayer. The epitope on the type X collagen molecule is located within the triple helical domain, 19 nm from the COOH-terminal domain.

Secondary antibodies were FITC-conjugated AffiniPure goat anti-mouse IgG (H + L chains; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and FITC-conjugated AffiniPure goat anti-rabbit IgG (H + L chains; Jackson ImmunoResearch Laboratories, Inc.). Sterna incubated in the absence of a primary antibody were negative controls (Hirsch and Svoboda, 1993; Hirsch et al., 1994, 1996). In addition, single-labeled FITC and TRITC samples scanned with the

opposite excitation and barrier filter sets were also negative for crossover.

### **In Situ Analysis of Apoptosis**

In situ nucleotide end labeling protocols, also known as TUNEL, for the detection of internucleosomal breakdown of nuclear DNA have been previously described (Tilly, 1994; Gavrieli et al., 1992). Briefly, our tissue samples were fixed, permeated, rehydrated, and digested with proteinase K before incubation with biotin-dUTP and Tdt enzyme (Boehringer Mannheim Corp., Indianapolis, IN).

Apoptotic nuclei were visualized with avidin-FITC (Molecular Probes). All nuclear material was labeled by incubating samples in 0.5  $\mu\text{g/ml}$  propidium iodide (Molecular Probes) for 5 min. Sternal samples ( $n = 3$ ) were then viewed on the CLSM as described previously. Negative controls were incubated in nucleotide end labeling mixture without probe, washed, detected with avidin-FITC, and viewed on the CLSM. Positive controls were incubated in 100  $\mu\text{g/ml}$  DNase 1 (Boehringer Mannheim) for 30 min at 37°C prior to nucleotide end labeling, hybridized, washed, double labeled with avidin-FITC and propidium iodide, and viewed on the CLSM.

### **Determination of Apoptotic Ratio**

Tissues fixed for in situ analysis of apoptosis were viewed on the CLSM. All nuclei were stained red with propidium iodide. Apoptotic nuclei were double labeled and appear yellow with the overlapping of FITC (green) and rhodamine (red) labels. The number of apoptotic nuclei were counted and the ratio of apoptotic nuclei to total number of nuclei was determined. Samples were viewed in each of three regions: cephalic, middle, and caudal. In each region, three counts ( $n = 30\text{--}50$  cells) were made in separate areas within the region at each of three depths: 10, 30, and 50  $\mu\text{m}$ . This gave a total of nine counts per region per sample ( $n = 3$ ). The ratios obtained from each field within each region were calculated and averaged.

### **Confocal Laser Scanning Microscopy**

The specimens were analyzed with the Leica (Deerfield, IL) upright confocal laser scanning microscope equipped with an argon ion laser as described previously (Hirsch and Svoboda, 1993; Hirsch et al., 1996). The tissue was viewed with a 50x P1 Fluotar ( $n.a. = 1.0$ ) water immersion lens with a working distance of 100  $\mu\text{m}$  (Leica).

Merged images were computer generated from two optical sections in the same focal plane recorded from the FITC or TRITC photo multiplier tubes. The images were electronically colored so that the individual images were green or red. Combined images maintained red or green color in pixels that did not overlap. Pixels that contained information from both images were yellow.

Confocal images were analyzed, enhanced, and stored on optical discs. Black and white or pseudocolour

images were computer generated with a minimum of computer enhancement and arranged with Adobe Photoshop and Pagemaker (Adobe Systems, Inc., Mountainview, CA), then printed on a Kodak 8650 dye sublimation printer. All control tissue was collected, analyzed, enhanced, and photographed with the same conditions as experimental tissue.

## **RESULTS**

Whole in ovo day-14 sterna were removed and dissected free of all perichondrial membranes and placed in culture medium modified from Quarto et al. (1992). Sterna were also cultured with integrin subunit specific antibodies to determine if cell-matrix communication was necessary for sternal development and chondrocyte maturation. For all experimental conditions the following parameters were measured: terminal chondrocyte differentiation as determined by the deposition of type X collagen into the interstitial matrix (Figs. 1 and 3), growth as determined by measuring sternal length (Fig. 2), and cell size and actin distribution as determined by measuring the diameter of chondrocytes stained with phalloidin (Figs. 4 and 5). Ultrastructural studies demonstrated similarities and/or differences between cells in control media compared to cells cultured with  $\beta 1$  integrin antibody (Fig. 6). Overall cell viability was assessed with an assay that determined the number of nuclei with fragmented DNA. An apoptosis ratio was determined for each developmental region of sterna after incubation in control media compared to those cultured in the presence of anti- $\beta 1$  integrin (Fig. 7).

To determine the number of days necessary for cephalic chondrocytes to terminally differentiate, whole sterna were removed from organ culture daily and stained for type X collagen. In ovo (Fig. 1A) type X collagen was prominently detected in the pericellular matrix of the hypertrophic chondrocytes in the cephalic region after 17 days of incubation. Type X collagen was first observed in cephalic, central regions of the 14-day organ cultured sterna (OCS) after 6 days in optimal medium. However, it took 8 days in organ culture (Fig. 1B), for type X collagen to be qualitatively comparable to a day 17 in ovo sterna (Fig. 1A). A higher concentration of type X collagen in pericellular regions with less staining in the interstitial matrix was observed in OCS similar to in ovo sterna. No type X collagen was observed in caudal, proliferative regions even after 2 weeks of organ culture, indicating that the chondrocytes within the cultured sterna maintained the normal developmental pattern.

### **Integrin Antibodies Inhibit Chondrocyte Terminal Differentiation**

Experiments were performed to test the hypothesis that integrin subunit antibodies can interfere with the terminal chondrocyte differentiation process. Embryonic sterna were cultured with a control IgG or a specific integrin subunit antibody ( $\beta 1$ ,  $\alpha 2$ , or  $\alpha 3$ ) to determine if blocking cell-matrix interactions would

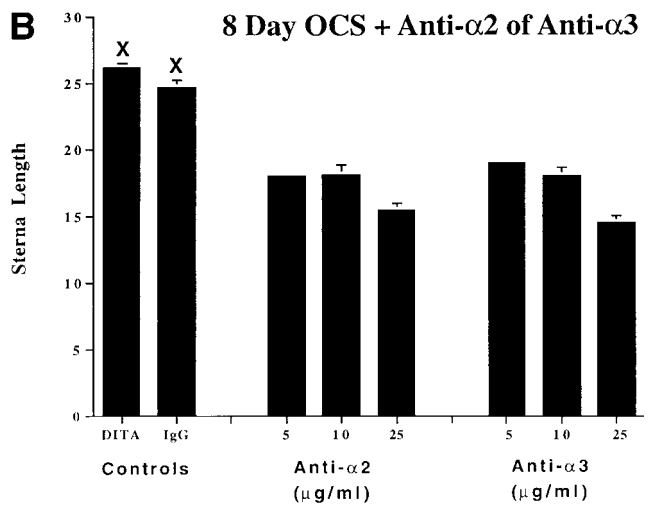
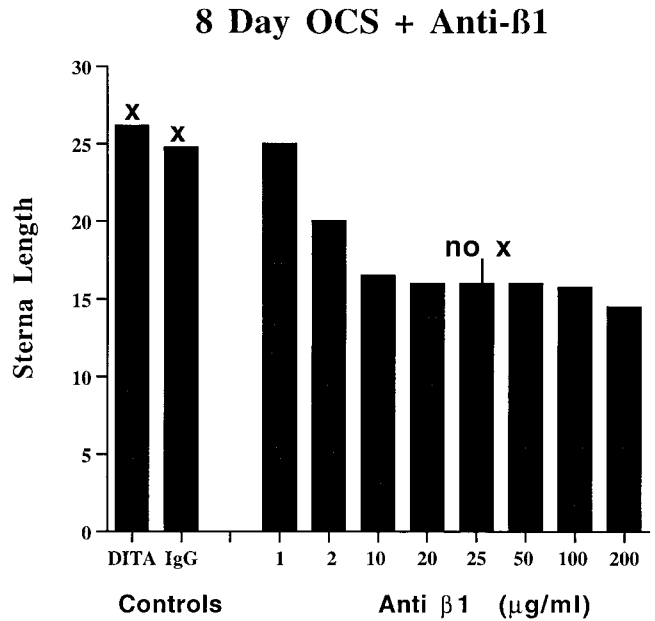
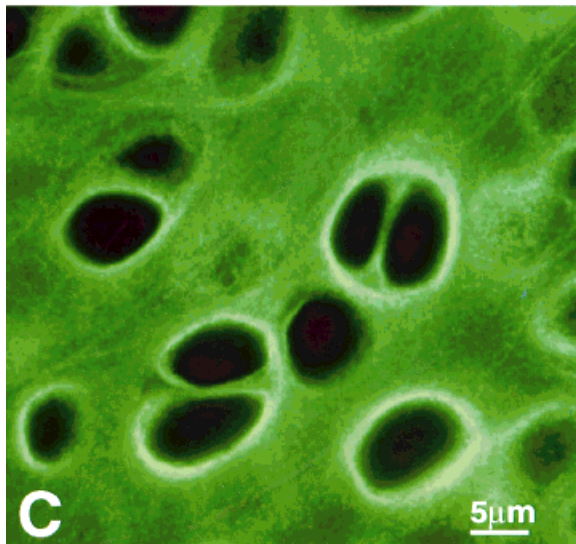
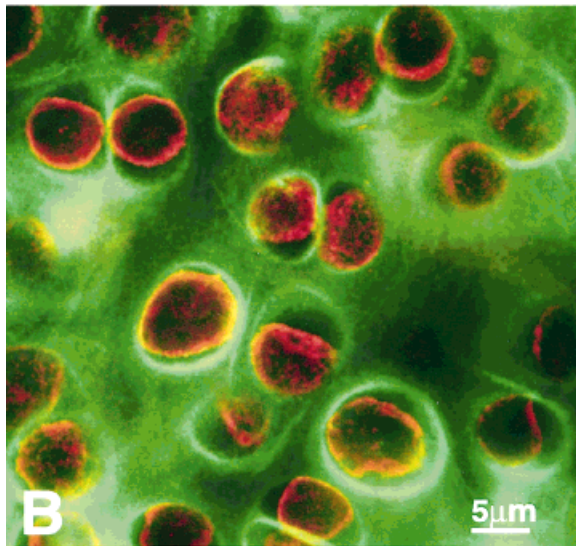
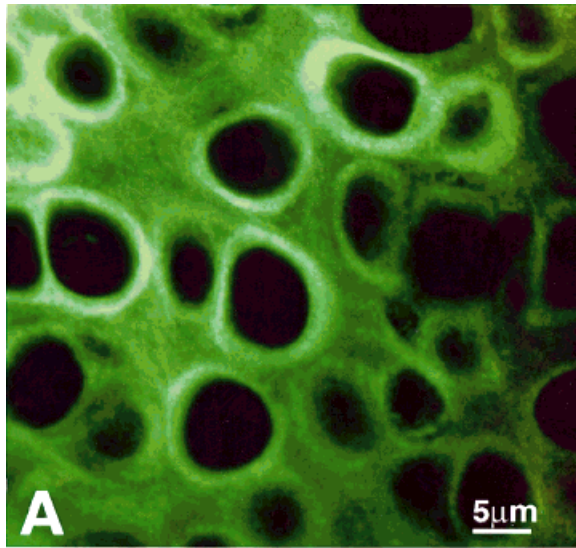


Fig. 2. Sternal growth is inhibited by integrin antibodies. Sterna grown with control anti-mouse IgG or integrin anti-β1 (W1B10), anti-α2 (P1E6), anti-α3 (P1B5) antibodies were compared to DITA controls. DITA and IgG controls were not significantly different from each other and both produced type X collagen in medial cephalic regions (X). Anti-β1 integrin antibody (β1) inhibited sternal growth in a dose-dependent fashion. Sterna grown with at least 25 µg/ml β1 integrin antibodies were significantly smaller than controls and did not secrete type X collagen (A). Sterna grown with 10 µg/ml anti-α2 or anti-α3 integrin were also growth arrested compared to controls, but they did produce some type X collagen (B). The amount of type X collagen produced, however, was less than controls. In the presence of 25 µg/ml anti-α2 or anti-α3 integrin, some type X collagen was still detected.

Fig. 1. Type X collagen distribution in control sternum. Single confocal optical sections of sternum were single (A,C) or double (B) labeled for type X collagen (green) and F-actin (red). Medial cephalic regions from day-17 in ovo sternum (A), control 8-day organ-cultured sternum (B) and IgG control-treated sternum (C). The highest concentration of type X collagen was observed in the pericellular matrix with less in the interstitial matrix. In single-labeled tissue (A,C), negative areas represent the lacunae. Scale bar = 5 µm.

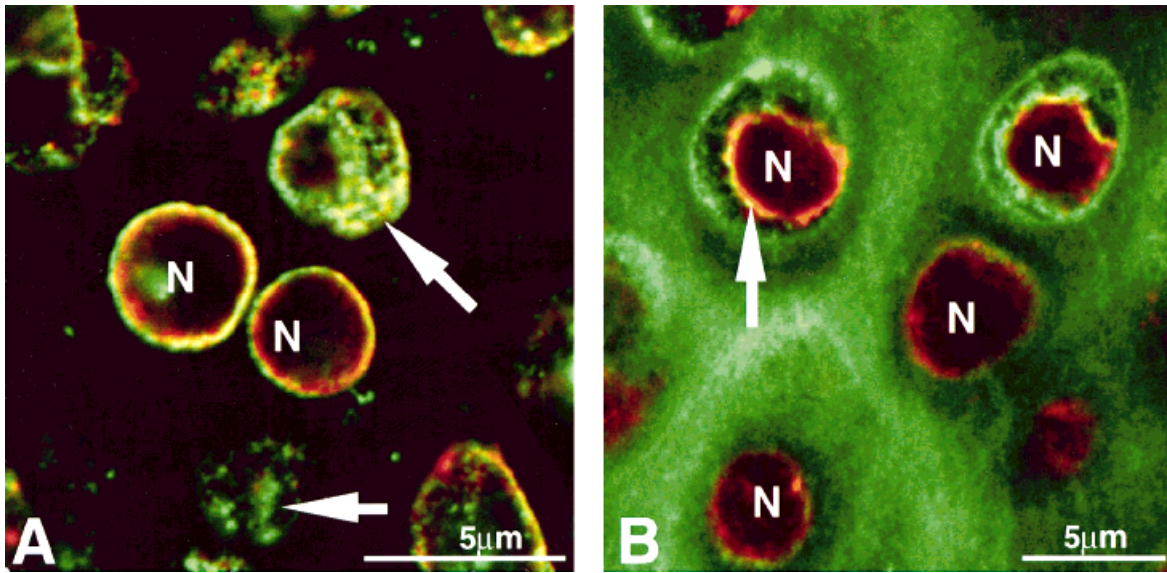


Fig. 3. Higher magnification of single confocal optical sections of type X collagen (green), integrin subunits (green), and F-actin (red) distribution in the hypertrophic zone of sterna cultured in the presence of anti- $\beta 1$  (A) or anti- $\alpha 3$  (B). The integrin subunits,  $\beta 1$  (A) or  $\alpha 3$  (B) integrin were detected in a punctate pattern associated with chondrocyte plasma membranes (arrows) throughout the tissue and could be distinguished from type X collagen by staining pattern as the anti-integrin antibodies were only seen in association with cell membranes and not the matrix (see Fig. 1) (Hirsch et al., 1996). Antibodies that blocked  $\beta 1$  integrin

inhibited type X collagen deposition into the interstitial matrix as this area is negative (A), whereas antibodies directed against the  $\alpha 3$  subunit decreased, but did not inhibit type X collagen deposition into interstitial matrix in the hypertrophic zone (B). The type X collagen was not seen intracellularly as the tissue was not permeabilized before secondary antibody incubation (B). Note that the chondrocytes from antibody treatment groups were more spherical than controls (Fig. 1). Scale bars = 5  $\mu$ m; N = nucleus.

also block terminal differentiation. In the presence of control IgG, hypertrophic chondrocytes produced type X collagen in medial, cephalic regions (Fig. 1C), similar to sterna grown in control medium (DITA) (Fig. 1B). Control OCS (n = 31) or control medium with IgG (n = 8), were not significantly different in overall sternal length (Fig. 2).

Sterna cultured in increasing concentrations of anti- $\beta 1$  integrin (n = 25) demonstrated a dose-dependent decrease in type X collagen deposition into interstitial matrix (Figs. 2 and 3A) and final overall sternal length. Addition of the anti- $\beta 1$  to defined media did not completely block chondrocyte terminal differentiation at concentrations less than 25  $\mu$ g/ml, although sternal growth was significantly decreased at 10  $\mu$ g/ml compared to controls (Fig. 2). At antibody concentrations greater than or equal to 25  $\mu$ g/ml, the deposition of type X collagen was blocked as the matrix surrounding the chondrocytes was negative (Fig. 3A) but sternal growth was not significantly different from that observed with 10  $\mu$ g/ml anti- $\beta 1$  (Fig. 2). The positive FITC staining in the confocal image (Fig. 3A, arrows) was the secondary antibody binding to the anti- $\beta 1$  integrin subunits on the chondrocyte membranes similar to immunostaining described previously in this tissue (Hirsch et al., 1996). This staining pattern was found throughout the tissue, even in the prehypertrophic and proliferative zones. In confocal z series images, the staining was located to the

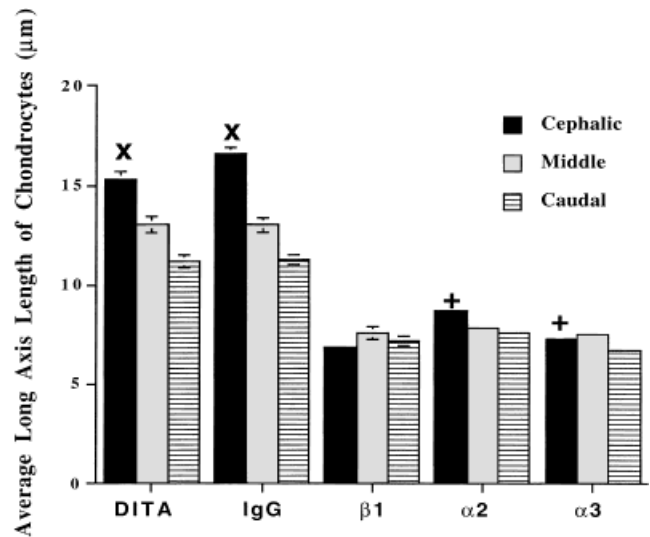


Fig. 4. Integrin antibodies decreased cell size. Chondrocyte diameters in medial cephalic (hypertrophic zone), middle (prehypertrophic zone), and caudal (proliferative) regions at 20  $\mu$ m below the surface were measured from confocal images of phalloidin stained tissue. Cell diameters were significantly decreased in all regions of sterna cultured with integrin antibodies: 25  $\mu$ g/ml anti- $\beta 1$  (W1B10), 10  $\mu$ g/ml anti- $\alpha 2$  (P1E6), or 10  $\mu$ g/ml anti- $\alpha 3$  (P1B5) compared to controls (DITA, IgG). Solid bars = cephalic hypertrophic chondrocytes; shaded bars = middle prehypertrophic chondrocytes; horizontal lines = caudal proliferative chondrocytes; X = all cells in these regions secreted type X collagen; + = some of the cells in these regions secreted type X collagen.

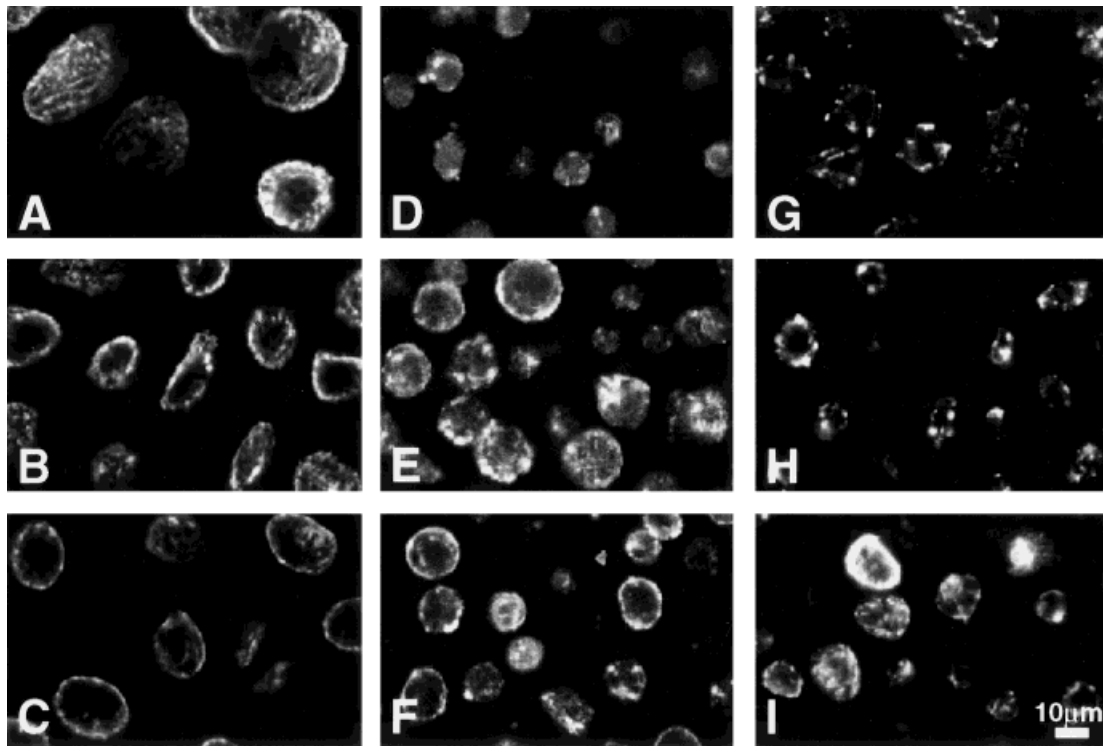


Fig. 5. Integrin antibodies disrupted F-actin. Low magnification single confocal optical sections of phalloidin stained chondrocytes in medial cephalic (A,D,G), middle (B,E,H), and caudal (C,F,I) regions of sternal lateral plates from controls (A–C), anti- $\beta$ 1 (D–F), and anti- $\alpha$ 2 (G–I) treated tissues. All images were acquired on the same day with the same magnification and microscope settings. Mouse IgG (A–C) had no effect on the F-actin distribution compared to DITA control or in ovo sterna (Hirsch and Svoboda, 1993; Hirsch et al., 1996). Hypertrophic chondrocytes in medial cephalic (hypertrophic) regions (A) were larger than middle (prehypertrophic) (B) and caudal (proliferative) (C) chondrocytes. In contrast, the addition of 25  $\mu$ g/ml anti- $\beta$ 1 (W1B10) (D–F), 10  $\mu$ g/ml anti- $\alpha$ 2 (P1E6)

(G–I), or 10  $\mu$ g/ml anti- $\alpha$ 3 (data not shown) had a dramatic effect on cell size and F-actin distribution. Significant decreases in cell size were observed in medial cephalic regions when sterna were cultured with anti- $\beta$ 1 (D) or anti- $\alpha$ 2 (G). Filamentous actin bundles normally observed in control chondrocytes (A) were not apparent, but instead actin was observed in condensed foci (D,G). The distribution of F-actin was less disturbed in chondrocytes from middle and caudal regions of sterna grown with anti- $\beta$ 1 (E,F) or anti- $\alpha$ 2 (H,I). In the presence of the anti-integrin antibodies, chondrocytes adopted an extremely round morphology (D–I) compared to controls (A–C). Scale bar = 10  $\mu$ m.

cell periphery, indicating that it was on the cell surface and not within the cells. Double labeling with rhodamine phalloidin for actin confirmed that the FITC label extracellular (Fig. 3A, center cells). The tissue was not detergent extracted before the immunohistochemistry, therefore, it is not detecting intracellular type X collagen. Other researchers have reported that transcription of type X collagen mRNA occurs immediately prior to the appearance of intracellular and extracellular type X collagen protein (LuValle et al., 1989, 1992; Schmid and Linsenmayer, 1990; Linsenmayer et al., 1991). Based on these studies, the transcription of type X collagen was probably inhibited in sterna grown with  $\beta$ 1 integrin antibody, but it needs to be confirmed.

The doses of blocking antibodies in this study were fourfold less than the amount of anti- $\beta$ 1 (anti-human clone AIIB2) needed to block mammary epithelial cell differentiation in vitro (100  $\mu$ g/ml, Howlett et al., 1995). In contrast, a single high dose of the chick anti- $\beta$ 1 integrin (100  $\mu$ g/ml) on the first day of culture resulted in a sternum that was significantly shorter than controls, but contained chondrocytes that secreted type X

collagen (data not shown). These results suggest that consecutive signals over time may be responsible for chondrocyte terminal differentiation, whereas sternal growth may only be dependent on signals during a specific time in development. Integrin antibodies bound to receptors on chondrocyte plasma membranes throughout the tissue and were detected with secondary antibodies conjugated to FITC (Fig. 3A).

Culturing sterna with increasing concentrations (1  $\mu$ g/ml to 30  $\mu$ g/ml,  $n = 23$ ) of anti- $\alpha$ 2 (P1E6) or anti- $\alpha$ 3 (P1B5) integrin did not completely block deposition into interstitial matrix of type X collagen (Fig. 3B). Mammary epithelial cells required 100  $\mu$ g/ml of anti- $\alpha$ 2 (P1E6) placed in type I collagen matrix and media to partially block differentiation, and the same concentration of anti- $\alpha$ 3 (P1B5) had no effect (Howlett et al., 1995). Attachment assays with freshly isolated chondrocytes also required 10  $\mu$ g/ml anti- $\alpha$  integrin to block attachment greater than 50% on purified collagen substrates (Durr et al., 1993). Sterna cultured in 10  $\mu$ g/ml anti- $\alpha$  integrin (either P1E6 or P1B5) were significantly shorter than control sterna (Fig. 2B), but

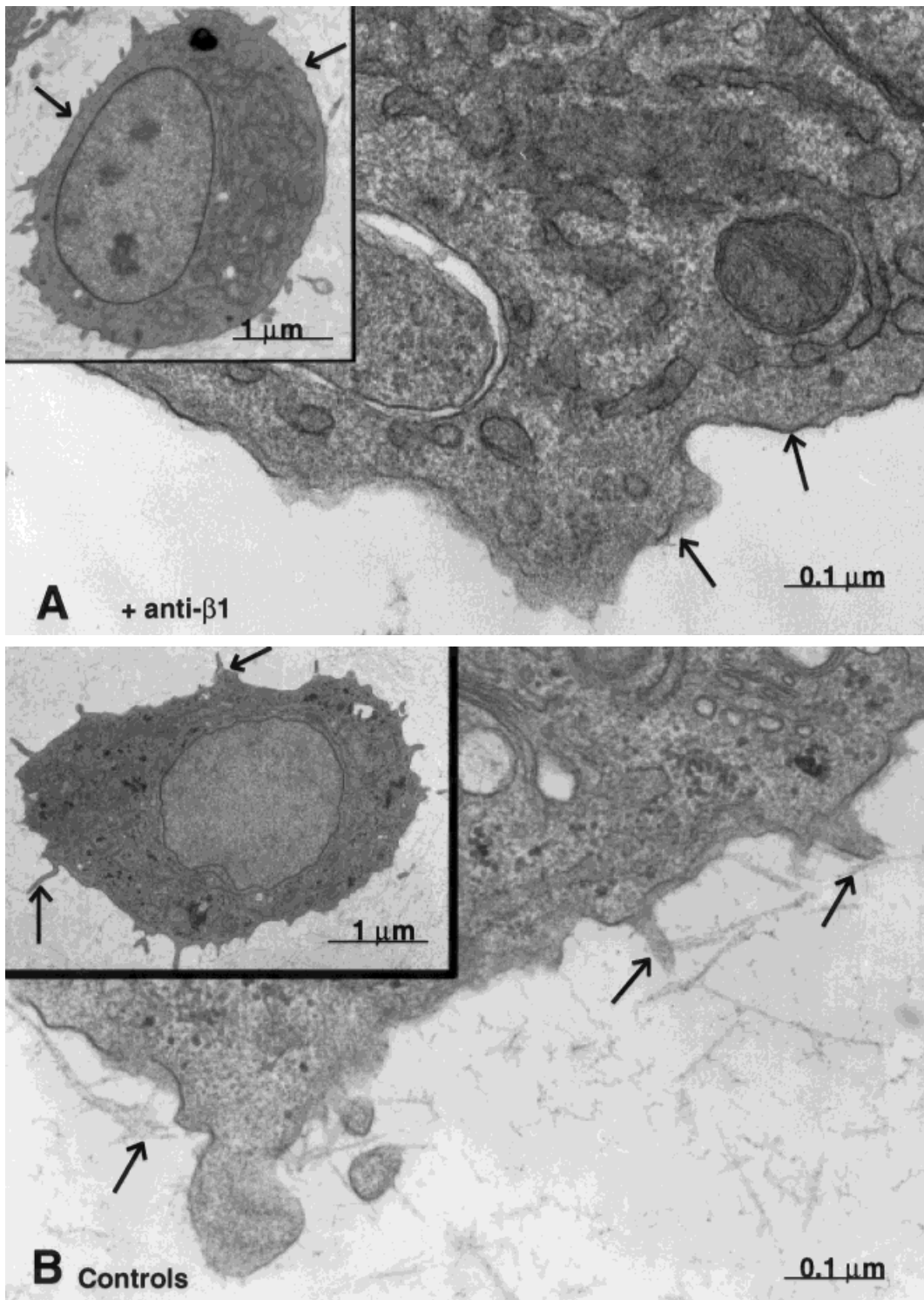


Fig. 6. Integrin antibodies decreased cell-matrix interactions. Chondrocytes from the hypertrophic region of anti-β1 treated tissue (A) and controls (B) were completely surrounded by ECM molecules (insets, A and B). Both treatment groups had chondrocytes with large spherical nuclei and contained numerous ER, Golgi, and mitochondria profiles. In control tissue, collagenous molecules appeared to be closely associated

with chondrocyte plasma membranes (B, arrows) and the cells had many filopodia projections into the matrix (inset, B, arrows). Chondrocytes treated with 25 μg/ml anti-β1 integrin had very few collagen molecules directly interacting with the plasma membrane (A, arrows) and few filopodial extensions (inset, A, arrows). Scale bar = .1 μm; inset = 1 μm.

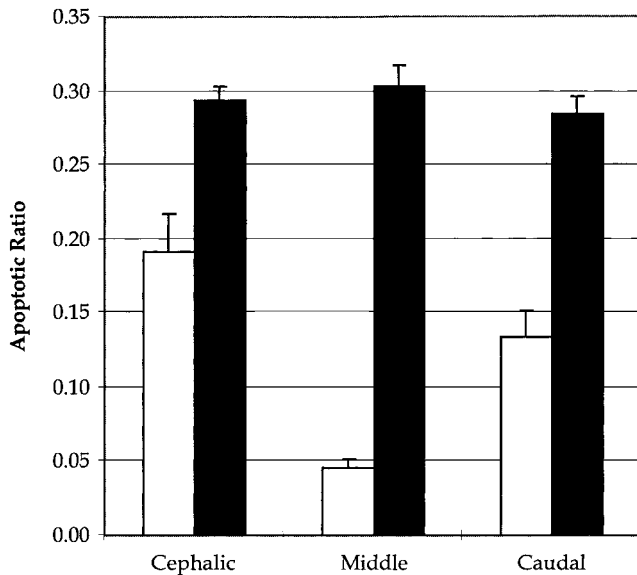


Fig. 7. Apoptotic ratio: Controls vs. anti- $\beta$ 1 integrin treated. Whole sterna were cultured in 30  $\mu$ g/ml anti-mouse IgG (controls; white bars) or 25  $\mu$ g/ml anti-chick  $\beta$ 1 (W1B10) integrin (black bars) for 8 days and double-labeled for apoptotic nuclei and all nuclear material using in situ nucleotide end labeling and propidium iodide, respectively. Apoptotic ratios, the number of apoptotic nuclei to total number of nuclei, were obtained for each treatment group at three sternal regions: cephalic (hypertrophic), middle (prehypertrophic), and caudal (proliferative). The counts were performed in triplicate at three depths of the tissue and averaged. In all three regions, the apoptotic ratio was significantly larger in anti- $\beta$ 1 integrin-treated sterna than controls ( $P < 0.05$ ). In the controls, the largest apoptotic ratio was observed in the cephalic region.

were slightly larger than sterna cultured in 25  $\mu$ g/ml anti- $\beta$ 1 (Fig. 2A).

The deposition into interstitial matrix of type X collagen was anti- $\alpha$  integrin dose dependent similar to the anti- $\beta$ 1 experiment; however, anti- $\alpha$  only partially inhibited type X deposition (Fig. 3B) even at the highest doses in this study. The area of positive type X staining was smaller and less intense than controls in the anti- $\alpha$  treated tissues (compare Figs. 1B to 3B). The secondary antibody also labeled the cell surface associated anti- $\alpha$  integrin (Fig. 3B, arrow), but not the type X collagen intracellularly. In non-hypertrophic regions, the secondary antibody labeled the cell surface  $\alpha$  integrin subunits similar to previous experiments (Hirsch et al., 1996), but did not label the interstitial matrix. These results suggest that chondrocyte terminal differentiation was dependent on more than one collagen integrin receptor. This was further supported by the results that sterna cultured with high concentrations of anti- $\beta$ 1 integrin demonstrated no type X collagen deposition into the interstitial matrix (Fig. 3A). This dose may have blocked both the  $\alpha$ 2 $\beta$ 1 and  $\alpha$ 3 $\beta$ 1 receptors (and possibly the  $\alpha$ 1 $\beta$ 1 if it is also present on chondrocyte plasma membranes), resulting in complete inhibition of type X collagen deposition into interstitial matrix. This conclusion is supported by experiments with dermal fibroblasts that don't contract collagen gels in the presence

of anti- $\alpha$ 2 (P1E6) alone. However, the combination of antibodies against  $\alpha$ 2 and  $\beta$ 1 contracted the gels more than  $\beta$ 1 alone (Langholz et al., 1995). In the current study the anti- $\alpha$  integrins did have an effect on sternal growth, cell size, and shape. Sterna cultured in 10  $\mu$ g/ml anti- $\alpha$  integrin were significantly shorter than control sterna (Fig. 2B), but were slightly larger than sterna cultured in 25  $\mu$ g/ml anti- $\beta$ 1 (Fig. 2A).

#### Cell Sizes Determined With Phalloidin Staining

Chondrocyte cell size (diameter) was measured in OCS on days 6 and 8 and compared to in ovo sternum chondrocytes from the same regions (Hirsch et al., 1996). Terminally differentiated chondrocyte areas were immunohistochemically identified with type X collagen (Fig. 1). Analysis of variance was used to determine significant differences in average cell sizes. Hypertrophic chondrocytes in medial cephalic regions of control OCS and in ovo sternum were similar in diameter, and were significantly larger than chondrocytes from middle and caudal regions.

Cell size analysis was determined in sterna cultured in control medium (DITA), IgG, or anti-integrin (Fig. 4). Data were collected at 20  $\mu$ m below the surface as in ovo and cultured control sterna demonstrated few changes beyond this depth (Hirsch et al., 1996). Chondrocytes from sterna cultured with IgG demonstrated no significant difference in cell diameter in any sternal region compared to sterna grown in control medium alone, DITA (Fig. 4, solid bars = hypertrophic cephalic chondrocytes; hatched bars = prehypertrophic middle chondrocytes; striped bars = proliferative caudal chondrocytes). In contrast, chondrocyte diameter was significantly inhibited in all regions of sternum cultured with an integrin antibody (Figs. 4 and 5). The average diameter of cells in cephalic regions of sternum grown with anti- $\alpha$ 2 or anti- $\alpha$ 3 were slightly larger than those grown with anti- $\beta$ 1, and some type X collagen was detected (+, Figs. 4 and 5). This was consistent with the theory that the anti- $\beta$ 1 blocks both the  $\alpha$ 2 $\beta$ 1 and  $\alpha$ 3 $\beta$ 1 integrin receptors, resulting in increased inhibition of chondrocyte terminal differentiation. No significant difference in average cell diameters in middle or caudal regions was observed between anti- $\beta$ 1, anti- $\alpha$ 2, or anti- $\alpha$ 3 (Figs. 4, 5E,F and 5H,I). However, they were significantly smaller than cells from respective areas of controls (Figs. 4 and 5B,C).

Similar to in ovo sternum chondrocytes (Hirsch and Svoboda, 1993; Hirsch et al., 1995), cells from sterna grown with IgG antibody demonstrated microfilament bundles of F-actin near cell surfaces. Actin was also associated with plasma membranes (appeared as rings by confocal microscopy) in optical sections through the center of the cells that included the nuclear profile (Fig. 5A-C). Cells in the medial cephalic region (Fig. 5A) had matured into hypertrophic chondrocytes as determined by cell size and deposition of type X collagen (Figs. 1B and 4). In contrast, sterna cultured with integrin

antibodies had altered chondrocyte shape and disrupted F-actin network (Fig. 5D–I).

Sterna cultured in anti- $\beta$ 1 integrin (Fig. 5D) had significantly smaller hypertrophic region chondrocytes than controls (Fig. 5A). The anti- $\beta$ 1 treated chondrocytes did not have organized actin filaments and did not produce type X collagen (Fig. 3A). In middle and caudal regions (Fig. 5E,F), the change in cell shape from oval to round was also observed. Similar to the data collected for cell size (Fig. 4) and sternal growth (Fig. 2), the addition of an  $\alpha$  integrin antibody did affect the F-actin distribution and the cell shape but not as extensively as the  $\beta$ 1 integrin antibody. In the presence of the  $\alpha$ 2 integrin antibody, chondrocytes in medial cephalic regions demonstrated few bundles of F-actin (Fig. 5G), while middle (Fig. 5H) and caudal (Fig. 5I) chondrocytes still demonstrated an association between F-actin and plasma membranes. In all regions, however, chondrocytes from sterna grown with the  $\alpha$ 2 integrin were more round than controls (Fig. 5G–I). Similar results were observed from sterna grown with the  $\alpha$ 3 integrin antibody (data not shown).

#### Ultrastructural Analysis of Organ Cultured Chondrocytes

Electron microscopy was used to compare hypertrophic chondrocyte ultrastructural morphology between sterna cultured in control media (Fig. 6B) to those cultured with integrin antibodies (Fig. 6A). The hypertrophic chondrocytes from the medial cephalic region of an 8-day control OCS (Fig. 6B, inset) were completely surrounded by ECM molecules and ultrastructurally similar to cells from in ovo sternum cartilage. The large, spherical nucleus contained heterochromatin, and mitochondria were observed throughout the cytoplasm. In addition, fibrillar collagenous-like molecules appeared to be closely associated with the chondrocyte plasma membranes (Fig. 6B, arrows). All cells observed had uneven cell peripheries with many short filopodia projecting into the surrounding ECM. The chondrocytes also contained the normal organelles for protein synthesis and were surrounded by an abundant ECM (Hirsch and Svoboda, 1993, 1994). Chondrocytes from other sternal areas had similar characteristics (data not shown).

Similar to cells from in ovo sternum or controls (Fig. 6B), chondrocytes from sternum grown with integrin antibodies possessed the organelles necessary for protein synthesis and secretion (Fig. 6A, inset). Golgi apparatus and mitochondria were observed in the cytoplasm with numerous profiles of rough ER in perinuclear regions of medial cephalic chondrocytes. Although the cells were similar to control chondrocytes they were smaller and had fewer filopodia extensions (Fig. 6A, inset). Similar EM results were observed in sterna cultured with anti- $\alpha$ 2 or anti- $\alpha$ 3 integrin (data not shown).

Consistent with cell size data obtained with confocal microscopy, the diameter of chondrocytes from sterna

grown with an integrin antibody were smaller (Fig. 6A, inset) than control chondrocytes (Fig. 6B, inset). In contrast to the ellipsoidal shape of chondrocytes from control sterna, chondrocytes treated with an integrin antibody were round and had fewer short filopodia projecting into the surrounding ECM (Fig. 6A), although the collagenous ECM was still abundant surrounding the cells (Fig. 6A, inset). Anti- $\beta$ 1 integrin also affected chondrocyte-ECM interactions (Fig. 6A) as few collagen fibrils appeared to interact directly with some areas of chondrocyte plasma membrane (Fig. 6A). This result was consistent with previous studies that reported cultured epithelial or fibroblast cells become round and detached from substrates when cultured with antibodies or peptides that block integrin-substrate interactions (Meredith et al., 1993; Frisch and Francis, 1994; Re et al., 1994; Howlett et al., 1995; Boudreau et al., 1995).

#### Blocking $\beta$ 1-Integrin Increased Apoptosis

Cell matrix attachment through integrin receptors has been shown to influence differentiation and may be a factor in cell survival as other laboratories have demonstrated that blocking cell-matrix attachment increased programmed cell death in other cell types (Meredith et al., 1993; Frisch and Francis, 1994; Re et al., 1994; Howlett et al., 1995; Boudreau et al., 1995). We hypothesized that chondrocytes lacking matrix attachment may have increased programmed cell death, apoptosis, compared to control chondrocytes.

Sterna for apoptosis analysis were cultured in 25  $\mu$ g/ml anti- $\beta$ 1 integrin ( $n = 3$ ), while control sterna were cultured in 30  $\mu$ g/ml anti-mouse IgG ( $n = 3$ ) for 8 days. Apoptotic nuclei were visualized using in situ nucleotide end labeling and avidin-FITC. All nuclear DNA was visualized using propidium iodide. In the controls, the greatest apoptotic ratio was found in the cephalic hypertrophic region (0.18–0.22 Fig. 7). The middle prehypertrophic (.05) and caudal proliferative (0.10–0.15) regions had lower apoptotic ratios; however, the caudal region had an apoptotic ratio higher than the middle region. These results suggest that there may be regional differences, but not tissue depth differences, within control sternum as three depths from each specimen were analyzed (10, 30, and 50  $\mu$ m from the surface). The cephalic region had more apoptotic chondrocytes than both the middle and caudal regions in control tissue.

In sterna cultured in the presence of anti- $\beta$ 1 integrin, apoptotic ratios were between 0.25 and 0.30 in all regions, significantly higher than controls (Fig. 7). These results demonstrate that blocking outside-in matrix signaling through the  $\beta$ 1 integrin receptor caused an increase in the number of apoptotic cells (for review, see Ruoslahti and Reed, 1994). In comparison, mammary epithelial cultures treated with 100  $\mu$ g/ml anti- $\beta$ 1 induced apoptosis in 20.9% (=0.209 ratio) after 2 days and 59.9% after 6 days (Howlett et al., 1995).

Representative apoptosis staining in the cephalic, middle, and caudal regions of the  $\beta$ 1-integrin antibody treated sterna and the controls show the dramatic difference between groups (Fig. 8). Non-apoptotic nuclei appeared red with propidium iodide staining. Apoptotic nuclei appeared yellow due to overlap of propidium iodide and avidin-FITC (green) labels. An increase in yellow apoptotic cells was seen in all regions of the  $\beta$ 1-integrin antibody treated sterna (Fig. 8A–C) compared to controls (Fig. 8D–F). In each region, the non-apoptotic, red nuclei appeared smaller in the anti- $\beta$ 1-integrin treated group compared to controls. Sizes of fully condensed apoptotic nuclei appeared consistent between the anti- $\beta$ 1 integrin treated sterna and the controls.

In a representative confocal field of the cephalic region of a control sternum (Fig. 8D) some yellow, fragmented condensed DNA was seen (arrow). Non-fragmented DNA had the normal round morphological distribution of intact nuclei surrounding the yellow apoptotic portion of the nuclei. This suggests that these nuclei were in the beginning stages of apoptosis when DNA fragmentation and condensation had begun in some, but not all nuclei. An example of the middle region (prehypertrophic) of a control sternum contained one apoptotic nucleus (Fig. 8E, arrow). The fragmented DNA is similar in size to non-apoptotic nuclei suggesting that nuclear fragmentation has begun throughout the nucleus but complete condensation and formation of apoptotic bodies (blebbing) has not occurred. In the caudal region (proliferating) of a control sternum, many small apoptotic bodies containing condensed nuclear material were seen near an area approximating the space of 4 nuclei (Fig. 8F, arrows). These apoptotic bodies appeared to be the remnants of at least 3 degraded nuclei. The apoptotic bodies are very small compared to intact nuclei and appear too closely associated to represent apoptotic bodies of unique nuclei. These may demonstrate apoptotic bodies that have begun to drift away from the original location of the chondrocytes prior to their being engulfed by other cells. We theorize that the higher apoptotic ratio in the caudal region may be due to injury during periderm removal in preparation for organ culture.

## DISCUSSION

This study demonstrates that cell-matrix attachment in a whole cartilage developmental model is necessary not only for differentiation but also survival. Previous experiments have demonstrated the role of integrin subunits on freshly isolated or cultured chondrocyte attachment to purified matrix components (type I and II collagen or FN) (Durr et al., 1993; Enomoto et al., 1993; Loeser et al., 1995) with some of the same antibodies used in the present study. However, this is the first demonstration of the requirement for cell-matrix attachment in an intact cartilage differentiation model that maintains cell-matrix and cell-cell relationships. We demonstrate that blocking cell-matrix inter-

actions with specific integrin subunit antibodies changes cell and nuclear shape and increases apoptosis. We hypothesize that the cell shape change is directly related to the disruption of the actin cytoskeleton, a direct result of blocking the integrin linked signal transduction pathway (Sastry and Horwitz, 1993; Parsons, 1996). It has been established in many cell types that integrin receptor attachment to ECM substrates stimulates the Rho, Rac, and Cdc42 superfamily of small GTPases to regulate the actin cytoskeleton (Nobes and Hall, 1995; Ridley, 1996; Ridley and Hall, 1994; Chrzanowska-Wodnicka and Burridge, 1996; Parsons, 1996). Our future experiments will examine and dissect these possible second messenger pathways in the cartilage model.

## Chondrocyte Survival and Differentiation Are Integrin Mediated

The current experiments were designed to test the hypothesis that normal cell-matrix interactions via integrins were necessary for all chondrocytes to survive and differentiate normally. To test this hypothesis, whole cartilage was cultured in defined media and shown to develop normally as type X collagen was deposited in the interstitial matrix in appropriate sternal regions. In addition, the cells were morphologically similar to *in ovo* chondrocytes. Type X collagen was first detected in the interstitial matrix after 6 days in organ culture, and was comparable to day 17 *in ovo* sternum (Fig. 1A) after 8 days (Fig. 1B). The distribution of type X collagen surrounding lacunae and in the interstitial matrix was consistent with previously reported studies (Schmid and Linsenmayer, 1990; Linsenmayer, 1991; LuValle et al., 1992). In addition, type X collagen was not detected in inappropriate areas such as middle or caudal sternal regions. Chondrocytes from cultured specimens were generally larger in diameter, but ultrastructural analysis demonstrated that both non-hypertrophic chondrocytes and hypertrophic chondrocytes contained the organelles necessary for protein synthesis and secretion (Fig. 6).

After the system was established, we tested the hypothesis by blocking the cell-matrix interaction with integrin subunit specific antibodies. All antibodies were affinity purified and had been used in previous experiments to block cell attachment to collagen substrates (Enomoto et al., 1993; Durr et al., 1993; Loeser, 1993; Howlett et al., 1995). As doses of antibodies varied in these previous experiments, a dose response curve was established for this tissue using sternal length and deposition into interstitial matrix of type X collagen as effector parameters (Fig. 2). The amount of antibody needed to block  $\beta$ 1 integrin was substantially less in this tissue than mammary epithelium (Howlett et al., 1995). The doses used to block  $\alpha$ 2 and  $\alpha$ 3 integrin were also the same or less than other groups utilized on isolated chondrocytes to block attachment to specific collagen substrates (Enomoto et al., 1993; Durr et al., 1993). Most importantly, terminal differentiation as

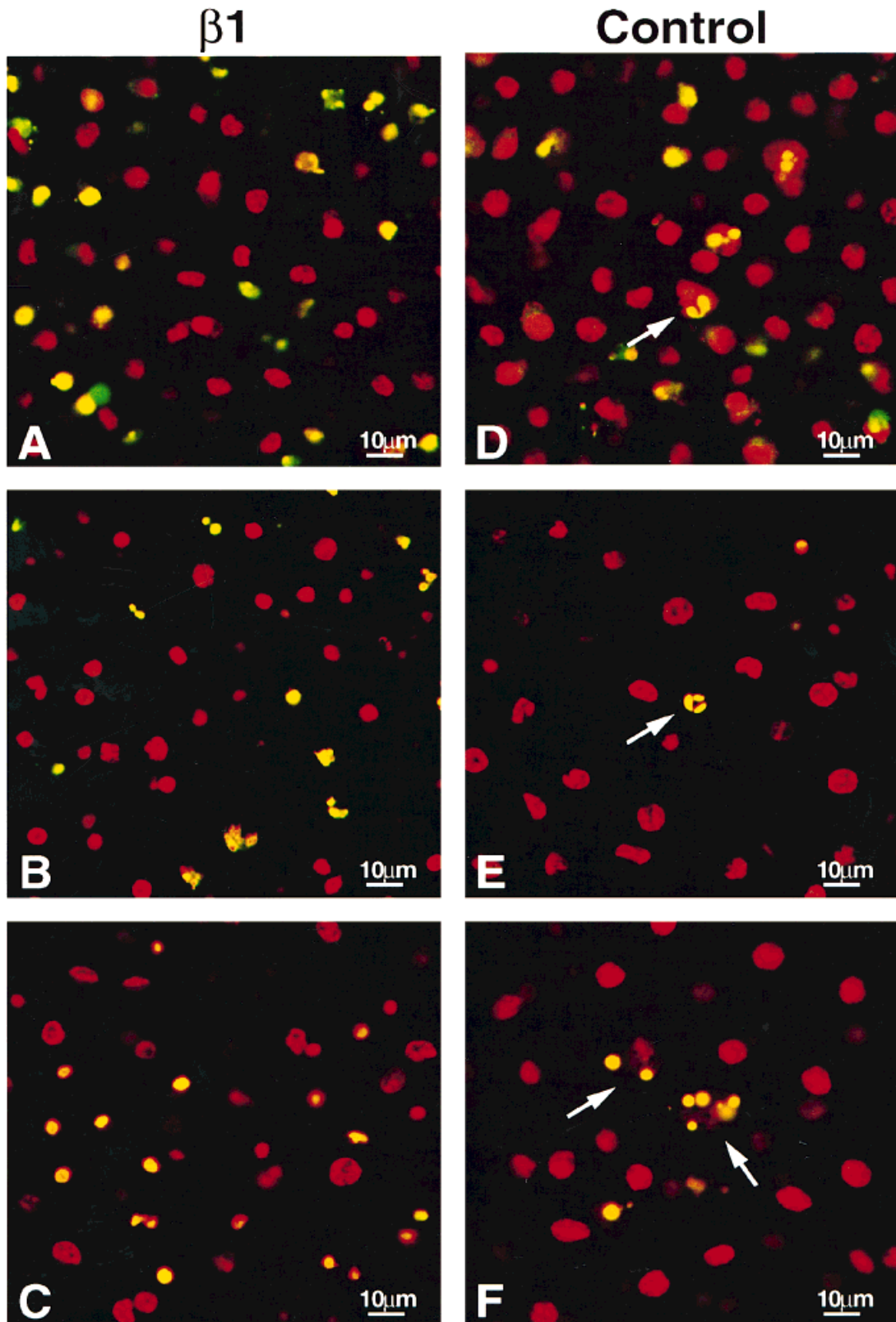


Fig. 8.

measured by type X collagen deposition into interstitial matrix was inhibited in sterna cultured with integrin antibodies. In addition, sterna cultured with anti- $\beta 1$  integrin were significantly shorter than control sterna or sterna cultured with anti- $\alpha$  integrin. Sterna that were organ cultured with the anti- $\alpha 2$  or anti- $\alpha 3$  integrin were not significantly different from each other, but were significantly shorter than control sterna (Fig. 2). One property that may explain the decreased sternal length was that the cell diameters in all sternal regions were smaller than control cells in the presence of all integrin antibodies (Fig. 4). The smaller cell diameter was quantitated using fluorescent tagged phalloidin and confocal microscopy. Furthermore, the cells were round and the actin cytoskeleton was disrupted in chondrocytes from all integrin antibody treated tissue (Fig. 5). Although the cell morphology was markedly affected, the cells increased secretion of metalloprotease inhibitors and metalloproteases, indicating that they were metabolically active (Hirsch et al., 1995).

Another factor that contributed to the shorter sterna was that the apoptotic ratio was significantly increased in all regions (0.30) of anti- $\beta 1$  treated sternum. In addition, the non-apoptotic nuclei in the anti- $\beta 1$  treated sterna were smaller than controls throughout the tissue, correlating with the smaller cell diameter (Fig. 7). This apoptotic ratio is less than the effect of blocking  $\beta 1$  integrin in mammary epithelial cells (Howlett et al., 1995); however, the doses and clones of antibodies were significantly different in the two studies.

Ultrastructural analysis demonstrated that the addition of integrin antibodies to OCS may have inhibited the interaction of ECM molecules with chondrocyte plasma membranes. Collagen molecules that appeared to directly contact plasma membranes in control chondrocytes (Fig. 6B) were decreased in some immediate pericellular regions in chondrocytes cultured with inte-

grin antibodies (Fig. 6A). It was also observed from both EM and confocal analysis that chondrocytes from sterna grown with integrin antibodies were generally rounder in shape throughout the tissue and the actin was disrupted. The anti- $\beta 1$  treated cells had fewer filopodial extensions. These three morphological criteria are consistent with the theory that the Rho and Rac signal pathways are disrupted (Ridley, 1996).

The integrin  $\beta 1$  antibodies have been mapped to three major epitope sites (Takada and Puzon, 1993). All anti- $\beta 1$  antibodies that block or bind and activate ligand interactions with the receptor were mapped to the extracellular domain from the amino terminal end to residue 218. The clone, W1B10 (Hayashi et al., 1990) is a second-generation antibody from the CSAT antibody developed in Dr. Horwitz's laboratory and mapped to residues 1–189 (Takada and Puzon, 1993). This clone is a chicken specific antibody and was necessary for this study as the anti-human antibodies have low reactivity to chicken tissue (Takada and Puzon, 1993). The anti- $\beta 1$  antibodies that don't block or activate the receptor are directed near the transmembrane domain in the carboxyl terminal region of the  $\beta 1$  extracellular domain (Takada and Puzon, 1993). Other blocking antibodies have been previously used to demonstrate that the subunits were expressed on chondrocytes and that they blocked substrate attachment in vitro (Enomoto et al., 1993; Durr et al., 1993; Loeser, 1993). The first matrix attachment blocking antibody isolated (CSAT) was also used to demonstrate that cultured myoblast differentiation into myotubes was inhibited including cell fusion, terminal differentiation, and meromyosin expression (Menko and Boettiger, 1987).

In contrast, human umbilical endothelial cells responded to blocking cell-matrix attachment using a variety of techniques by increasing programmed cell death (Meredith et al., 1993; Frisch and Francis, 1994; Re et al., 1994) similar to mammary epithelial cells (Howlett et al., 1995; Boudreau et al., 1995). The cells changed shape, usually becoming rounder or not making aggregated structures before the onset of apoptosis (Meredith et al., 1993; Frisch and Francis, 1994; Re et al., 1994; Howlett et al., 1995; Boudreau et al., 1995). Interestingly, vascular endothelial cells could be rescued with anti- $\beta 1$  antibodies that activated the integrin signal transduction pathway (Meredith et al., 1993).

Synovial fibroblast (Werb et al., 1989) and dermal fibroblast (Langholz et al., 1995) also respond to integrin antibodies by changing cell shape and decreasing collagen synthesis. These cells not only stopped making collagen, but increased expression of the proteases that degrade the matrix components including collagenase (Werb et al., 1989; Langholz et al., 1995).

In this study, antibodies directed against either  $\beta 1$ ,  $\alpha 2$ , or  $\alpha 3$  integrin subunits changed cell shape, cell size, and F-actin distribution. In addition, the tissue incubated with anti- $\beta 1$  had increased programmed cell death and decreased type X collagen deposition into

Fig. 8. Blocking  $\beta 1$ -integrin receptor increased apoptosis. Low magnification single optical sections of nuclei from control (D–F) or anti- $\beta 1$  treated sterna (A–C). Fragmented DNA was used as a marker for apoptosis. Intact DNA was labeled with propidium iodide (red). Intact, red nuclei in the control sterna appeared larger than the intact nuclei of the  $\beta 1$ -integrin antibody treated sterna reflecting the smaller cell size recorded in the previous experiment. Apoptotic DNA was immunolabeled with FITC (green) and appeared yellow in overlapping labels. An increase in yellow or apoptotic cells was seen in the hypertrophic region of the  $\beta 1$ -integrin antibody treated sterna (A) compared to the hypertrophic region of the controls (B). An increase in the number of apoptotic nuclei was seen in the prehypertrophic region of the  $\beta 1$ -integrin antibody-treated sterna (C) compared to controls (D) as well as in the proliferative region (E and F, respectively). The beginnings of nuclear condensation were demonstrated in an apoptotic nuclei in the cephalic region of a control sternum (B). Yellow condensed DNA was seen within the red nucleus. An apoptotic nucleus halted prior to condensation was seen in the middle region of a control sternum (D). The apoptotic area was similar in size to other non-apoptotic nuclei suggesting condensation had not taken place. Apoptotic bodies formed by the breakdown and nuclear blebbing were demonstrated in the caudal region of a control sternum (F). These apoptotic bodies were smaller than whole condensed nuclei and appeared to have broken apart. Scale bar = 10  $\mu$ m.

interstitial matrix, indicating that the cells did not terminally differentiate.

Interestingly, recent reports have suggested that expression of Indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP) and PTHrP receptors were necessary for the regulation of cartilage development (Vortkamp et al., 1996; Lanske et al., 1996). These studies suggested that Ihh produced by pre-hypertrophic chondrocytes stimulate synthesis of PTHrP by the periarticular perichondrium. The PTHrP then signals back to the PTHrP receptors on pre-hypertrophic chondrocytes, participating in negative feedback inhibition, required to control the rate of cartilage differentiation. It should be noted that results presented from the current investigation were obtained from sterna grown in organ culture without a perichondrium. Type X collagen deposition into interstitial matrix into medial cephalic matrix increased gradually over time. In addition, type X collagen was not observed in caudal regions, even after long-term cultures (greater than 2 weeks), suggesting that other mechanisms may also be involved in regulating the rate of cartilage differentiation. In addition, preliminary results with sterna grown in organ culture with a perichondrium demonstrated type X collagen deposition into interstitial matrix after 8 days. However, the sterna did not grow in length as extensively when compared to sterna grown without perichondrium (data not shown). Recently our laboratory has begun to investigate the effects of PTHrP on terminal chondrocyte differentiation in organ cultured sterna grown with or without a perichondrium, and in the absence or presence of an integrin receptor antibody.

In conclusion, these experiments constitute the first demonstration that chondrocytes in situ depend on cell-matrix attachment via integrin molecules to maintain viability and normal cell morphology, including cell shape and size. Furthermore, cephalic chondrocytes need this attachment complex to terminally differentiate, as determined by inhibited cell hypertrophy and type X collagen deposition into interstitial matrix. Future experiments need to examine the synthesis and production of collagens including types I, II, IX, X, and XI as well as possible control mechanisms in this developmental model. Overall, the involvement of integrin signaling (cell-matrix interactions) provides clues to a mechanism for terminal chondrocyte differentiation that may act antagonistically or synergistically with other mechanisms to regulate development of the skeletal system.

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