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Different Roles for Fibronectin in the Generation of Fore and Hind Limb Precartilage Condensations

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Fibronectin expression and spatiotemporal distribution were examined in relation to the distinctive patterns of mesenchymal condensation and chondrogenesis seen in high-density serum-free cultures of chicken wing and leg bud precartilage cells. More fibronectin protein was produced on a per cell basis by leg than by wing mesenchyme, both in freshly isolated tissue and during the prechondrogenic condensation period in culture, where the difference was twofold. The quantitative difference in fibronectin expression in freshly isolated wing and leg mesenchyme was also seen at the level of total and poly(A)$^+$ RNA. During the condensation phase, fibronectin was distributed in the wing and leg mesenchymal cultures in a way that prefigured the eventual distribution of cartilage in these cultures: in wing cultures condensations were broad and flat, and rich in diffusely organized fibronectin; in leg cultures, condensations were compact and spheroidal, and contained abundant deposits of fibronectin. In addition, the leg condensations were connected by long fibronectin-rich fibers. Transient treatment with TGF-β early during the culture period led to increase in fibronectin production and expansion of condensations in both wing and leg cultures. Leg mesenchyme was more responsive to transforming growth factor-β than wing mesenchyme with respect to fibronectin production, and this was reflected in a greater enhancement of cartilage formation in later cultures. Treatment of cultures with monoclonal antibody 304 directed against the amino-terminal heparin-binding domain of fibronectin inhibited condensation formation and reduced chondrogenesis in wing mesenchyme, but left these two processes unchanged in leg mesenchyme, despite disruption by the antibody of the leg-specific fibronectin fibers. These studies indicate that for both wing and leg mesenchyme the morphology, extent, and spatiotemporal regulation of precartilage condensation and subsequent chondrogenesis closely parallels the deposition of fibronectin. But whereas the interaction between cells and fibronectin in wing bud mesenchyme is mediated in part by the protein's amino-terminal domain, this domain does not appear to be involved in analogous interactions in leg bud mesenchyme.

INTRODUCTION

The onset of chondrogenesis during vertebrate limb development is marked by increased expression and accumulation of fibronectin at the sites of future skeletal elements (Dessau *et al.,* 1980; Kulyk *et al.,* 1989; Tomasek *et al.,* 1982; Kosher *et al.,* 1982). Fibronectin deposition is accompanied by aggregation or condensation of the precartilage mesenchymal cells both *in vivo* (Dessau *et al.,* 1980; Tomasek *et al.,* 1982; Kosher *et al.,* 1982) and *in vitro* (Frenz *et al.,* 1989a,b; Downie and Newman, 1994).

Two types of studies have provided evidence that fibronectin is causally involved in the condensation process. In one set of experiments, heparin-coated polystyrene latex beads mixed with precartilage cells accumulated at sites of condensation by a mechanism that could be inhibited by an antibody directed against the 29-kDa amino-terminal heparin-binding domain of fibronectin (Frenz *et al.,* 1989a). This suggested that limb precartilage cells, which have heparan sulfate proteoglycans on their surfaces (Gould *et al.,* 1992) could increase their packing density in fibronectin-rich regions by a similar adhesion-based process. In another set of experiments, both the antibody against the fibronectin 29-kDa amino-terminal domain and a peptide corresponding to a repeated motif in that domain important for heparin binding (Jaikaria *et al.,* 1991) were found to inhibit condensation formation *in vitro* (Frenz *et al.,* 1989b). Inhibition of mesenchymal condensation by these agents also decreased the amount of cartilage that eventually formed from these cells (Frenz *et al.,* 1989b). This result, along with studies in which chondrogenesis was inhibited following interference with direct cell–cell adhesive interactions within

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condensations (Widelitz *et al.,* 1993; Oberlander and Tuan, 1994), supports the idea that condensation formation or related events are required in order for chondrogenesis to proceed (Newman, 1977, 1988; Solursh *et al.,* 1978; Hall and Miyake, 1993).

The studies described above were performed with precartilage cells derived from the embryonic chicken fore limb, or wing bud. More recently, we have found that cells of the embryonic hind limb, or leg bud, form condensations *in vitro* that have a distinctly different appearance from those formed by wing cells. In contrast to wing mesenchyme which formed broad, flat condensations, leg mesenchyme formed condensations that were more compact, hemispherical, apparently richer in fibronectin, and interconnected by arrays of long fibronectin-containing fibers. Unlike the wing condensations, leg condensations failed to expand and converge with adjacent condensations; rather, they remained highly compact, giving rise to separate nodules of cartilage rather than the uniform sheet of cartilage formed by the wing tissue (Downie and Newman, 1994).

The finding that fibronectin differed in amount and organization between wing and leg mesenchyme *in vitro,* together with the observation that the enhancement of chondrogenesis by transforming growth factor-β (TGF-β) (previously shown to be closely tied to the enhancement of fibronectin gene expression by this factor (Leonard *et al.,* 1991)) was significantly greater in leg mesenchyme than in wing mesenchyme (Downie and Newman, 1994), led us to explore the relationship between fibronectin expression and chondrogenesis in these two types of limb forming tissue. We now report that leg bud mesenchyme produces substantially more fibronectin than wing bud mesenchyme both *in vivo* and *in vitro* and that the difference in fibronectin production between the two tissues is accentuated by exposure to TGF-β. The spatiotemporal deposition of fibronectin in micromass cultures is coincident with the development of precartilage condensations and is altered with transient exposure to TGF-β. The TGF-β-induced changes in fibronectin organization and in precartilage condensation morphology are limb type-specific and correlate with changes in pattern and amount of cartilage produced in treated cultures. Unexpectedly, a monoclonal antibody directed against the 29-kDa amino-terminal domain of fibronectin, which was found to inhibit condensation formation and chondrogenesis in wing mesenchyme previously (Frenz *et al.,* 1989b) and in the present study, failed to disrupt condensations or decrease chondrogenesis in leg mesenchyme, despite its capacity to interfere with the formation of fibronectin fibrils in this tissue. These results provide additional evidence for intrinsic differences between wing and leg bud mesenchyme and indicate that the role of fibronectin differs in the formation of precartilage condensations in these two tissue types.

MATERIALS AND METHODS

Cell culture. Fertile White Leghorn chicken eggs were obtained from Avian Services, Inc. (Frenchtown, NJ). Primary cultures were prepared by separately pooling mesenchyme from the myoblast-free distal 0.3 mm (Newman *et al.,* 1981; Brand *et al.,* 1985) of stage 24 (Hamburger and Hamilton, 1951) wing and leg buds. Cells were dissociated in 1% trypsin-EDTA (Sigma), filtered through Nytex 20-μm monofilament nylon mesh (Tetko, Briarcliff Manor, NY), and washed with and resuspended in medium for plating at 2.5×10^5 cells per 10-μl spot. Much of the limb bud ectoderm remained in sheets after trypsinization and was therefore removed in the filtration step; for most experiments reported there was no additional attempt to remove the ectoderm. Moreover, earlier studies had shown that the distinctive chondrogenic patterns of wing and leg mesenchyme in micromass culture persisted when the ectoderm was specifically removed prior to dissociation of the mesoderm (Downie and Newman, 1994). Cell spots were deposited in Costar 24-well tissue culture plates and allowed to attach for 45 min before wells were flooded with 1 ml of serum-free defined medium (DM (Paulsen and Solursh, 1988): 60% Ham's F-12, 40% Dulbecco's modified Earle's medium (DMEM), 5 μg/ml insulin (Sigma), 10 n*M* hydrocortisone (Sigma), 50 μg/ml L-ascorbic acid, 5 μg/ml chicken transferrin (Sigma)). Media were changed daily.

Cultures that were prepared to study the effects of TGF-β were treated with 1 ng/ml TGF-β1 (R & D Systems, Minneapolis, MN) for 5 hr on Day 1 (24 hr) after plating (Leonard *et al.,* 1991) and otherwise maintained in DM. Inhibition studies used anti-fibronectin monoclonal antibody from clone 304 (a gift of Dr. Steven Akiyama, NIH; Newman *et al.,* 1987). Antibody was included in the culture medium at a concentration of 10 μg/ml from the time of plating. Cultures were maintained for 6 days except where noted.

Quantitative Alcian blue staining of cultures. Cultures were fixed in 10% formalin, 0.5% cetylpyridinium chloride for 5 min, washed with 3% acetic acid, pH 1.0, for 1 min, and then stained overnight with Alcian blue 8GS (Electron Microscopy Sciences; 0.5% in 3% acetic acid) at pH 1.0 (Lev and Spicer, 1964). Cartilage patterns were visualized after washing stained culture spots with 3% acetic acid, pH 1.0, to remove unbound stain. For quantitative studies the bound dye was extracted with 8 *M* guanidinium chloride and measured spectrophotometrically using an EIA reader with a 600-nm filter (Hassell and Horrigan, 1982; Leonard *et al.,* 1989). The amount of Alcian blue bound and extracted under these conditions reflects the relative content of highly sulfated proteoglycan in cohort cultures (Leonard *et al.,* 1989).

Indirect immunofluoresence. Limb mesenchymal cultures were maintained for 2–3 days and then fixed with 2% paraformaldehyde for 20 min, washed with phosphate-buffered saline (PBS), and incubated at room temperature for 10 min with 1% Tergitol NP-40 (Sigma) in PBS. After a 5-min wash in PBS, cultures were incubated for 20 min at room temperature with 2% casein (Sigma) in PBS and then overnight at 4°C with either rabbit anti-fibronectin (A101, Telios, San Diego, CA) or monoclonal anti-fibronectin, clone B3D6 (Gardner and Fambrough, 1983; Developmental Studies Hybridoma Bank, Iowa City). Unbound primary an-

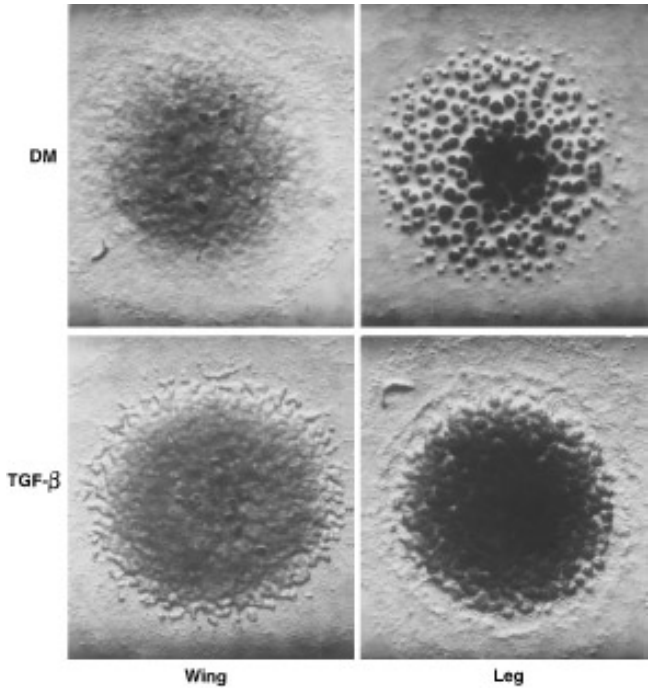


FIG. 1. Morphology of representative Alcian blue stained, 6-day, serum-free micromass cultures prepared from mesenchyme isolated from the distal tips of stage 24 wing and leg buds. DM, wing and leg cell cultures grown in serum-free DM. TGF-β, wing and leg cell cultures grown in serum-free DM and treated on Day 1 after plating with TGF-β1 (1 ng/ml, 5 hr). Macroscopic image; cell spot diameter approximately 5 mm.

tibody was removed by washing cultures with PBS. Fibronectin was visualized by incubating cultures for 1 hr with fluorescein isothiocyanate (FITC)- or Texas Red-conjugated goat anti-rabbit (GAR) or goat anti-mouse (GAM) IgG (Zymed, San Francisco, CA) and viewing through a Bio-Rad Model MRC 1000 confocal microscope or a Zeiss IM 35 inverted microscope with the appropriate fluorescence filters. Controls were incubated without primary antibodies, or with unrelated antibodies of the same isotype.

Western blot analysis. Freshly isolated distal limb tips and Day 2 cell cultures were solubilized in 2× TEGS buffer (0.02 *M* Tris, pH 7.5, 0.02 *M* EDTA, pH 7.3, 20% glycerol, 0.04% lithium dodecyl sulfate, 0.015 *M* dithiothreitol), at a ratio of four tips or two cultures per 40 μl buffer. The tissue was sonicated using a cup horn attachment (Heat Systems, Inc., Farmingdale, NY) until there was no visible cell debris and heated at 100°C for 2 min. Samples were loaded into 7.5% SDS–polyacrylamide gels and electrophoresed for 1 hr (Laemmli, 1970). Proteins were transferred to PVDF membrane (Millipore) by electroblotting for 1 hr in Caps/MetOH buffer (10 m*M* Caps, pH 11.0, 10% MetOH). Blots were blocked for 1 hr at 37°C in casein blocking buffer (20 m*M* Tris base, 154 m*M* NaCl, 0.02% w/v Na azide, 2% w/v casein) then incubated overnight with rocking at room temperature with anti-fibronectin monoclonal antibody B3D6 diluted in casein blocking buffer. Blots were washed with TTBS (Tris-buffered saline, 0.05% Tween), incubated for 1 hr with alkaline phosphatase (AP) conjugated GAM IgG, washed twice with TTBS, and then with TBS.

Fibronectin was visualized by developing blots with AP Immunoblot Assay Developer (Bio-Rad). Relative amounts of protein were quantitated by densitometry (Quick Scan Jr., Helena Laboratories, Beaumont, TX) and normalized against total protein loaded. Some blots were double stained with anti-actin monoclonal antibody, clone 1501 (Chemicon, Temecula CA) to provide a visual reference for protein loading. Amounts of fibronectin accumulated per culture were determined by comparison of densitometric traces of sample blots with a standard dilution series prepared as above with purified chicken fibronectin (gift of Dr. Steven Akiyama).

Northern blot analysis. Total RNA was isolated from freshly harvested distal limb tips of chicken embryos using the RNAzol method as per manufacturer's instructions (Tel-Test Inc., Friendswood, TX). All samples had 260-nm/280-nm absorbance ratios of 1.9–2.0. RNA samples were denatured with formaldehyde/formamide (Sambrook *et al.*, 1989). Samples to which ethidium bromide (EtBr) had been added were electrophoresed on 0.8% agarose gels containing 6.6% formaldehyde in 10% Mops buffer (0.2 *M* Mops, 5 m*M* Na acetate, 1 m*M* EDTA, Sambrook *et al.*, 1989). Gels were rinsed in distilled H_2O, incubated in 50 m*M* NaOH for 30 min, and then neutralized in excess Tris buffer for 30 min.

After Polaroid photography of the gel using ultraviolet transillumination, RNA was directly transferred to nitrocellulose (GeneScreen Plus, NEN Products, Boston, MA) by capillary flow using 20× SSC as the reservoir (1× SSC: 3 *M* NaCl, 3.3 *M* sodium citrate, pH 7.0) for 12–14 hr. Blots were baked for 2 hr at 80°C *in vacuo* and stored at room temperature until use. A 400-bp chicken fibronectin cDNA, pchfn01 (gift of Dr. Richard Hynes, Norton and Hynes, 1987), was labeled with ^{32}P using the Multiprime DNA labeling kit (Amersham, Arlington Heights, IL) to a specific activity of 2×10^8 cpm/μl. Labeled cDNA was denatured in 100 μl prehybridization buffer (5× SSPE, 5× Denhardt's solution (Sambrook *et al.*, 1989), 50% formamide, 1% SDS) and incubated with nitrocellulose membranes which had been prehybridized for 2 hr at 42°C in a shaking water bath.

Hybridization was performed overnight in a shaking water bath at 42°C. Blots were washed with 2× SSPE, 20 min at room temperature and exposed to Kodak XAR-5 film at −80°C using two DuPont Cronex Lightning Plus intensifying screens. Northern Blot signals were quantitiated by scanning densitometry (Quick Scan Jr.).

Poly(A)$^+$ RNA was isolated using the mRNA STAT isolation kit (Tel-Test). First, total RNA was isolated from fresh wing and leg tissues as above, then applied to prepacked oligo(dT)-cellulose columns as per manufacturer's instructions. Poly(A)$^+$ RNA was eluted, air dried, and solubilized in DEPC-treated water. Electrophoresis, transfer, and hybridization were as described above.

Photography of cell cultures. Macroscopic images were obtained with a Nikon binocular dissecting microscope.

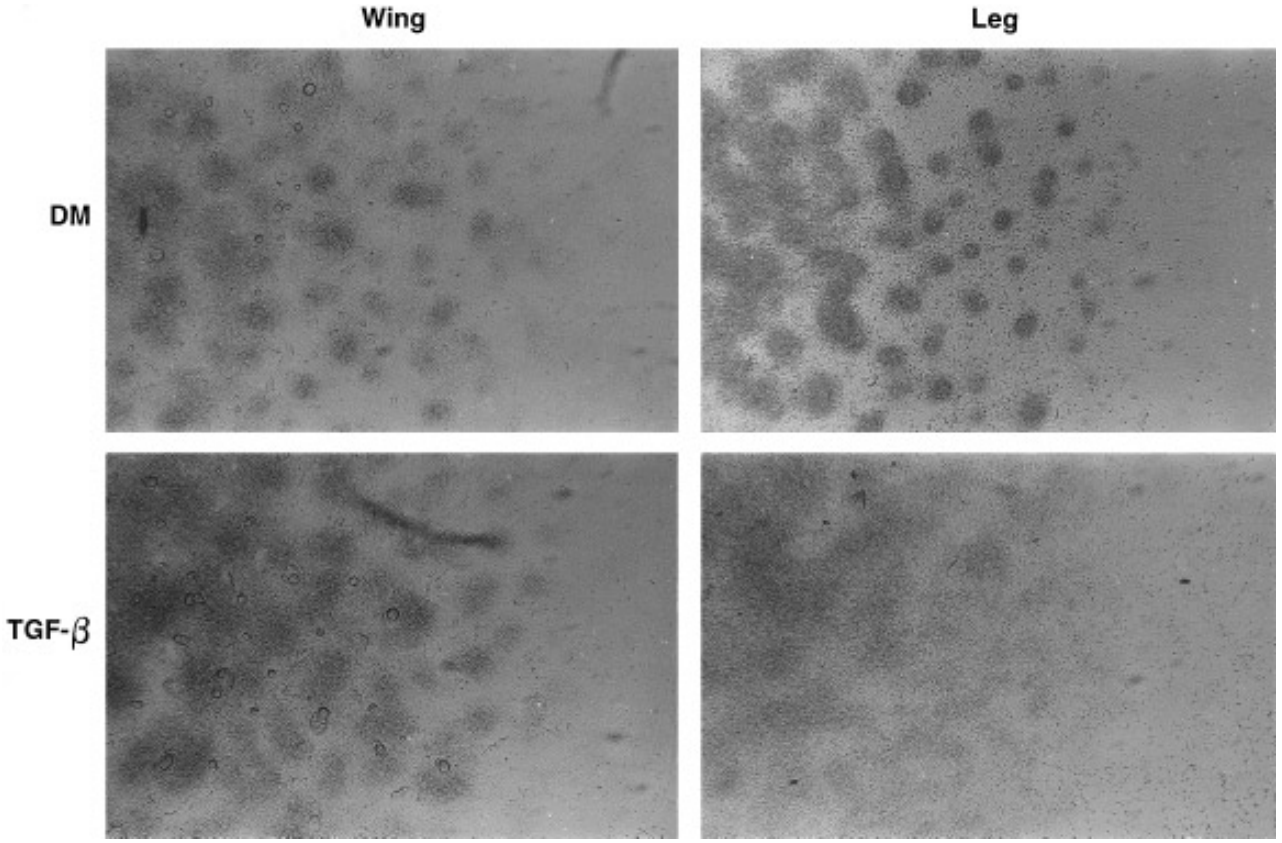


FIG. 2. Precartilage condensations in living $2\frac{1}{2}$-day wing and leg cell cultures prefigure the respective patterns of cartilage that will form by Day 6. The edges of the cultures are shown in these pictures so as to better compare condensation morphologies. In the central regions of wing cultures at this stage condensations are already becoming confluent (see Fig. 7 of Downie and Newman, 1994). DM, wing and leg mesenchyme grown in serum-free defined medium (DM). TGF-β, wing and leg mesenchyme grown in DM and treated on Day 1 with TGF-β1 (1 ng/ml, 5 hr). Condensations are seen as dark patches with Hoffman Modulation-contrast microscopy. Compare with cultures shown in Fig. 1 which were stained $3\frac{1}{2}$ days later. Objective magnification 4×.

Bright-field, phase-contrast, and Hoffman Modulation-contrast microphotographs were obtained with a Zeiss IM 35 inverted microscope. When used as described (Frenz *et al.*, 1989b; Leonard *et al.*, 1991) Hoffman optics permits the visualization of precartilage condensations as dark foci, the intensity of which correlates with cell packing density as assessed by phase contrast microscopy.

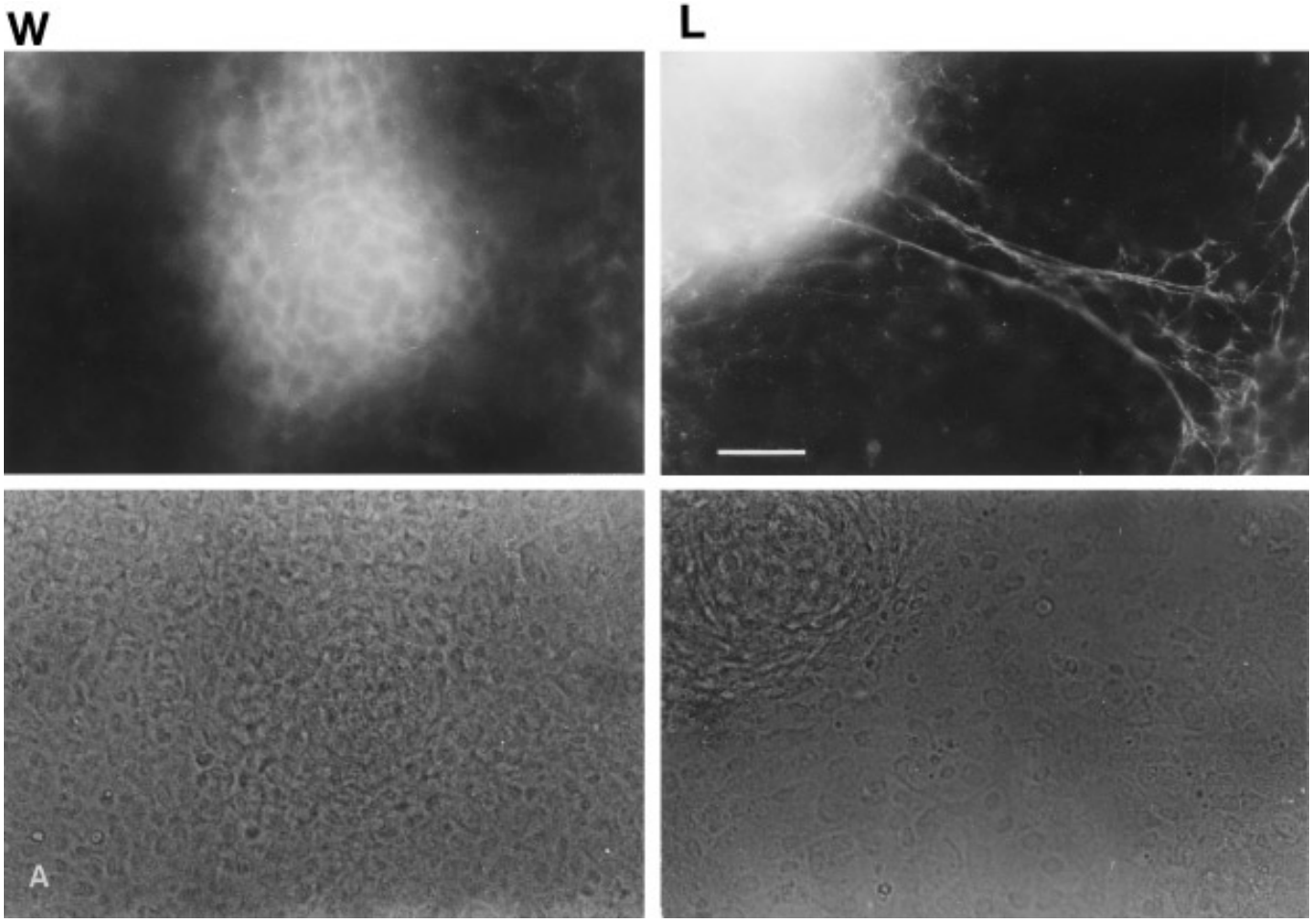
Statistical analysis. All experiments were conducted using companion wells of treated and untreated wing and leg cell cultures. Each single experimental or control sample represents the mean of three to six wells. Results were analyzed by Student's matched pair *t* test.

RESULTS

TGF-β-Induced Changes in Chondrogenic Pattern in Leg Mesenchyme Are Prefigured by Early Changes in Condensation Morphology

Previous studies showed that whereas wing bud precartilage mesenchyme from stages 24–26 chicken embryos dif-

FIG. 3. (A) Fibronectin is organized differently in precartilage condensations that form in cultures of wing and leg mesenchyme. (Top) Immunofluoresence micrograph of $2\frac{1}{2}$-day wing and leg cell cultures. Note the fibronectin-rich fibrils radiating from leg cell condensations. (Bottom)Corresponding phase-contrast images of wing and leg cell condensations. Objective magnification 40×; bar 50 μm. W, wing; L, leg. (B) TGF-β treatment alters the organization of fibronectin in leg cell cultures but not wing cell cultures. (Top) Immunofluoresence micrograph of $2\frac{1}{2}$-day wing and leg cell cultures. Note the enlarged fibronectin-rich condensations and shorter leg-specific fibronectin fibrils corresponding to reduced distance between condensations. (Bottom) Corresponding phase-contrast images of wing and leg cell condensations. Objective magnification 40×.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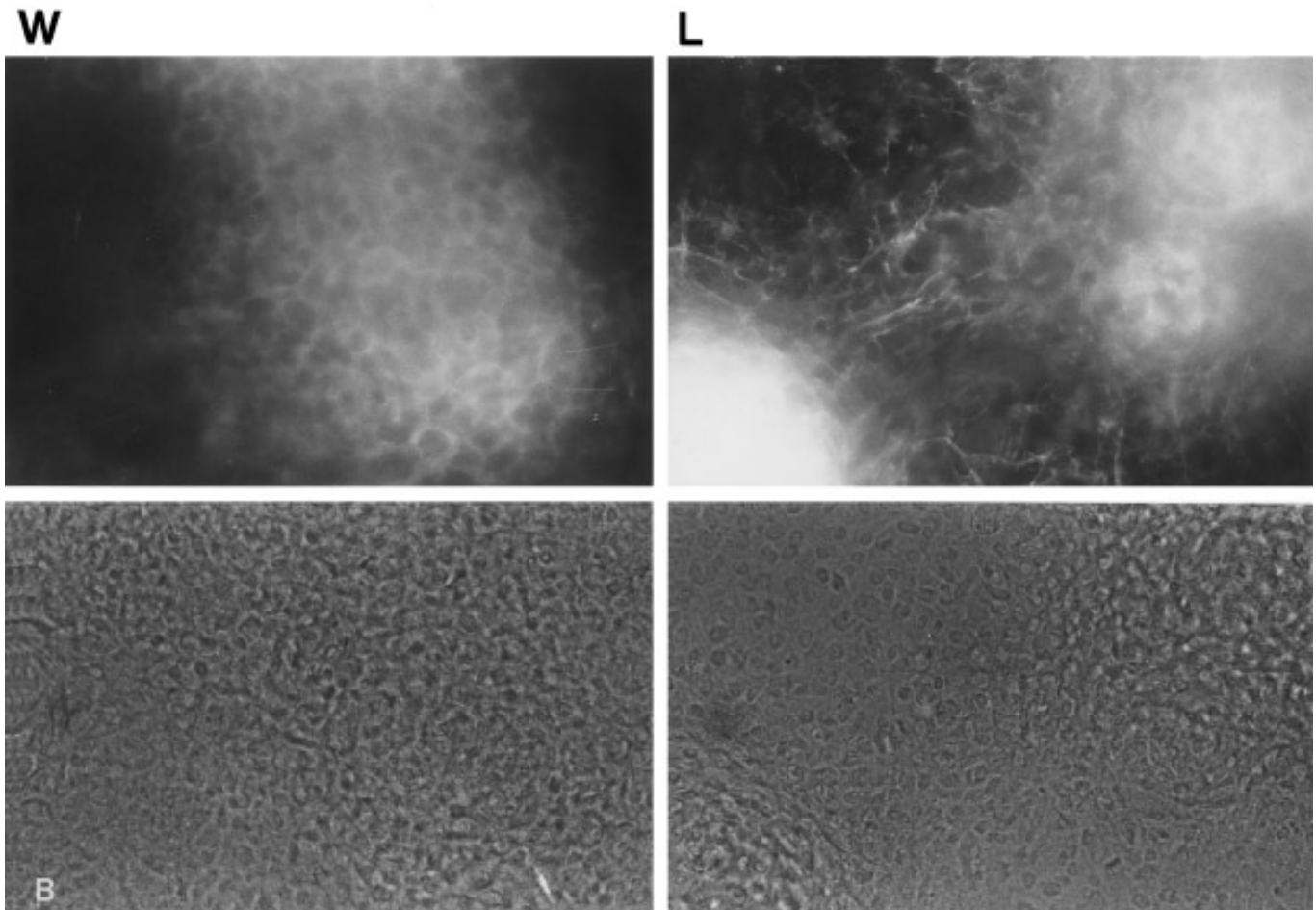
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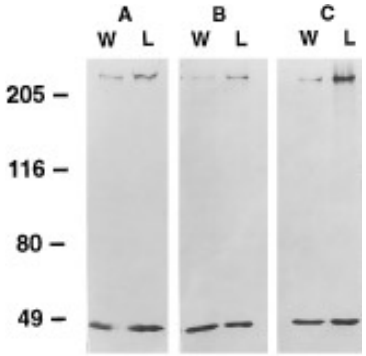

FIG. 4. Immunoblot analysis of fibronectin in freshly isolated distal limb tips (stage 24 embryos) and wing and leg cell cultures. (A) Tissue from freshly isolated distal tips of stage 24 chicken limb buds. (B) Cultures grown in DM for $2\frac{1}{2}$ days. (C) Cultures grown in DM for $2\frac{1}{2}$ days and treated on Day 1 after plating with 1 ng/ml TGF-β1. Blot was double-stained for fibronectin (220 kDa) and actin (43 kDa) which was used to visualize total cell loading. Leg:wing ratio of fibronectin in A was about 1.5 when normalized for loading differences indicated by the actin staining (see Table 1). W, wing; L, leg.

TABLE 1

Accumulation of Fibronectin in Freshly Isolated and Cultured Fore and Hind Limb Precartilage Mesenchyme

	Fresh[a]	DM[a]	TGF-β[a]
Wing	0.27 ± 0.01	0.22 ± 0.02	0.33 ± 0.03
Leg	0.39 ± 0.03*	0.44 ± 0.06*	0.77 ± 0.07*
Leg:wing	1.46	2.01	2.36

Note. Cultures were grown for $2\frac{1}{2}$ days in defined medium (DM); those indicated by TGF-β received 1 ng/ml of the factor for 5 hr on the day after plating.

[a] Data represent arbitrary densitometric units from scans of Western blots. Equivalent loading of lanes was controlled for by DNA measurements and verified by immunostaining of actin. Values are mean ± SEM of three or more experiments.

* Significantly different from wing ($P < 0.05$).

ferentiated into a continuous sheet of cartilage in serum-free culture, leg precartilage mesenchyme from the same range of stages differentiated in a nodular pattern (Downie and Newman, 1994). An example of a pair of stage 24 cultures is shown in Fig. 1, along with companion cultures that were treated transiently with TGF-β1 on the day after plating. As noted previously, TGF-β treatment caused a modest increase in the accumulation of Alcian blue stainable proteoglycan, without affecting the pattern of staining in wing cultures. In leg cultures, in contrast, TGF-β treatment altered the chondrogenic pattern by inducing accumulation of stainable matrix by the internodular cells (Fig. 1).

Because our previous studies indicated that differences in leg and wing mesenchyme could be detected several days before overt chondrogenesis in the form of differences in packing density of the respective precartilage mesenchymal condensations and differences in the distribution and organization of fibronectin, we sought to determine whether the perturbations induced in the chondrogenic pattern by TGF-β could be similarly detected during the condensation stage. After $2\frac{1}{2}$ days of development in culture wing and leg precartilage condensations were visible by Hoffman Modulation-contrast microscopy (Fig. 2). The morphology and packing density of the condensations prefigured the cartilage distribution of the corresponding differentiated tissues. Wing cell cultures produced mesenchymal condensations that were broad and flat (Fig. 2) and which coalesced after 3 days in culture, leading to a continuous sheet of cartilage that stained moderately with Alcian blue (Fig. 1). In contrast, leg cell cultures produced mesenchymal condensations that were compact and nodular (Fig. 2) and gave rise to discrete nodules of cartilage that stained intensely with Alcian blue (Fig. 1).

Treatment of wing and leg cell cultures with TGF-β for 5 hr on Day 1 after plating produced changes in the morphology of the precartilage condensations seen a day and a half later, which in turn corresponded to the changes in cartilage pattern seen in the treated cultures in Fig. 1. TGF-β-treated wing cell condensations were broader and more numerous than those of control cultures (Fig. 2). Leg condensations underwent a more dramatic change in treated cultures, appearing broad and nearly confluent, rather than small and discrete as in the corresponding controls (Fig. 2).

Relationship between Condensation Morphology and Fibronectin Organization

Using confocal and conventional immunofluorescence techniques, we had previously demonstrated differences be-

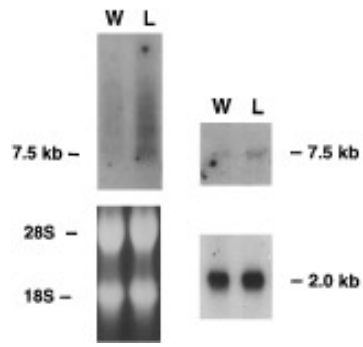

FIG. 5. Northern blot analysis of fibronectin RNA extracted from freshly isolated distal tips of wing and leg buds. W, wing; L, leg. (Left) Total RNA extracted from stage 24 wing and leg tissues (top) and corresponding EtBr-stained 18S and 28S rRNA (bottom). (Right) Poly(A)$^+$ RNA isolated from stage 24 leg tissues showing fibronectin (top) and actin (bottom) RNAs.

tween the extracellular organization of fibronectin in cultures of wing and leg precartilage mesenchyme grown in DM (Downie and Newman, 1994). The differences in fibronectin pattern in those cultures correlated with the different patterns of cartilage produced (Downie and Newman, 1994). We were therefore interested in whether treatment with TGF-β, which is known to induce expression of fibronectin and other extracellular matrix macromolecules (Massagué, 1990) exerted its effects on the cartilage pattern of leg cell cultures through changes in the accumulation and organization of fibronectin.

Wing and leg cell cultures were fixed and stained with a monoclonal antibody against fibronectin after $2\frac{1}{2}$ days of growth (Fig. 3). Wing cells grown in DM (Fig. 3A, top left) produced fibronectin that was diffusely organized around the cells in the central region of the precartilage condensations (Fig. 3A, bottom left). In leg cell cultures, fibronectin staining was more intense in the condensations, suggesting the presence of higher concentrations of the glycoprotein (Fig. 3A, top right). In addition, fibronectin-rich fibrils, some as long as 300 μm, spanned the nonstaining regions between leg cell condensations. No fibronectin-rich fibrils were seen in wing cell cultures; instead, the fibronectin staining was diffuse and amorphous (Fig. 3A, top left).

The effects of TGF-β treatment on fibronectin were most pronounced in leg cell cultures where enlarged cellular condensations, which stained intensely for fibronectin, encompassed a substantial portion of the microscopic field (Fig. 3B, top right). The leg-specific fibronectin fibrils seen in control cultures were shorter, corresponding to the narrower intercondensation spaces (Fig. 3B, top right). Fibronectin was more broadly distributed in condensations of treated wing cell cultures (Fig 3B, top left) corresponding to the expansion of the condensations themselves in these cultures (Fig. 2).

Relative Abundance of Fibronectin in Wing and Leg Mesenchyme in Vivo and in Vitro

The relative amounts of fibronectin accumulated in freshly isolated wing and leg tissues and in cultures were determined by immunoblot analysis: A representative blot double-stained for fibronectin (220 kDa) and actin (43 kDa) contained equal cell numbers of freshly isolated stage 24 wing and leg mesenchyme (Fig. 4A), wing and leg cultures grown for $2\frac{1}{2}$ days in DM (Fig. 4B), and $2\frac{1}{2}$-day wing and leg cultures treated with TGF-β1 for 5 hr on Day 1 (Fig. 4C). Fibronectin was more abundant in leg than in wing tissues at all stages, ranging from 46% greater in fresh tissues isolated before condensations had begun to form to twice as abundant during the period of condensation at $2\frac{1}{2}$ days in culture. In the TGF-β-treated cultures, fibronectin was 136% more abundant in leg than in wing tissues when condensations were forming (Table 1). On the basis of immunoblot analysis of a standard dilution series of chicken fibronectin, we estimate that freshly isolated wing bud precartilage mesenchyme contained 100–200 ng fibronectin per 10^6 cells.

Fibronectin RNA in Freshly Isolated Wing and Leg Tissues

Northern blot analysis of RNA in freshly isolated wing and leg mesenchyme from stage 24 embryos indicated the presence of approximately 2.5-fold as much fibronectin RNA in leg mesenchyme as in wing mesenchyme (Fig. 5). The fibronectin RNA ratios in poly(A)$^+$ RNA samples from wing and leg tissues were similar to those in total RNA samples, suggesting that processing of fibronectin pre-mRNA was not likely to be the cause of the differences between wing and leg in fibronectin messenger RNA amounts. The greater amount of fibronectin mRNA in leg tissue than in wing tissue was consistent with the greater amount of fibronectin protein in freshly isolated leg mesenchyme and leg cell cultures compared with the corresponding wing preparations (Fig. 4).

Effect of Blocking a Fibronectin Functional Domain

The role of fibronectin in the condensation process was addressed in an earlier study (Frenz *et al.*, 1989b) in which monoclonal antibody 304, which recognizes the amino-terminal domain of fibronectin (Newman *et al.*, 1987) inhibited condensation formation in wing cell cultures grown in serum-containing medium. In the present study we used immunofluoresence and confocal microscopy to examine the effects of monoclonal antibody 304 on wing and leg cells after 2 days of serum-free culture in DM or in DM containing 10 μg/ml Mab 304 (Fig. 6).

The disruption of cell–fibronectin interactions in Mab 304-treated wing cultures was evident in the decrease in localized concentrations of fibronectin and by condensations in which cells were more loosely organized (Fig. 6A, left panels). Moreover, accumulation of highly sulfated proteoglycan at 6 days was reduced by 50% in antibody-treated wing cell cultures (Fig. 7; Table 2), similarly to the effects of Mab 304 on serum-containing wing cultures (Frenz *et al.*, 1989b). In Mab 304-treated leg cell cultures the amount of fibronectin in the condensations was also decreased relative to controls, but the condensations themselves remained discrete and compact (Fig. 6A, right panels). The amount of highly sulfated proteoglycan accumulated in leg cell cultures treated with Mab 304 for 6 days did not differ from controls (Fig. 7; Table 2). Thus, in contrast to the results with wing cell cultures, Mab 304 affected neither condensation morphology nor chondrogenesis in leg cell cultures. Strikingly, however, the long fibronectin fibrils characteristic of the leg cell cultures were almost completely disrupted by treatment with Mab 304 (Figs. 6A, lower right, and 6B).

DISCUSSION

We have shown that the synthesis and spatiotemporal distribution of fibronectin in cultures of both fore and hind limb precartilage mesenchyme are closely correlated with the formation and organization of precartilage condensa-

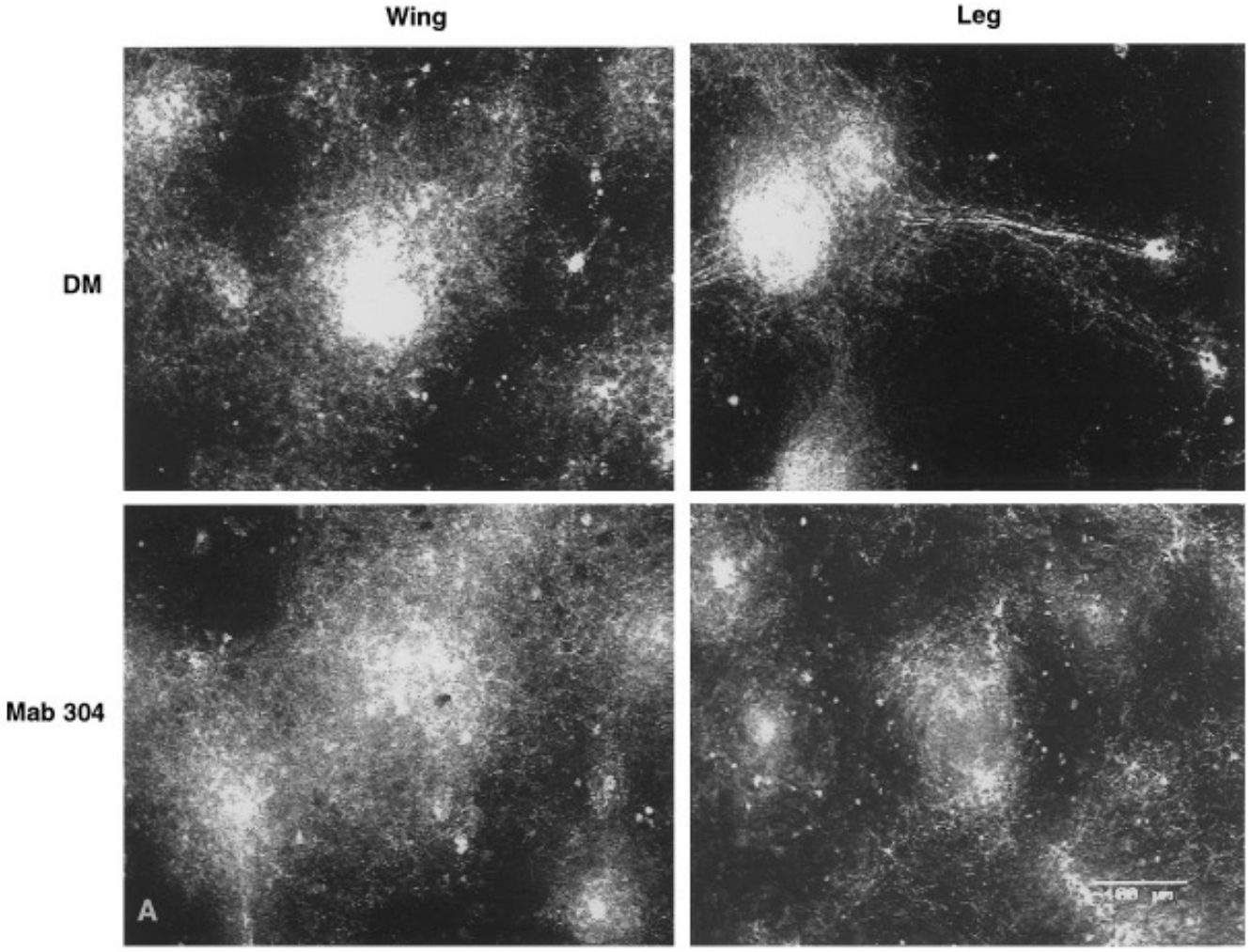

FIG. 6. Antibody to the amino-terminal domain of fibronectin disrupts fibronectin in cultures of wing and leg precartilage mesenchyme. (A) Extended focus confocal immunofluoresence images of wing and leg mesenchyme after 2 days of culture in DM (top panels) and in DM containing anti-fibronectin antibody Mab 304. (B) Higher magnification confocal immunofluorescence image of long fibronectin-rich fibers in control (top) and Mab 304-treated (bottom) leg cell cultures.

tions. We have also found that the extent of fibronectin gene expression, both in untreated wing and leg cultures and in cultures treated with TGF-β, are predictive of the amount of cartilage that will form in these cultures several days later. Finally, while our results confirmed that the amino-terminal heparin-binding domain of fibronectin plays the key role in mediating precartilage condensation in serum-free culture of wing mesenchymal cells that it was previously found to play in serum-containing culture of this cell population (Frenz *et al.,* 1989a,b), blocking this fibronectin domain had no effect on leg mesenchymal condensation.

Limb bud precartilage mesenchymal cells exhibit intrinsic pattern forming capability in high-density culture despite their being removed from *in situ* sources of morphoregulatory activity. In particular, the wing and leg micromass cultures described here lacked a microvascular system, a potential source of morphogenetic nonuniformity in the embryonic limb (Caplan and Koutroupas, 1973; Feinberg and Saunders, 1982; Feinberg *et al.,* 1983). These cultures were also essentially devoid of a myogenic subpopulation of cells whose presence might subdivide centers of chondrogenic activity (Newman *et al.,* 1981; Downie and Newman, 1994) and contained only minimal amounts of limb ectoderm, a source of FGF and Wnt-7A activities (Niswander *et al.,* 1993; Fallon *et al.,* 1994; Parr and McMahon, 1995; Yang and Niswander, 1995). Moreover, endogenous gradients in the distribution of Hox A-D gene products (Duboule, 1992) and Sonic hedgehog protein or peptides (Riddle *et al.,* 1993) were completely disrupted by the cell dissocia-

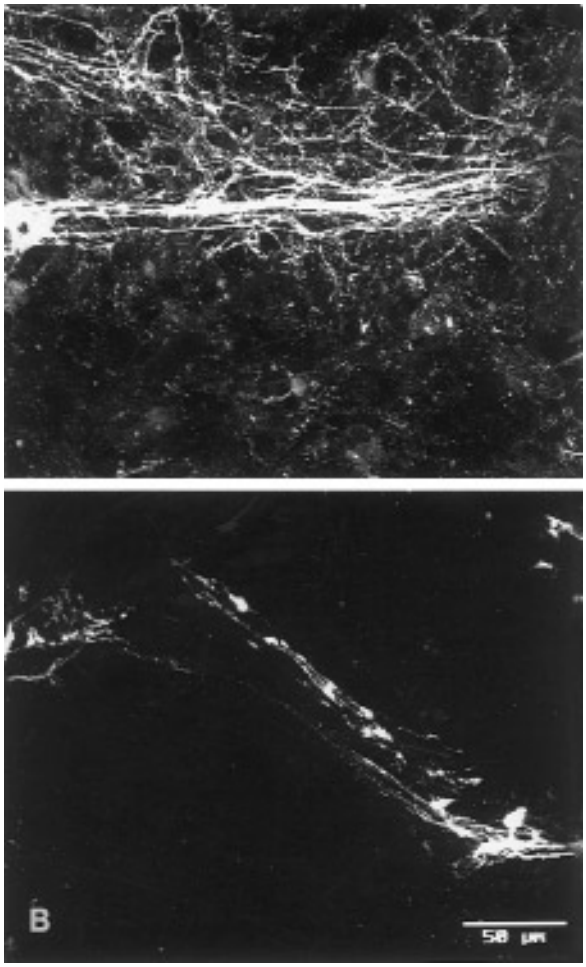

FIG. 6—*Continued*

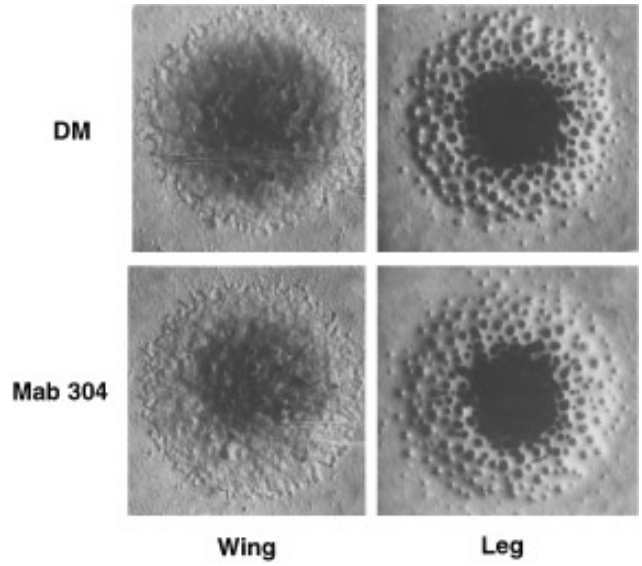

FIG. 7. Antibody against fibronectin amino terminus reduces accumulation of cartilage-specific proteoglycan in wing, but not leg precartilage mesenchyme. Cultures were grown for 6 days and stained with Alcian blue, pH 1.0, as described under Materials and Methods. Left, top panel, control wing micromass culture; bottom panel, Mab 304-treated wing micromass culture; Right, top panel, control leg micromass culture; bottom panel, Mab 304-treated leg micromass culture.

tion procedures used to prepare the cultures. The formation of expanding or confined precartilage condensations, leading to a uniform sheet or nodular pattern of cartilage depending on the limb type origin of the mesenchyme, is therefore a ''self-organizing'' property of these tissues (Newman, 1993).

One important aspect of this self-organizing capacity is the production of fibronectin, which, in wing mesenchyme, as shown here and in earlier studies (Frenz *et al.*, 1989a,b), is functionally involved in establishing foci of condensation. Because the function-blocking Mab 304 was present continuously from the time of plating in the treated leg cultures, we do not believe that its failure to disrupt leg mesenchymal condensations was due to lack of access to the fibronectin amino terminus by virtue of the presence of other extracellular matrix components. Nor do we think it likely that fibronectin plays no role at all in mediating leg condensation formation. The coincident distribution of fibronectin with, and its abundance in, condensing leg mesenchyme and the upregulation and expansion of its domain of expression as internodular cells are recruited into condensations in response to TGF-β strongly suggest that it is intimately involved in this process. We suggest rather, that a different domain, or domains, of fibronectin may be involved in mediating leg condensation. Attempts to explore the possible role in leg condensations of the integrin-binding domain of fibronectin by using RGDS-containing peptides (which were previously found to be ineffective in inhibiting wing condensations (Frenz *et al.*, 1989b)), were unsuccessful, since these peptides caused the micromasses to detach in

TABLE 2

Effects of Antibody Against Fibronectin Amino Terminus on Accumulation of Proteoglycan in Cultures of Fore and Hind Limb Precartilage Mesenchyme

	DM[a]	DM + mAb 304[a]
Wing	0.188 ± 0.03	0.081 ± 0.01*
Leg	0.232 ± 0.03	0.257 ± 0.05

Note. Cultures were grown for 6 days in defined medium (DM) in the absence or presence of 10 μg/ml mAb 304.

[a] Data represent OD_{600} of GuHCl-extracted Alcian blue stain from stage 24 cultures. Values are mean ± SEM of three experiments.

* Significantly different from control ($P < 0.05$).

the serum-free culture conditions employed in this study. We are currently approaching this question by introducing dominant-negative forms of fibronectin into the precartilage extracellular matrix.

Whatever the extracellular determinants of leg precartilage condensation may be, it is significant that the prominent fibronectin-rich fibers that span the distance between the tightly organized condensations in the developing leg mesenchyme are not necessary for the formation or maintenance of those condensations: blocking their assembly by treatment with Mab 304 (see also McDonald *et al.,* 1987) had no effect on the pattern of condensations or on the subsequent pattern or extent of chondrogenesis (Figs. 6C, 7; Table 2). These fibers, which resemble those seen in sections of early avian hind limbs (Hurle *et al.,* 1989), may be a consequence of tensions generated by the formation of the highly compact leg condensations rather than a determinant of their morphology.

Because blocking the amino-terminal domain of fibronectin did not completely eliminate condensation formation, or reduce chondrogenesis by more than about 50%, even in wing mesenchyme (Frenz *et al.,* 1989b; this study, Fig. 7, Table 2) it is likely that other domains of fibronectin or other cell–matrix or cell–cell interactions contribute to condensation formation in both fore and hind limb mesenchyme. We note, for example, that like fibronectin, N-cadherin been shown to play a part in mediating condensation formation in fore and hind limb mesenchyme (Oberlander and Tuan, 1993) and it is upregulated in this tissue in response to TGF-β (Tsonis *et al.,* 1994). Tenascin, which is similarly distributed (Mackie *et al.,* 1987; Pacifici *et al.,* 1993), and may modulate fibronectin activity (Chiquet-Ehrisman *et al.,* 1988), is also a candidate for mediating differential morphogenetic effects in wing and leg mesenchyme.

Leg mesenchyme produced more fibronectin than wing mesenchyme, both in freshly isolated tissue where condensations had not yet begun to form and in foci of condensation in $2\frac{1}{2}$-day cultures, and was more responsive than wing mesenchyme to the fibronectin-inducing effects of TGF-β (Fig. 4). This is consistent with the idea that TGF-β or a related activity is an endogenous regulator of fibronectin production and hence condensation formation in limb bud mesenchyme (Newman, 1988, 1993; Leonard *et al.,* 1991) and that leg precartilage cells are inherently more responsive than wing precartilage cells to this activator of chondrogenesis (Downie and Newman, 1994). Indeed, the wing–leg differences we have observed may have arisen, in part, from intrinsic differences in the level of production of such an activator. Because of the positive autoregulation of TGF-βs (van Obberghen-Schilling *et al.,* 1988) even small differences of this sort could become quite pronounced at particular locations within a tissue mass. The fact that leg condensations remained confined to small domains unless stimulated by exogenous TGF-β, in contrast to wing condensations, which expanded and eventually fused, suggests that leg cells may also be more active than wing cells in producing a diffusible inhibitor of chondrogenesis from incipient centers of cartilage formation. The identity of such an inhibitor is presently unknown, but evidence for its existence in the embryonic mouse limb has recently been presented by Lee and co-workers (1994), who suggest that it acts to prevent the potentially chondrogenic cells of the interdigital mesenchyme from differentiating into cartilage. Systems of diffusible activators and inhibitors are capable of giving rise not only to patterns of expanding or discrete foci similar to those seen in the micromass cultures described here, but to patterns of bars like those seen in the developing limb (Ouyang and Swinney, 1991; Newman and Frisch, 1979; Newman, 1993). Our results may therefore be relevant not only to an understanding of the differences between fore and hind limb development, but to their similarities.

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