

Morphogenetic Differences between Fore and Hind Limb Precartilaginous Mesenchyme: Relation to Mechanisms of Skeletal Pattern Formation

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Accepted October 29, 1993

Using a serum-free culture system, we have investigated morphogenetic differences between fore and hind limb precartilaginous mesenchymal cells derived from stages 21-26 chicken embryos. Across all stages, wing and leg cultures were intrinsically different in the amount and spatial organization of cartilage that they produced. By stage 24, leg cells began to produce a nodular pattern of cartilage while wing cells continued to produce sheets of cartilage. This pattern difference persisted throughout the later stages and was not due to differences in cell survival as judged by DNA content or to the presence of distinctive cell subpopulations in either tissue, as determined by flow cytometry. Chondrogenesis in wing and leg precartilaginous cell cultures was affected differently by 10% fetal bovine serum, TGF- β 1 (1 ng/ml for 5 hr on the day after plating), and retinoic acid (5 ng/ml). In wing cultures, the extent of chondrogenesis was significantly enhanced by serum or by a combination of TGF- β 1 and retinoic acid, but the cartilage pattern was not altered with any treatment. In leg cultures, the extent of chondrogenesis was enhanced by TGF- β 1 alone, inhibited by retinoic acid alone, and the cartilage pattern was changed from nodular to sheet-like by treatment with TGF- β 1. Wing and leg cultures also differed from each other in relative cohesivity and in condensation morphology and organization of fibronectin during the early phase of differentiation. Wing cells produced broad, flat condensations containing diffusely organized fibronectin, whereas leg cells elaborated an extensive network of long fibronectin-rich fibrils connecting very compact, fibronectin-rich condensations. These intrinsic and induced differences in fore and hind limb mesenchyme provide insight into the mechanisms that are common to the formation of all limb skeletal elements and those that distinguish skeletal elements from different limb regions and limb types. © 1994

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Limb type differences in morphogenetic capability are thought to be present when the limb field is first established and to reside in the mesenchymal component of the limb bud (Saunders, 1948; Saunders *et al.*, 1959; Zwillling, 1968; MacCabe *et al.*, 1973; Stephens *et al.*, 1989). Grafting experiments on early stage chicken embryos, for example, have shown that the capacity to form either wing or leg structures is established before limb outgrowth begins and cannot be altered by heterotopic transplantation of either the mesenchymal or ectodermal components of the developing limb (Saunders *et al.*, 1959; Zwillling, 1968; MacCabe *et al.*, 1973; Stephens *et al.*, 1989).

The skeletons of all vertebrate limbs are initially established by the apportionment of precartilaginous mesenchyme into chondrogenic and nonchondrogenic domains (Newman, 1977; Newman, 1988). Position-dependent chondrogenesis is regulated by processes which are common to all limb types and limb skeletal elements as well as by processes that differ between limb types and individual limb skeletal elements. Common processes include those, such as local accumulation of fibronectin (Dessau *et al.*, 1980; Tomasek *et al.*, 1982; Kosher *et al.*, 1982; Glant *et al.*, 1985), that promote precartilaginous mesenchymal condensation (Frenz *et al.*, 1989a, b; Hall and Miyake, 1992). Distinguishing processes include the effects of growth and differentiation factors, such as retinoids and their receptors (Ide and Aono, 1988; Eichele, 1989; Maden *et al.*, 1989), and *Hox* gene products (Mackem and Mahon, 1991; Duboule, 1992; Coelho *et al.*, 1992; Morgan *et al.*, 1992), which vary in concentration across individual limb buds and between fore and hind limbs. Common and distinguishing processes presumably account, respectively, for why limb skeletal elements resemble one another and why they are distinctive.

Both the common and distinguishing processes of chondrogenesis can be studied in high-density cultures of precartilaginous mesenchyme. But while there is an ex-

INTRODUCTION

During the development of tetrapod vertebrates, mesenchymal cells of the fore and hind limbs differentiate into cartilage in homologous, but distinct patterns.

tensive literature on common mechanisms of limb chondrogenesis *in vitro* (Newman, 1977; Solursh *et al.*, 1978; Paulsen *et al.*, 1988; Frenz *et al.*, 1989a, b; Kulyk *et al.*, 1989; Leonard *et al.*, 1989, 1991; Chen *et al.*, 1991; Tuan, 1991; Jiang *et al.*, 1993), there has been little attention to the basis of variation in morphogenetic activities in limb precartilaginous mesenchyme from different sources (however, see Aono and Ide, 1988). Here we report that chicken fore and hind limb precartilaginous mesenchyme, over a range of embryonic stages, exhibit distinctive patterns of chondrogenesis *in vitro*. The two cell populations also differ from each other in accumulation of cartilage-specific proteoglycan, responses to serum, TGF- β 1, and all-*trans*-retinoic acid and, prior to overt chondrogenesis, in the extracellular organization of fibronectin.

Our results suggest that intrinsic differences in fore and hind limb bud precartilaginous mesenchyme can modulate the activities of pattern-forming processes common to both tissues. These properties may contribute to differences in size and shape of corresponding fore and hind limb skeletal elements *in vivo* and may account for the well-known differences in susceptibility to teratogens of fore and hind limbs in a variety of species.

MATERIALS AND METHODS

Cell culture. Fertile eggs were obtained from Avian Services, Inc. (Frenchtown, NJ). Primary cultures were prepared by separately pooling mesenchyme from the distal 0.3 mm of stages 21–26 (Hamburger and Hamilton, 1951) wing and leg buds. Cells were dissociated in trypsin, washed, and resuspended for plating at 2.5×10^5 cells per 10- μ l spot, unless otherwise noted. 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 medium (DM (Paulsen and Solursh, 1988): 60% Ham's F12, 40% DMEM, 5 μ g/ml insulin, 100 nM hydrocortisone, 50 μ g/ml L-ascorbic acid, 5 μ g/ml chicken transferrin (Sigma)). Media were changed daily. For some experiments, ectoderm was removed from isolated distal tips. Tips were incubated for 20 min in cold calcium- and magnesium-free Earle's balanced salt solution (CMF EBSS, Gibco) containing 2% trypsin (Sigma). The trypsin solution was removed and the tips were incubated at 37°C for 20 min in CMF EBSS containing 1% EDTA (Sigma). Tips were then transferred to cold CMF EBSS containing 10% fetal bovine serum (FBS, Celsis Silver; GIBCO) and the ectoderm was dissected free with fine needles. The mesodermal cells were dissociated and resuspended for plating as described above. Some cultures received the following treatment. Serum: cultures were maintained in medium

containing 10% fetal bovine serum. Transforming growth factor- β (TGF- β): cultures were treated with 1 ng/ml TGF- β 1 (R & D Systems, Minneapolis, MN) for 5 hr on Day 1 after plating (Leonard *et al.*, 1991) and otherwise maintained in DM. Retinoic acid: cultures were maintained with 5 ng/ml all-*trans*-retinoic acid (RA; Sigma) in DM. RA/TGF- β : cultures were maintained with 5 ng/ml RA in DM and received 1 ng/ml TGF- β 1 for 5 hr on Day 1 after plating. Cultures were maintained for 6 days except where noted.

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 culture spots with 3% acetic acid, pH 1.0, to remove unbound Alcian blue. Content of highly sulfated proteoglycan was determined by extraction of the bound dye with guanidinium chloride (8 M) and spectrophotometric quantitation using an EIA reader with a 600-nm filter (Hassell and Horrigan, 1982; Leonard *et al.*, 1989).

Flow cytometry. The forward and right angle light scatter of unstained freshly prepared stage 24 wing and leg mesenchymal cells were measured in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). More than 3 million cells from each tissue were analyzed. Data were acquired and processed using Consort 32 software (Becton-Dickinson).

DNA determination. The DNA content of cell cultures was determined by the Hoechst dye binding method (Labarca and Paigen, 1980) using a Hoefer TKO 100 spectrofluorometer. Cultures were harvested in 50 μ l/well Labarca-Paigen buffer (0.05 M NaPO₄, 2.0 M NaCl, pH 7.4) and sonicated using a Branson Model 250 sonifier with a cup horn attachment (Heat Systems, Farmingdale, NY) until no cell debris was visible (15 sec). The DNA content of prepared samples was determined in Labarca-Paigen buffer containing 1 μ g/ml of Hoechst 33258 reagent.

Indirect immunofluorescence. Limb mesenchymal cultures were maintained for 2–3 days and then fixed for 20 min with 2% paraformaldehyde, washed with phosphate-buffered saline (PBS: 130 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄), and incubated at room temperature for 10 min with 1% Tergitol Type NP-40 (Sigma) in PBS. After a 5-min wash in PBS, cultures were incubated for 20 min at room temperature with 2% casein in PBS and then overnight at 4°C with a rabbit polyclonal antibody to fibronectin (A101, Telios, San Diego, CA) in PBS containing 2% casein. Unbound primary antibody was removed by rinsing cultures with

PBS. Fibronectin was visualized by incubating cultures for 1 hr with FITC-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) and viewing through a Bio-Rad Model MRC 600 confocal microscope using the krypton-argon laser and the K1K2 filter set. Controls were incubated without primary antibodies or with unrelated antibodies of the same isotype.

Photography of cell cultures. Macroscopic images were obtained with a Nikon binocular dissecting microscope. Bright-field, phase-contrast, and Hoffman Modulation-contrast microphotographs were obtained with a Zeiss IM 35 inverted microscope.

Statistical analysis. All experiments were conducted with companion wells of wing and leg cell cultures and results were analyzed by Student's matched-pair *t* test. Each single experimental or control sample represents the mean Alcian blue or DNA measurement for three to six wells.

RESULTS

Patterns of Chondrogenesis and Accumulation of Proteoglycan in Wing and Leg Precartilaginous Mesenchymal Cultures

Precartilaginous mesenchyme was isolated from the distal tips of chick embryo fore and hind limb buds and grown in serum-free micromass culture. Six-day cultures stained with Alcian blue exhibited stage-dependent and limb-type-dependent patterns of cartilage. Precartilaginous cells isolated from both fore and hind limbs of early stage embryos (H.H. 21–23) differentiated into relatively uniform sheets of cartilage by Day 6 in culture (Fig. 1). In contrast, fore and hind limb cells isolated from later stage embryos (H.H. 24–26) produced chondrogenic cultures that could readily be distinguished from one another (Fig. 1). Wing cells from these later stages, like those of the earlier stages, differentiated into a diffusely staining, uniform sheet of cartilage, whereas leg cells differentiated into several dozen darkly staining cartilage nodules separated by flatter regions of tissue that failed to stain with Alcian blue (Fig. 1). High magnification views of characteristic regions of stage 24 wing and leg cell cultures (Fig. 2) confirmed that the internodular regions of the leg cultures were fully populated with cells. These limb-type differences in pattern were identical whether or not the ectoderm was removed from the limb tips prior to culturing the mesenchyme and were unchanged over a range of plating densities ($1.7\text{--}3.0 \times 10^5$ cells per $10\text{-}\mu\text{l}$ spot). Subsequent experiments were therefore performed with limb tips from which the ectoderm had not been removed, at a standard cell density of 2.5×10^5 cells per spot. When cultures derived from stages 24–26 limbs

were grown for up to an additional week there was no change in cartilage morphology: wing and leg cultures were as distinct from one another as they had been at 6 days (data not shown).

These results were not due to differences in cell survival over the 6-day culture period nor to heterogeneity of the initial populations. First, the DNA content of cultures derived from all of the embryonic stages studied indicated that there was a gradual increase in cell number of both the fore and hind limb cultures to an average of twice as many cells at Day 5 as at Day 1 (Figs. 3A, 3B, and 3C). Second, flow cytometry analysis of the cell populations derived from stage 24 wing and leg bud distal tips indicated that the two mesenchymal populations did not contain different proportions of distinct cell types (Fig. 4). Finally, no myoblasts were detectable in cultures prepared from stages 24–26 wing or leg distal tips, in agreement with fate mapping studies (Newman *et al.*, 1981; Brand *et al.*, 1985).

The amount of Alcian blue stainable matrix extracted with guanidinium chloride changed somewhat according to the embryonic stage of the donor, but leg cultures always accumulated more stainable matrix than wing cultures (Table 1), even after stage 23 when the leg cultures exhibited a nodular morphology (Fig. 1).

Effects of Serum, TGF- β , and Retinoic Acid

In preliminary experiments using serum-containing medium, we had been unable to establish chondrogenic cultures using leg bud mesenchyme. We therefore investigated the effects of added serum in the defined medium used in the present study. Fetal bovine serum (10%) enhanced the amount of Alcian blue staining of wing cell cultures by 5-fold (Table 2) without changing the uniform pattern of staining observed in the serum-free cultures (compare the wing serum panel of Fig. 5 to the stage 24 wing panel of Fig. 1). Although serum treatment did not lead to significant changes in leg cell cultures with regard to the amount of Alcian blue staining (Table 2), the nodular pattern was eliminated and the diameter of the chondrogenic area was reduced (compare the leg serum panel of Fig. 5 to the stage 24 leg panel of Fig. 1). In addition, serum had different effects on the growth and/or survival of wing and leg mesenchyme as monitored by DNA content after 5 days of continuous exposure (Table 3). Whereas the DNA content of wing cell cultures increased an average of 2.5-fold in the presence of serum, there was no significant increase in DNA content in leg cell cultures (Table 3).

The effects of serum led us to ask whether wing and leg mesenchyme would also respond differently to TGF- β and retinoids, two classes of molecules that are pres-

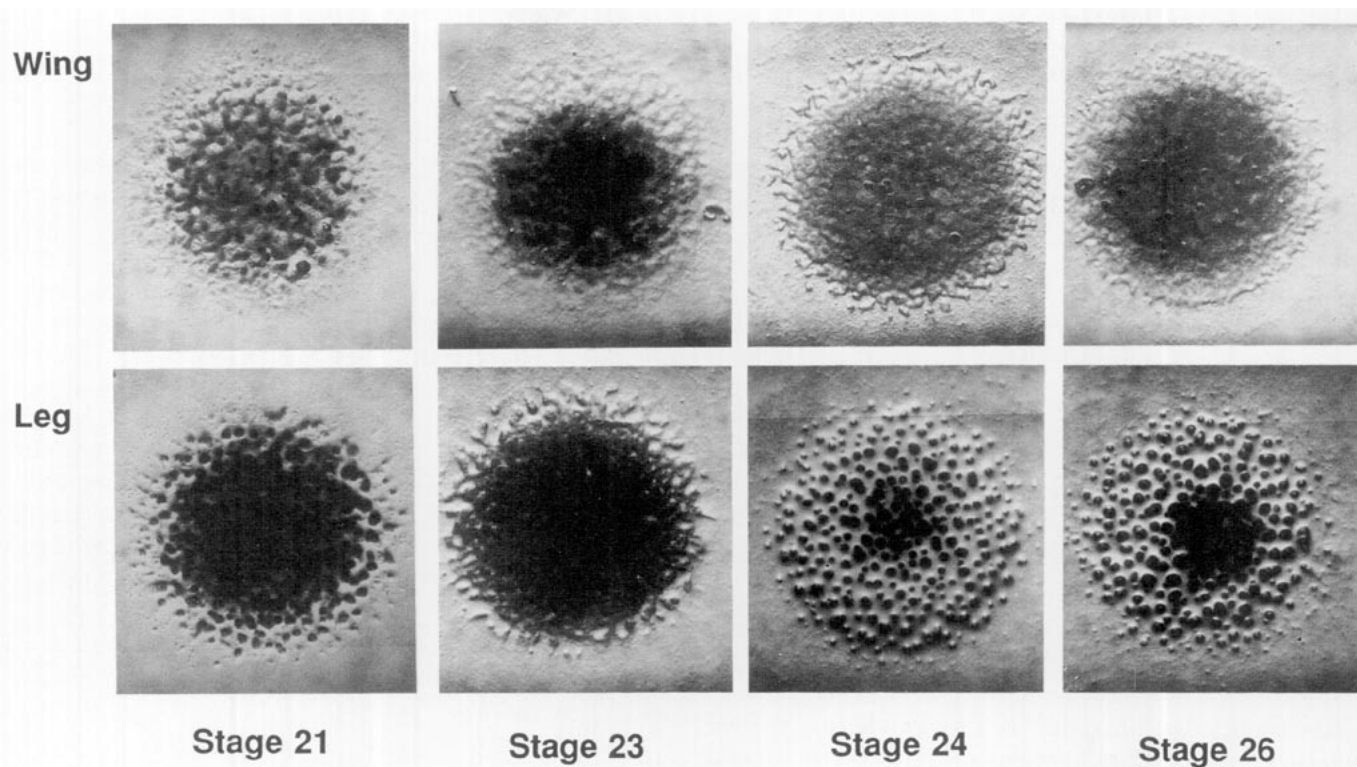


FIG. 1. Morphology of representative Alcian blue stained, 6-day, serum-free micromass cultures prepared from mesenchyme isolated from the distal tips of stages 21, 23, 24, and 26 wing and leg buds. Macroscopic image; cell spot diameter approx. 5 mm.

ent endogenously in limb mesenchyme (Heine *et al.*, 1987; Leonard *et al.*, 1991; Thaller and Eichele, 1987) and have been proposed to be involved in pattern formation. Serum-free wing mesenchymal cultures exposed transiently to TGF- β 1 contained about the same amount of proteoglycan (Table 2) and retained the diffuse, uniform

pattern of staining seen in controls (compare the wing TGF- β panel of Fig. 5 to the stage 24 wing panel of Fig. 1). In contrast, leg mesenchymal cultures exposed to TGF- β 1 contained an average of 55% more proteoglycan than controls (Table 2) and produced a sheet-like pattern of cartilage similar to that seen in wing cultures

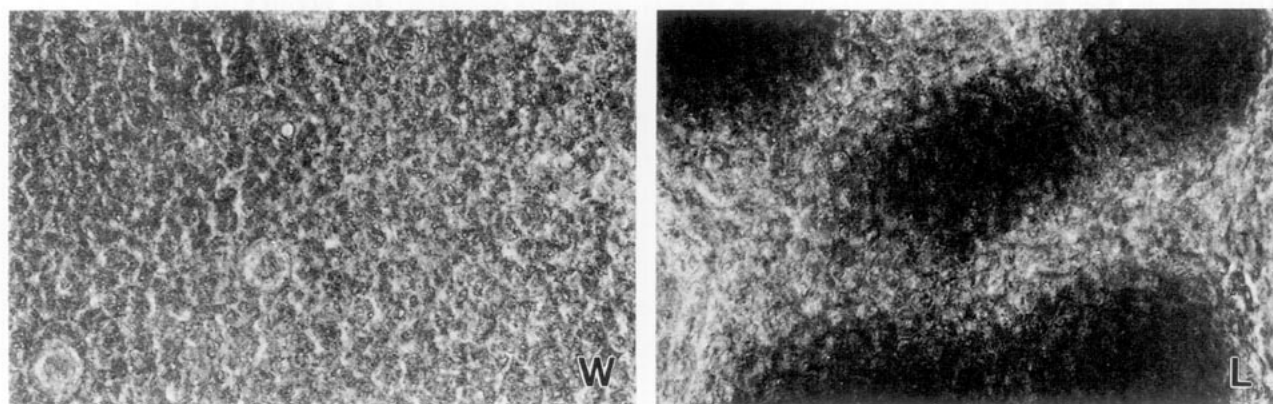


FIG. 2. Wing- and leg-derived cell cultures are continuous sheets of cells that produce different patterns of cartilage. High magnification photomicrographs of Alcian blue stained, 6-day, serum-free cultures of stage 24 wing (W) and leg (L) mesenchyme. The distribution of Alcian blue stainable matrix in wing cell cultures is diffuse and the intensity of staining is moderate across the sheet of cells. The distribution of Alcian blue stainable matrix in the leg cell cultures is nodular and staining is intense. Nonstaining internodular cells are visible. Phase-contrast image; objective magnification 32 \times .

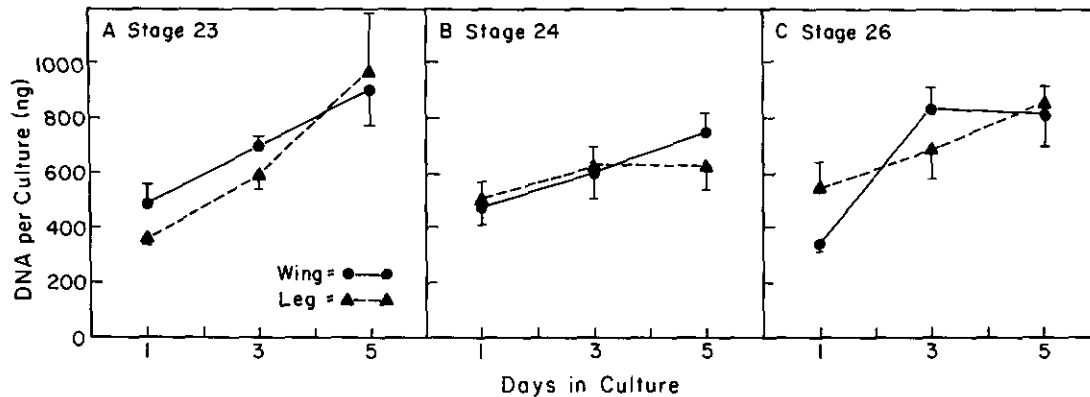


FIG. 3. DNA content of differentiating wing and leg precartilaginous cells from stages 23, 24, and 26 limb buds in serum-free culture. Values are mean \pm SEM. $N = 4$ in each case.

but not in leg control cultures. The internodular regions which failed to stain in untreated leg cultures (Fig. 1) accumulated Alcian blue staining matrix after transient exposure of the cultures to TGF- β 1 (Fig. 5, leg TGF- β panel).

Retinoic acid also had differential effects on wing and leg cell cultures. These effects varied with the concentration of retinoid (data not shown), but were always more pronounced in leg cell cultures than in wing cell cultures, and usually deleterious to the former. For ex-

ample, the proteoglycan content of wing cultures grown with continuous exposure to 5 ng/ml of all-*trans*-retinoic acid (RA) was not significantly altered from controls, while leg cultures contained 80% less proteoglycan than controls (Table 2). The patterns of Alcian blue staining in both wing and leg cultures remained the same as those in controls except that the intensity of staining of the peripheral nodules in the RA-treated leg cultures was greatly reduced (compare the RA panels of Fig. 5 to the stage 24 panels of Fig. 1). Also, while there

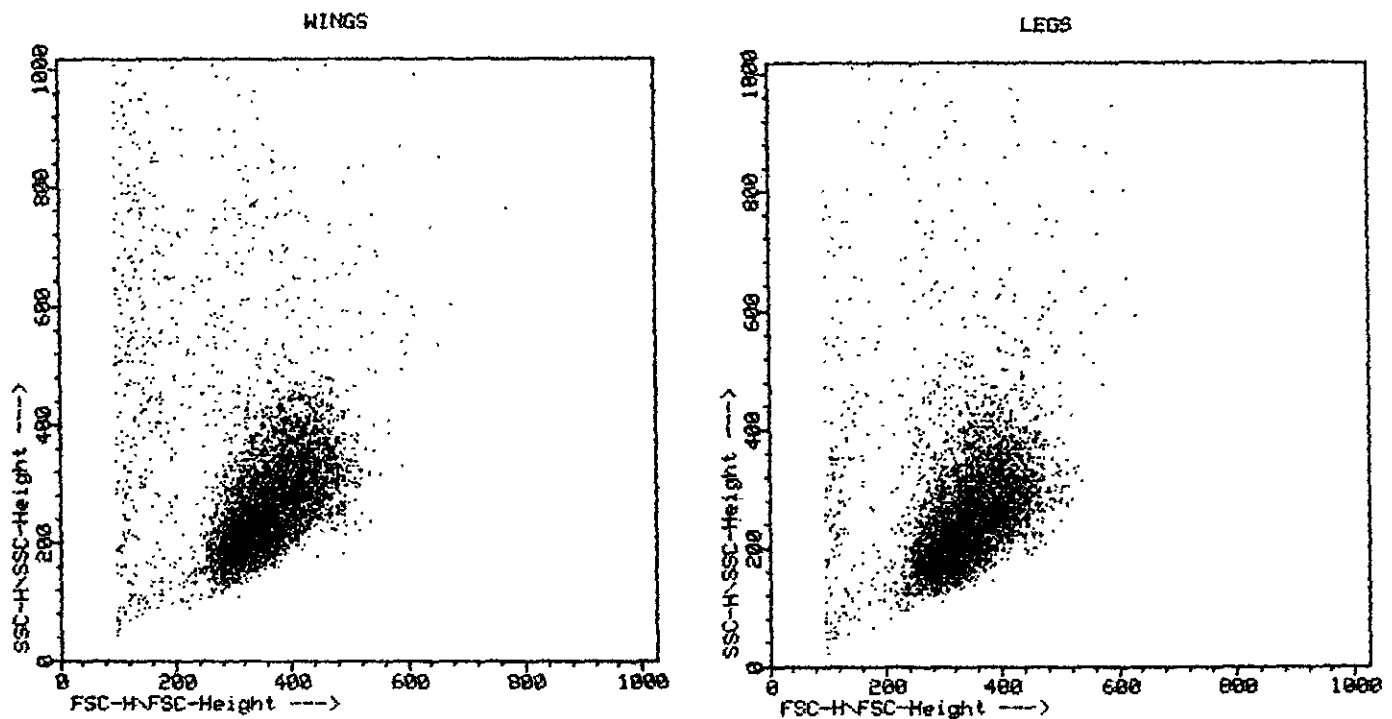


FIG. 4. Flow cytometry analysis of stage 24 wing and leg precartilaginous mesenchymal cells. The x axis represents forward light scatter (FSC) which is proportional to cell diameter. The y axis represents right angle light scatter (SSC) which reflects subcellular structure or granularity.

TABLE 1
LEG CELL CULTURES ACCUMULATE MORE PROTEOGLYCAN
THAN WING CELL CULTURES

		Alcian blue stain ^a	L:W
Stage 23	W	0.095 ± 0.013 ^b	4.5
	L	0.427 ± 0.057	
Stage 24	W	0.189 ± 0.020 ^b	1.4
	L	0.273 ± 0.021	
Stage 26	W	0.243 ± 0.043	1.4
	L	0.352 ± 0.049	

Note. W, wing; L, leg.

^a Data represent mean ± SEM of the OD₆₀₀ of extracted Alcian blue stain (see Materials and Methods). *N* = 7 for each data set.

^b Significantly different from leg (*P* < 0.0005).

was no significant change in DNA content in wing cultures exposed to retinoic acid, the DNA content in RA-treated leg cultures was significantly decreased (Table 3).

Wing cell cultures exposed to both TGF-β1 and RA accumulated a level of proteoglycan that was significantly greater than those in controls or cultures exposed to RA alone (Table 2), while the pattern of Alcian blue staining remained uniform (Fig. 5, wing RA/TGF-β panel). With leg cell cultures the quantity of Alcian blue staining in the presence of both TGF-β1 and RA was intermediate between the values obtained with either treatment alone, placing it nearly at the level of controls (Table 2) and the staining intensity of the peripheral

nodules was increased over that resulting from treatment with RA alone (compare the leg RA/TGF-β panel of Fig. 2 to the leg stage 24 panel of Fig. 1).

Stability of Chondrogenic Phenotypes and Evidence for Cohesivity Differences in Wing and Leg Mesenchymal Cultures

The results described above raised the question of whether the pattern differences in wing and leg cultures could have been determined in part by any component which was released by wing or leg cells and affected their neighbors. We therefore cocultured stage 24 wing and leg bud mesenchyme in the same 35-mm culture dish at decreasing distances from one another. Growth in a common medium led to no convergence in the morphology of the spot cultures. When cultures were deposited on the dish in a contiguous fashion an unambiguous interface was always formed (Fig. 6). The interface appeared as soon as the wing and leg morphologies were distinguishable (at about 2 days of growth) and it remained distinct even after 6 days of culture (Fig. 6). Interestingly, the curve of the interface was always convex in relation to the leg culture (Fig. 6, top), suggesting that this cell spot was more internally cohesive than the wing spot. This is consistent with a previous report, based on fusions of intact limb tissues with other tissue types, in which leg mesenchyme was indirectly inferred to be more cohesive than wing mesenchyme (Heintzelman *et al.*, 1978).

TABLE 2
ACCUMULATION OF HIGHLY SULFATED PROTEOGLYCAN IN TREATED WING AND LEG CULTURES

	Wings ^a	Treated:control (R/T:R)	Legs ^a	Treated:control (R/T:R)
Serum	1.272 ± 0.161 ^b	5.0	0.564 ± 0.134	1.3
Control	0.222 ± 0.031 (<i>N</i> = 20)		0.443 ± 0.066 (<i>N</i> = 20)	
TGF-β	0.230 ± 0.038	1.1	0.636 ± 0.092 ^b	1.6
Control	0.212 ± 0.034 (<i>N</i> = 20)		0.409 ± 0.072 (<i>N</i> = 20)	
RA	0.325 ± 0.088	1.2	0.089 ± 0.029	0.2
Control	0.268 ± 0.073 (<i>N</i> = 6)		0.441 ± 0.167 (<i>N</i> = 6)	
RA/TGF-β	0.387 ± 0.094 ^{c,d}	1.4 (1.2)	0.394 ± 0.116 ^e	0.9 (4.4)
Control	0.268 ± 0.073 (<i>N</i> = 6)		0.441 ± 0.167 (<i>N</i> = 6)	

Note. R/T:R = RA/TGF-β:RA.

^a Data represent OD₆₀₀ GuHCl-extracted Alcian blue stain from stage 24 cultures.

^b Significantly different from control (*P* < 0.0001).

^c Significantly different from control (*P* < 0.04).

^d Significantly different from RA alone (*P* < 0.002).

^e Significantly different from RA alone (*P* < 0.03).

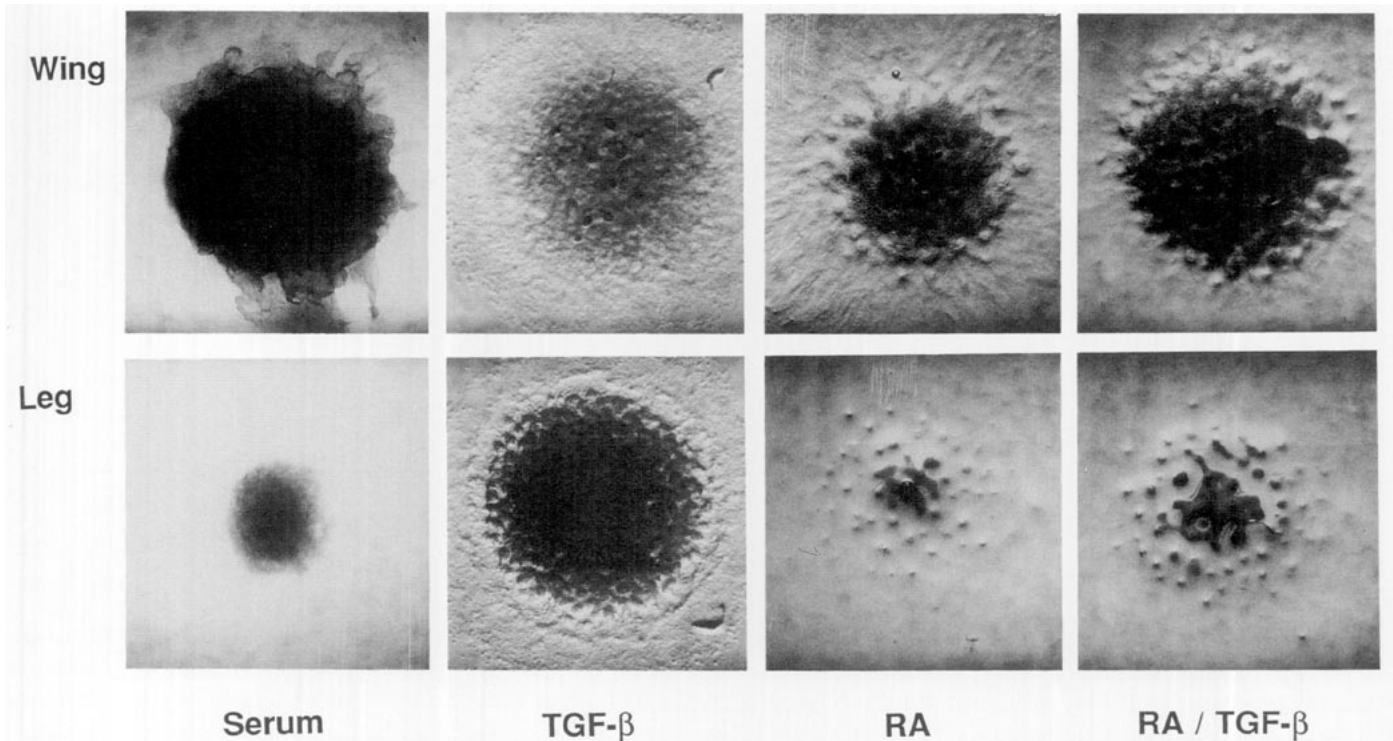


FIG. 5. Wing and leg precartilaginous cells respond differently to morphogenetic factors. Stage 24 wing and leg cell cultures, from left: grown in DM containing 10% FBS; grown in DM and treated on Day 1 with 1 ng/ml TGF- β 1; grown in DM containing 5 ng/ml all-*trans*-retinoic acid; grown in DM containing 5 ng/ml all-*trans*-retinoic acid and treated on Day 1 with 1 ng/ml TGF- β 1. Stage 24 wing and leg controls grown in DM alone are shown in Fig. 1. Macroscopic image; cell spot diameter approx. 5 mm.

Condensation Morphology and Fibronectin Organization in Wing and Leg Mesenchyme

At 2 days of development in culture, cartilage has not yet formed, but wing and leg precartilaginous condensations have assumed morphologies that anticipate the morphology of the corresponding differentiated tissue. Wing mesenchymal condensations are broad and flat (Fig. 7W) and will eventually coalesce, leading to a continuous sheet of cartilage. In contrast, leg mesenchymal condensations are compact and nodular (Fig. 7L) and remain discrete like the cartilage that will eventually arise from them.

Earlier studies indicated that precartilaginous mesenchymal condensations *in situ* (Tomasek *et al.*, 1982; Kosher *et al.*, 1982) and *in vitro* (Glant *et al.*, 1985; Frenz *et al.*, 1989a,b) are associated with, and are, in part, promoted by, the accumulation of fibronectin at specific sites in the mesenchyme. We were therefore interested in whether the distribution of fibronectin differed in the morphologically distinct wing and leg condensations. Figure 8a (top) shows a stereo pair of confocal micrographs of a 2½-day culture of stage 24 wing mesenchyme immunostained for fibronectin. A corresponding pair of

micrographs from leg mesenchyme is shown in Fig. 8a (bottom). In the wing tissue fibronectin was diffusely organized around the cells and was concentrated in the central region of the forming condensation. In the leg tissue fibronectin was even more abundant in the condensing regions, which radiated long fibronectin-rich fibers not seen in the wing cultures. The large-scale organization of fibronectin in the wing and leg cultures can be seen by low magnification conventional immunofluorescence (Fig. 8b). Fibronectin-rich fibers, some as long as 300 μ m, connect adjacent condensations in the leg cultures.

DISCUSSION

We have identified several properties which distinguish the development of fore and hind limb precartilaginous mesenchyme in serum-free culture. These include (i) morphology and pattern of differentiated cartilage, (ii) accumulation of Alcian blue stainable proteoglycan, (iii) tissue cohesivity, and (iv) organization of fibronectin during mesenchymal condensation. We have also found that these tissues respond differently with respect to growth and differentiation, to serum, retinoic

TABLE 3
EFFECTS OF TREATMENTS ON PROLIFERATION OF STAGE 24 WING AND LEG MESENCHYME *in Vitro*

	Wings ^a	Treated:Control	Legs ^a	Treated:Control
Serum	2797.6 ± 338 ^b		1313.7 ± 169	
Control	1142 ± 44.5 (N = 4)	2.5	1254 ± 141 (N = 4)	1.1
TGF- β	997.4 ± 209		1130.4 ± 163	
Control	1050.7 ± 161 (N = 7)	1.0	1037.4 ± 151 (N = 7)	1.1
RA	1444 ± 115		602.3 ± 27.8 ^b	
Control	1118 ± 15 (N = 2)	1.3	1319 ± 66.8 (N = 2)	0.5
RA/TGF- β	1199 ± 150		1310 ± 209	
Control	1118 ± 15 (N = 2)	1.1	1319 ± 66.8 (N = 2)	1.0

^a Values are expressed as ng DNA per culture on Day 5.

^b Significantly different from control ($P < 0.04$).

acid, and TGF- β , factors that contribute to the normal microenvironment during development *in vivo*. Our studies therefore provide evidence that fore and hind limb precartilaginous cells undergo chondrogenic processes common to the formation of all limb skeletal elements (e.g., condensation, differentiation), while exhibiting both intrinsic and induced variations on these processes.

Two possible interpretations of the patterning differences in post-stage 23 wing and leg cell cultures can be considered: a static one and a dynamic one. The static explanation would hold that the post-stage 23 leg cultures (but not the wing cultures) consist of two populations of cells, one more advanced and one less advanced along the chondrogenic pathway. The nodular distribution of cartilage in 6-day leg cultures would thus represent foci of differentiated cells founded by the more advanced population. Against this interpretation was our inability to detect any evidence of a bimodal distribution in the leg cell population by flow cytometry, or for that matter any differences at all in the profile of wing and leg cells (Fig. 4). Furthermore, the internodular cells in the leg cultures fail to "catch up" with the nodular cells over prolonged periods in culture, despite the fact that they are capable of undergoing chondrogenesis in the presence of an appropriate activator (Fig. 5).

We are therefore led to consider a dynamic explanation of our results. In post-stage 23 serum-free cultures, wing bud distal mesenchyme forms expanding circular condensations that eventually coalesce to form a sheet of cartilage. In contrast, leg bud distal mesenchyme forms circular condensations that remain spatially confined, resulting in discrete nodules of cartilage. It is reasonable to view these processes as the result of the in-

teraction of factors that stimulate and factors that inhibit chondrogenesis. In purely chemical systems in which there is a simple set of auto- and cross-regulatory interactions among diffusible substances in a confined region, stable or transient spots or stripes of chemical concentration can emerge (Castets *et al.*, 1990; Ouyang and Swinney, 1991; Lengyel *et al.*, 1993). These systems are "tunable"—the final patterns depending on the ratios of activators to inhibitors and the geometry and boundaries of the system. Such a "reaction-diffusion" framework can provide a dynamic model for how a uniform population of precartilaginous cells can become organized into the rods and nodules of cartilage that make up the typical limb skeleton *in vivo* (Newman and Frisch, 1979; Newman *et al.*, 1988) as well as patterns of cartilage *in vitro* (Leonard *et al.*, 1991; Newman, 1993).

Although the full complement of activators and inhibitors of limb chondrogenesis has not been characterized, members of the TGF- β family of growth and differentiation factors (Rizzino, 1988; Massagué, 1990) can promote chondrogenesis in limb

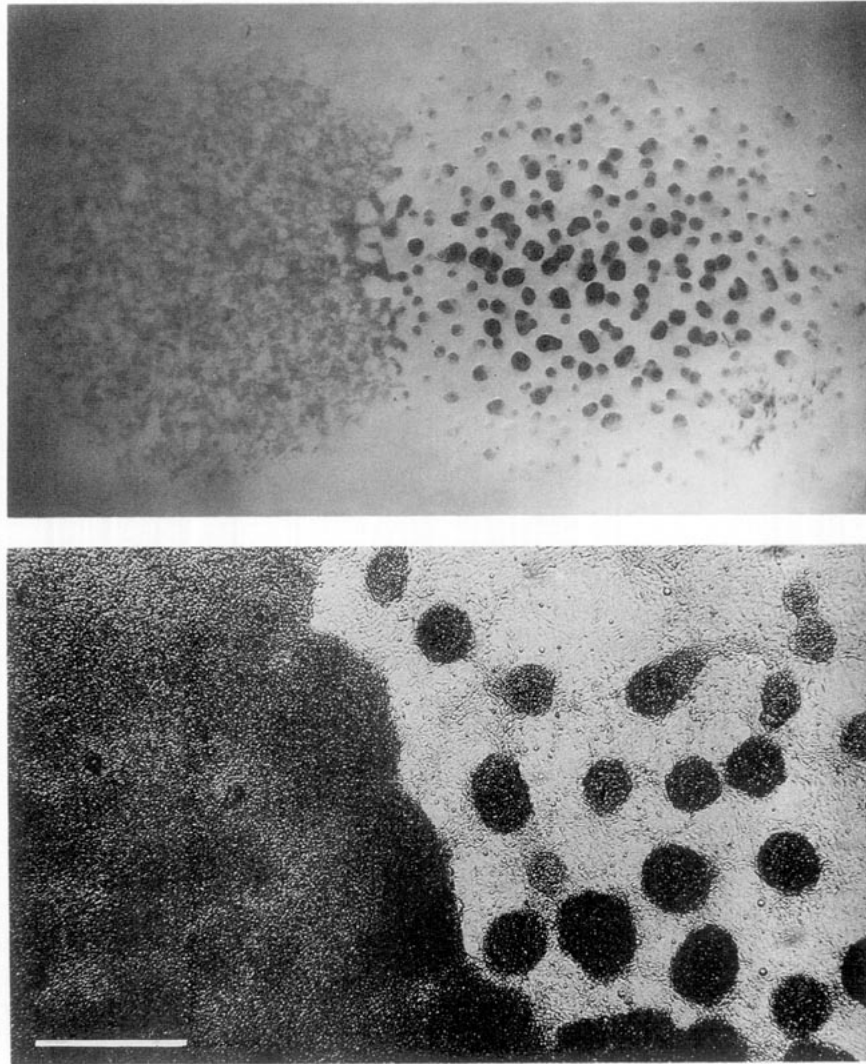


FIG. 6. Contiguous cocultures of stage 24 wing and leg cells retain their limb-type-specific morphologies and form a distinct interface. Top: stained coculture, after 6 days. Wing cell spot is on the left, leg cell spot is on the right. Note the nonstaining internodular cells in the leg cell culture. Macroscopic image; cell spot diameter approx. 5 mm. Bottom: high magnification view of wing-leg cell interface in a 6-day, unstained, living culture. Wing tissue on the left, leg tissue on the right. Bright-field image; objective magnification 4 \times ; bar, 0.5 mm.

Against this background we suggest that the differences in cartilage production and patterning that we have observed in wing and leg bud precartilaginous mesenchymal cultures may be due to different balances in their production of factors stimulatory and inhibitory to chondrogenesis and to differences in their responses to such factors. *In vivo*, wing and leg mesoblasts are spatially differentiated by gradients of nuclear proteins of the Hox A, C and D classes (Mackem and Mahon, 1991; Coelho *et al.*, 1992; Duboule, 1992; Morgan *et al.*, 1992) and by retinoic acid receptor proteins (Smith *et al.*, 1989; Dollé *et al.*, 1990), providing a possible basis upon which the effects of symmetrically distributed activators and inhibitors could be modulated in a regionally dependent

fashion, leading to asymmetries in the limb skeletons. Gradients in "polarizing activity" (Hinchliffe and Sansom, 1985) and responsiveness to the apical ectodermal ridge (Rowe and Fallon, 1981) probably reflect such molecular gradients.

The amounts and distributions of some of these graded molecules and activities also differ between fore and hind limbs (Mackem and Mahon, 1991; Rowe and Fallon, 1981; Hinchliffe and Sansom, 1985). In cultures of randomized wing or leg mesenchymal cells, any intralimb regional differences in susceptibility to morphogenetic factors would be averaged out, but differences between the limb types in intrinsic and induced pattern formation *in vitro* may be attributable to different

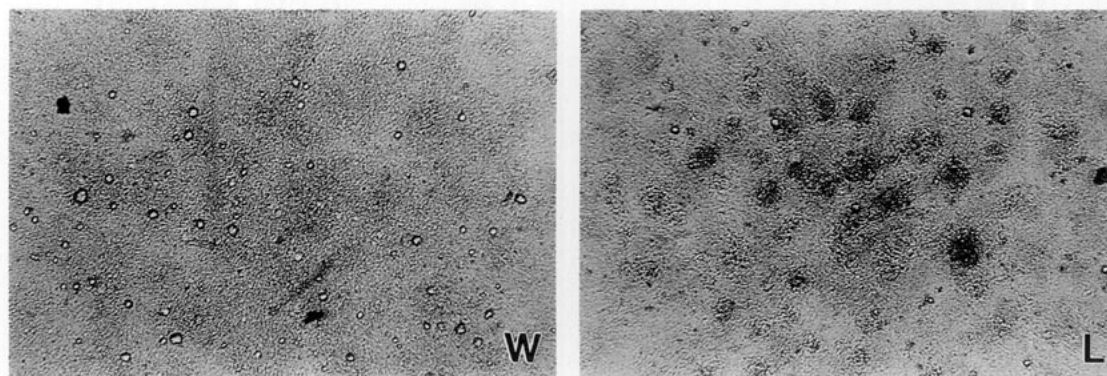


FIG. 7. Wing and leg precartilaginous condensations prefigure respective patterns of cartilage. W and L: stage 24 wing and leg cells after 2 days of culture in DM. Condensations are seen as dark patches with Hoffman Modulation-contrast microscopy. Objective magnification 4X.

characteristic ratios of homeobox proteins, retinoid receptors, or other response-mediators.

Serum, a complex mixture of factors, had widely divergent effects on the ability of wing and leg mesenchyme to differentiate and survive (Figs. 5 and 6). TGF- β 1 changed the pattern of chondrogenesis in leg cultures, causing cells that failed to differentiate in the serum-free micromass setting to become fully chondrogenic (Figs. 2 and 6). This is consistent with the proposed role of molecules of this class in determining the sites at which cartilage will actually form in a field of potentially chondrogenic cells (Newman, 1988; Kulyk *et al.*, 1989; Leonard *et al.*, 1991). Retinoic acid, in contrast, had little effect on the formation of cartilage in the uniformly chondrogenic wing cultures, but reduced the size and number of Alcian blue staining nodules in the leg cultures (an effect that could be overcome to some degree by addition of TGF- β 1; Fig. 6). This is consistent with a role for gradients of RA and its receptors in influencing the size and shape of individual cartilage elements and thus, the asymmetry of the developing limb (Ide and Aono, 1988; Paulsen *et al.*, 1988; Smith *et al.*, 1989).

In a previous study (Leonard *et al.*, 1991) we reported that transient treatment of wing mesenchyme with TGF- β in serum-containing culture enhanced proteoglycan accumulation by about 50%, a result not seen with wing mesenchyme in serum-free culture (Table 2). It was therefore of interest that the addition of RA to these cultures restored the ability of wing mesenchyme to respond to TGF- β in a fashion similar to that seen in the serum-containing culture system. Not only were the proteoglycan levels of the RA/TGF- β wing cultures elevated by 40% over their matched DM controls (Table 2), but the increased proteoglycan accumulation of RA/TGF- β wing cultures compared with that of matched RA controls was highly significant (Table 2). The reti-

noids present in serum (Napoli *et al.*, 1985) may therefore modulate the responsiveness of limb tissues to various treatments both *in vitro* and *in vivo*.

It is significant that wing and leg bud mesenchyme differ from one another substantially in their responses to all of the exogenous agents we have examined. Teratogens are known to differentially affect development of the fore and hind limbs of many vertebrate species, including humans (Bruyere *et al.*, 1983; Perrot *et al.*, 1987; Collins *et al.*, 1992; Sunil Kumar and Devi, 1992). We suggest that intrinsic differences between fore and hind limb mesenchymal populations may constitute a previously unrecognized basis for such susceptibilities.

The formation of an interface between confluent wing and leg micromass cultures (Fig. 6) indicates that any intrinsically produced activating or inhibiting molecules that may be responsible for the different appearances of the cartilage in these cultures are not *freely* diffusible. In particular, the high magnification view (Fig. 6, bottom) confirms that there is no zone of intermediate morphology at the boundary between the tissues. However, released molecules that reversibly bound to the extracellular matrix (such as TGF- β) could diffuse slowly enough so that their patterning influence was confined to domains comprising only a few cell diameters.

But while molecular mixing across the interface could be thus limited, the failure of cells to intermix across the boundary presents a problem of a different physical nature. Cells would normally be expected to mix across any boundary as a result of their random motility, unless they constituted adhesively distinct populations (Steinberg, 1970; Armstrong, 1989). But differential adhesion is relevant only when direct cell-cell contacts occur, as in epithelioid tissues (Steinberg, 1970; Armstrong, 1989). Since wing and leg mesenchymal cells were in neither direct homotypic nor heterotypic cell-cell con-

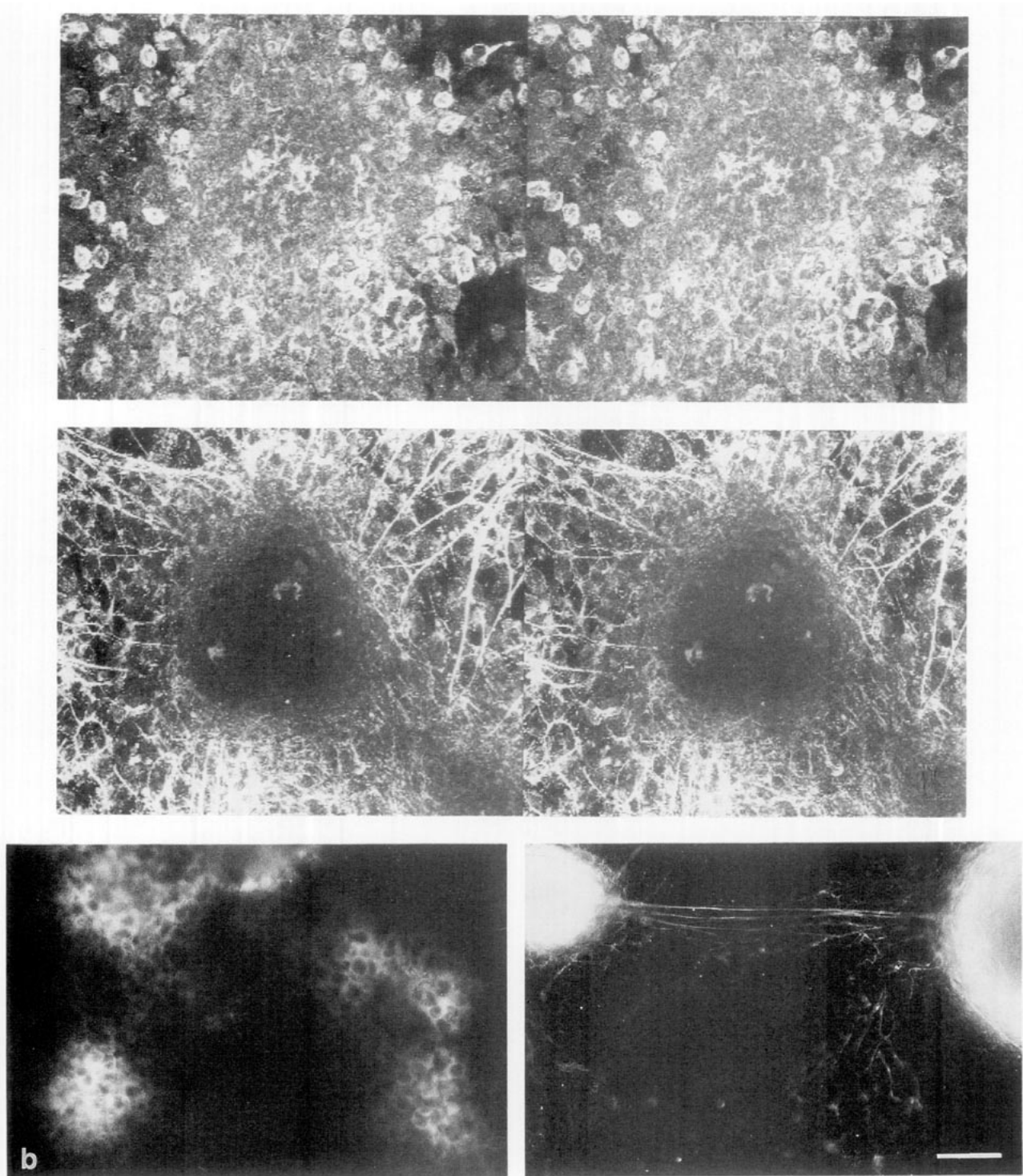


FIG. 8. (a) Fibronectin is organized differently in condensations of stage 24 wing and leg precartilaginous mesenchyme. Confocal stereo photomicrographs of Day 24 wing (top) and leg (bottom) precartilaginous condensations. Note the extensive network of fibronectin-rich fibrils in the leg cell condensations. The dark area at the center of the leg condensation represents a region of the optical section where the antibodies did not penetrate. Objective magnification 40 \times . (b) Conventional immunofluorescence image of field similar to 8a at lower magnification showing large scale organization of fibronectin-rich matrix of wing (left) and leg (right) cell cultures. Objective magnification 25 \times ; bar, 50 μ m.

tact after several hours of culture, the basis of their failure to subsequently mix must reside in the extracellular matrices of these tissues rather than the cell surfaces. It is therefore of interest that the lengths and extracellular organization of fibronectin fibrils differ markedly between wing and leg cultures (Fig. 8). On the basis of experimental studies with model extracellular matrices (Newman *et al.*, 1985; Forgacs *et al.*, 1989) and theoretical considerations (Forgacs *et al.*, 1989, 1991), we have suggested that the organization and density of extracellular fibers could provide the physical basis of boundaries of immiscibility in mesenchymal tissues.

We believe that the differences between wing and leg bud mesenchyme in cohesivity and fibronectin organization indicated in Figs. 6 and 8 are intrinsic to these tissue types and are not induced by the cell dissociation and reaggregation required for preparation of the cultures. In the first place, using a heterotypic tissue fusion technique with undissociated wing and leg mesenchymal tissues, Heintzelman *et al.* (1978) ascertained that leg mesenchyme was more cohesive than wing mesenchyme, a result also suggested by our experiments. Second, unusually long fibronectin fibrils like the ones seen in our cultures were identified *in situ* in sections of chick leg buds by Hurle *et al.* (1989). No such fibrils were seen in any of several published studies of *in situ* fibronectin distribution in wing buds (Dessau *et al.*, 1980; Kosher *et al.*, 1982; Tomasek *et al.*, 1982).

Our studies indicate that wing and leg precartilaginous mesenchymal tissues in culture undergo common processes of condensation-dependent chondrogenesis, but are intrinsically different in their responsiveness to certain microenvironmental factors and in the organization of their extracellular matrices. These results suggest, moreover, that the variations seen in differentiated cultures are based on molecular differences between fore and hind limb mesenchymal cells that are present prior to overt chondrogenesis. In work currently in progress, we are addressing the nature of these molecular determinants.

We thank Dr. Frank Traganos for the flow cytometry analysis, Mr. Charles Blanchard, Bio-Rad, Inc., for help with the confocal microscopy, and Dr. Mindy Steinholz for help with DNA assays. This work was supported by grants from the National Institutes of Health (HD22564) and the National Science Foundation (DCB-8819167).

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