Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 2804–2808, May 1985
Developmental Biology

Morphogenetic rearrangement of injected collagen in developing chicken limb buds

(connective tissue/morphogenesis/collagen)

DAVID STOPAK*†, NORMAN K. WESSELLS*, AND ALBERT K. HARRIS‡

*Department of Biological Sciences, Stanford University, Stanford, CA 94305; and ‡Department of Biology, University of North Carolina, Chapel Hill, NC 27514

Communicated by Clifford Grobstein, December 31, 1984

ABSTRACT **A fundamental question concerning the development of the extracellular matrix is what factors control the arrangement of collagen fibrils within a tissue and at the same time allow for the great diversity of geometric forms exhibited by collagen. In this report, we test the possibility that physical forces within the embryo serve to organize collagen fibers into regular patterns. In particular, we test the prediction that patterns of stress having this morphogenetic function are generated by cell traction, the contractile force exerted by cells to propel themselves. To study the effects of these mechanical forces on the extracellular matrix, type I collagen was fluorescently labeled and injected into developing chicken wing buds. When the injected limbs were allowed to develop and then examined histologically, the exogenous collagen was found incorporated within normal connective tissues of the wing. The labeled collagen became arranged according to its site of injection, forming parts of tendons, perichondria, cartilages, perineuria, and blood vessels. Since the injected collagen formed a gel within minutes of its injection, the subsequent incorporation of this preformed collagen within organized structures cannot be explained in terms of molecular self-assembly or other mechanisms occurring during collagen deposition. These results demonstrate that, within developing tissues, patterns of forces exist that are capable of physically rearranging collagen and determining its long-range order.**

During embryonic development, collagen somehow becomes organized into a wide variety of different spatial patterns whose mechanical properties lend structural support and give form to vertebrate tissues. Although much is known about the self-assembly of collagen molecules into fibrils (1, 2), comparatively little is known about how collagen fibrils become associated together into the diverse supramolecular arrangements found in the body. We have recently proposed that the cellular forces responsible for cell locomotion can have the additional morphogenetic function of organizing collagen fibers into regular patterns (3–7). Studies on cell traction have demonstrated that as cells spread on a flexible substratum (such as collagen or thin sheets of silicone rubber), each cell acts as a compressive element, drawing material inward toward itself and stretching material at distances of hundreds of micrometers beyond itself (3–5). Cells that are capable of exerting these kinds of strong tractional forces include fibroblasts (3–5, 8–11), glial cells (5), endothelial cells (12), and epithelial cells (13, 14). Other cell types such as transformed fibroblasts (5, 15, 16), chondrocytes (17), neurons (5), and leukocytes (4) exert relatively weaker tractional forces.

In collagen matrices, the compressive and tensile strains generated by cell traction bring about a rearrangement of collagen fibers. This realignment, in turn, affects cell behavior so that positive feedback cycles arise that spontaneously generate regular arrangements of cells and matrix (5–7). By controlling such simple factors as the initial cell distribution or the resistance of the collagen gel to distortion, a number of different connective tissue structures can be created in culture, including the pattern of dermal condensations, which make up the feather tracts of birds and pelage hair of mammals (6), the tendon and ligament attachments of muscles to long bones (5), and the arrangement of collagen into capsules or sheets, which cover many organs (5). Insofar as they have been tested *in situ*, the existence of these morphogenetic effects has been confirmed. During wound healing (18), in the development of the cardiac cushion of the heart (19), and during amphibian gastrulation (20), rearrangement of extracellular fibers has been observed to follow the migration of cells into the matrix.

To examine how the extracellular matrix becomes organized within embryos, we have developed a new experimental approach that is an extension of our earlier studies on the morphogenetic effects of fibroblast traction. Fluorescein isothiocyanate (FITC) was covalently coupled to collagen from rat tail tendons and injected into wing buds of 4-day-old chicken embryos. The embryos were allowed to develop for various periods of time and were then examined to determine whether this exogenous collagen could be reorganized by cells and become part of functional structures. We find that the injected collagen rapidly forms a gel that subsequently undergoes reorganization and becomes incorporated into various connective tissues of the limb. These rearrangements are consistent with the effects of cell traction previously observed in tissue culture.

MATERIALS AND METHODS

Embryo Culture and Injection. The injection of chicken embryos was more easily accomplished by raising embryos as shell-less culture (21). Fertile eggs (purchased from A & M Hatchery, Santa Rosa, CA) were raised in a forced-draft incubator for 3 days and were then cracked open (as if cracking an egg for frying) into 100 × 25 mm Petri dishes. The intact embryos were transfered to a 5% CO_2/95% air incubator and injected 24 hr later. For better survival during the initial 24 hr, additional egg white from unfertile eggs was added to the dishes. Although the rate of development is slower in shell-less cultures than *in ovo*, it continues normally up to the stages used in this study (days 4–9) (22). Embryonic age is given according to the standard Hamburger and Hamilton (23) series, rather than absolute days of development. Embryos were injected under a dissecting microscope by using a Gilmont (Great Neck, NY) micrometer syringe filled with 20 centistokes of silicone fluid (Sigma) (1 stoke =

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Abbreviation: FITC, fluorescein isothiocyanate.

†Present address: Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, CA 94143.

1×10^{-4} m^2/sec). Micropipettes were pulled to a diameter of 20–30 μm and mounted onto a Narishige three-way micromanipulator. Injections were not limited to any one specific location, although the region between the shoulder and elbow was most often chosen.

Preparation of Fluorescent Collagen. Collagen solutions were prepared from rat tail tendons dissolved in 0.5 M acetic acid by the method of Elsdale and Bard (24). FITC was covalently coupled to the collagen using standard methods for fluorescently labeling antibodies (25). The pH of the collagen solution was raised to 9.5 by dialysis in 0.25 M NaCl/0.05 M phosphate. FITC at a concentration of 0.08 mg per mg of protein was added to the collagen solution and mixed overnight. Unbound FITC was separated from the labeled collagen by gel filtration on a Sephadex G-25 column. After recovery from the column, the collagen was dialyzed against a solution of Dulbecco–Vogt's modified Eagle's medium (DME medium; GIBCO) diluted to 1/10th normal strength at pH 4.0. The concentration of collagen in these solutions was 2–3 mg/ml and between 1 and 3.5 mol of FITC was bound to 1 mol of collagen. Bovine serum albumin at a concentration of 2 mg/ml was also labeled by the same procedure.

To prepare a collagen solution that would form fibrils after injection, the solution was neutralized and made isotonic by adding NaOH and 5× DME medium in the following proportions: 60% FITC-collagen/20% 5× DME medium/10% 0.15 M NaOH/10% H_2O or fetal calf serum. The coupling of FITC to monomeric collagen does not interfere with its precipitation into a gel. Under phase-contrast optics, the appearance of these fluorescent collagen matrices is normal, and when used in cell culture there is no apparent difference in cell behavior or morphology when compared to unlabeled gels (6). Before injection, a sample was removed to determine the gelation time, which, in all cases, occurred within 10 min at 37°C.

Histology. The injected wings were dissected from the embryos, fixed, and embedded in paraplast using the cold ethanol fixation method of Sainte-Marie (26). All specimens were sectioned longitudinally at a thickness of 7 μm, and they were mounted in glycerol. The tissue sections were examined under epifluorescence and phase-contrast optics using a Nikon Optiphot microscope.

RESULTS

At the time of injection on day 4 of development (stage 24 or 25), the wing bud contains a loose undifferentiated mesenchyme. The cartilagenous elements are just beginning to form, and the extracellular matrix is still poorly developed with only small amounts of type I collagen present (27, 28). A total of 40 embryos were injected with ≈1 μl of fluorescent collagen. Fluorescent bovine serum albumin was injected into another 15 embryos as a control to show that soluble proteins could not be passively trapped in the extracellular matrix to form geometric arrangements. Unlike collagen, which remained at the site of injection, fluorescent bovine serum albumin diffused away quickly, spreading throughout the entire wing within 2 hr of injection. In contrast, collagen remained localized at the initial injection site for as long as the embryos were maintained. Although the pH and ionic strength of the collagen solution was adjusted to be the same for each injection, there was variability in how quickly a gel was formed. The appearance of the collagen immediately after injection reflected this variability. When the collagen gelled immediately upon injection, it formed a compact mass of fluorescent material that was acellular and discontinuous from the surrounding tissue (Fig. 1*A*). This same appearance of a cohesive gelled material was observed when collagen was allowed to precipitate inside the needle before injection. When the collagen remained in solution for several minutes after its injection, the label was distributed in an intercalated pattern within the extracellular spaces (Fig. 1*B*). The network of collagen that formed outlined the boundaries of cells and was spread over a large area, yet it still had strong fluorescence. In some embryos, a combination of both of these patterns was observed.

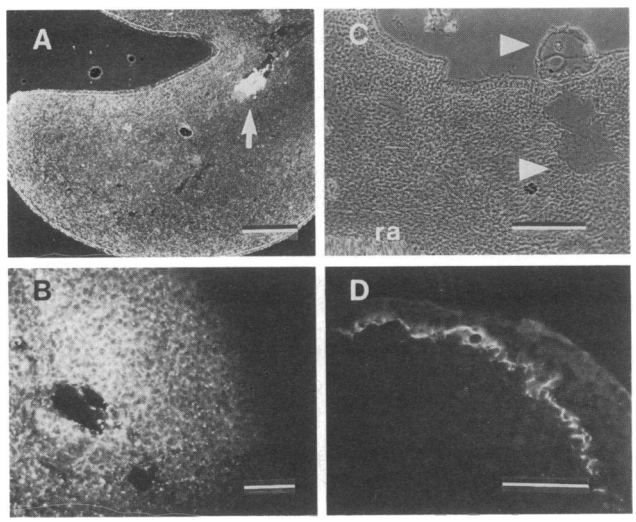

FIG. 1. (*A*) Fluorescent collagen (arrow), which gelled immediately after its injection into the wing, is shown in this combined dark field and fluorescence micrograph. The embryo was injected with 1 μl of collagen at stage 24 and was fixed 10 min afterwards. (Bar = 200 μm.) (*B*) Intercalated distribution of labeled collagen is shown in this fluorescence micrograph. Serial sections show that the injection site is spherical. The embryo was injected at stage 25 and was fixed 30 min afterwards. (Bar = 50 μm.) (*C*) Injected collagen (arrowheads) is located in the dorsal mesenchyme and dermis anterior to the radius (ra) and forms a cohesive mass that bulges outward, the epithelium forming a cap over the bulge. No differentiated structures have formed in the area near the injection site, and no rearrangement of the collagen is detectable. Viewed with phase contrast optics. Embryo developed to stage 30 (6.5 days). (Bar = 100 μm.) (*D*) Transverse section through an injection site in the dermis is shown in this fluorescence micrograph. Compare distribution and organization of label shown here with that in *B*; in this case, however, the injection site was circular rather than spherical. Embryo survived until stage 29 (6 days). (Bar = 50 μm.)

Alterations in the distribution and organization of the injected collagen were observed as early as 24 hr after injection (beginning on day 5 of development, stage 27) and up to 9 days (stage 35). In most cases, more than one type of tissue within a given embryo contained labeled collagen. Table 1 summarizes the distribution, pattern of organization, and

Table 1. Distribution and organization of injected collagen

Tissue type	No. of embryos	Pattern of distribution
Loose mesenchyme	12	Networks; cohesive mass; irregular patches
Dermis	9	Networks; cohesive mass
Perichondrium	7	Elongated patches; parallel strands
Cartilage		
Diaphysis	3	Networks
Epiphysis	3	Irregular patches
Tendon	4	Elongated patches; parallel strands
Blood vessel	4	Strands on surface
Perineurium	4	Strands on surface
Skeletal muscle	2	Irregular patches; fine strands

Data are from 28 embryos allowed to develop from stage 27 to 35.

frequency of occurrence of labeled collagen from 28 embryos that had developed from stage 27 to 35. In 5 of these embryos, no remodeling of the labeled collagen was observed. Labeled collagen became incorporated as part of the structure of normal connective tissues in the remaining 23 embryos. No abnormalities in the morphology of tissues containing the injected collagen were detected.

Much of the developing limb consists of an uncondensed loosely organized mesenchyme from which differentiated structures arise. Label was found most frequently in loose undifferentiated mesenchyme and in the dermis. In the 5 embryos where no remodeling was observed, the injection site was located in this loose mesenchyme (Fig. 1*C*). In each of these cases, the fluorescent collagen formed a cohesive mass upon injection, which remained acellular and discontinuous from the surrounding tissue. However, when an intercalated distribution of label was found in these same tissues, the injected collagen was distributed into a pattern similar to the distribution of type I collagen found in loose mesenchyme and dermis in normal development (28) (Fig. 1*D*). As development progressed, the effect of growth was to expand the material contained within the injection site, so that the diffuse networks of label became spread apart with larger spaces between the strands of label (compare Fig. 1 *B* and *D*). Because the label initially consisted of networks, it was difficult to assess whether any changes had occurred in their organization apart from this expansion.

Inside the diaphysis of cartilage, the label also was spread apart; in addition, the fluorescence was more diffuse and less intense. Fig. 2*A* shows a section in which the initial injection site includes two different tissues, the perichondrium and the cartilage matrix of the humerus. The label in the cartilage matrix is diffuse with no preferred orientation, and its appearance suggests that the initial network has become greatly expanded. In contrast, the label in the perichondrium has become elongated parallel to the long axis of the bone rudiment, and its initial organization into a network is no longer distinct. The intensity of the label in the perichondrium is also brighter than it is in the diaphysis. Together, these differences suggest that whereas in the cartilage matrix there has been an expansion of the injected collagen, in the perichondrium there has been an accumulation of label in a preferred orientation. The interpretation that growth of the cartilage matrix produces expansion of the label is confirmed when the injection site is found in epiphyseal cartilage or still differentiating cartilage near joints (Fig. 3*B*). In these cartilage matrices, which have undergone little growth, the expansion of the label occurred to a much lesser extent than in the diaphyses.

The most dramatic rearrangements were observed when

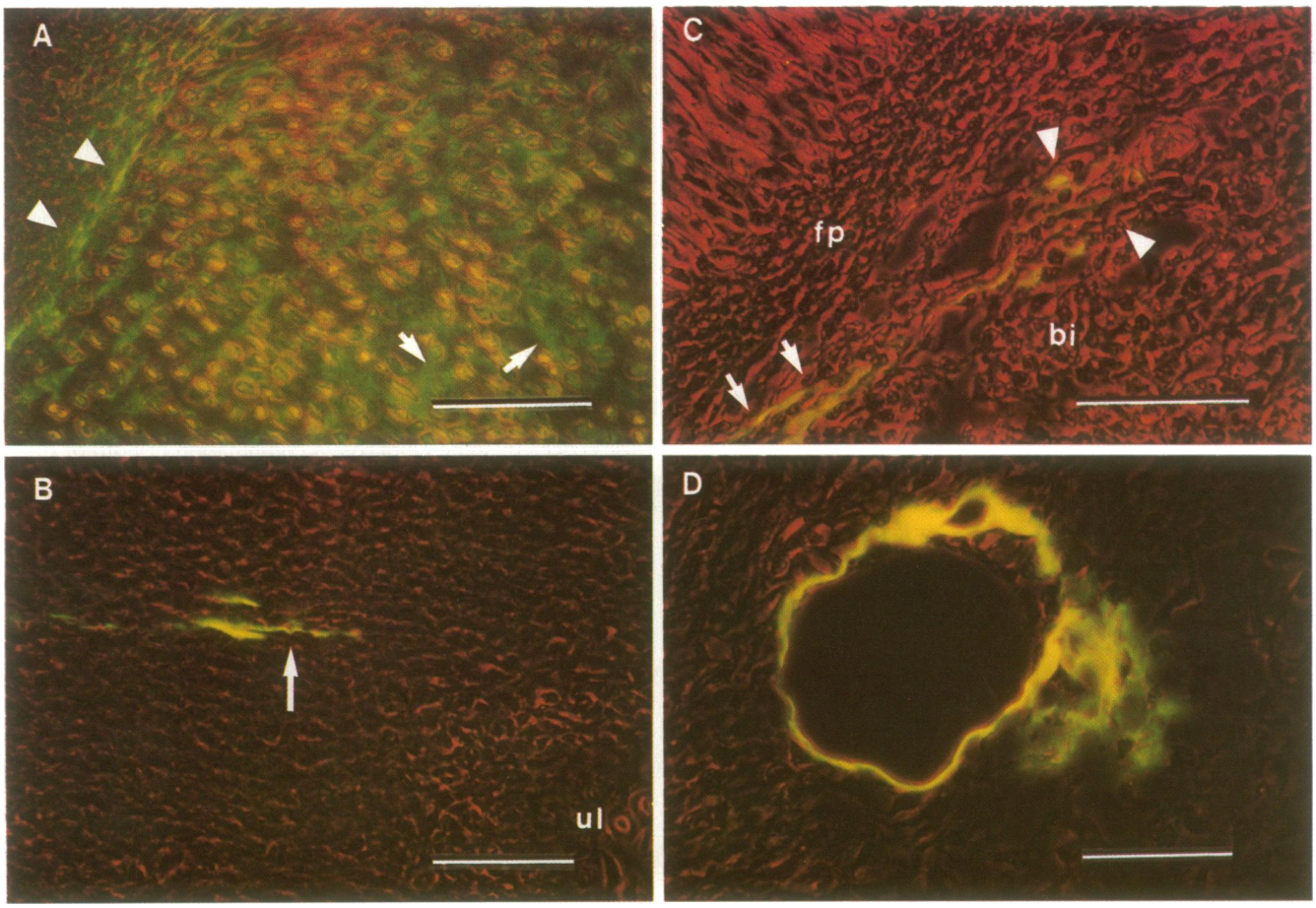

FIG. 2. (*A*) Labeled collagen is found in both the perichondrium (arrowheads) and the cartilage matrix (arrows) of the humerus and is viewed with combined phase-contrast and fluorescence optics. Initial distribution of injected collagen was comparable to that shown in Fig. 1*B*. Embryo was fixed at stage 30. (Bar = 100 μm.) (*B*) Labeled collagen is located in the prospective tendon region of the ventral flexors with the ulna (ul) visible to the right. In previous sections, the ulna extended across the field, but in this section only its proximal portion remains. Muscle mass of the ventral flexors is out of the field of view to the left. The strands of fluorescent collagen have a wavy form (arrow) and extend in and out of the plane of focus. Embryo survived until stage 31 (7 days). (Bar = 50 μm.) (*C*) Biceps muscle (bi) and humerus are shown in a combined fluorescent and phase-contrast view. Inside the muscle, the labeled collagen (arrowheads) is divided into numerous irregular strands. In the external fascia of the muscle (perimysium) and as it approaches the perichondrium (fp), the label (arrows) is organized into parallel elongated strands. Embryo survived to stage 30 (6.5 days). (Bar = 100 μm.) (*D*) Labeled collagen was accumulated onto the outer wall of blood vessels. Fluorescence intensity of the label is greater in the blood vessel wall than in the adjacent loose mesenchyme where it is not organized into any particular pattern. Embryo survived to stage 34 (8 days). (Bar = 50 μm.)

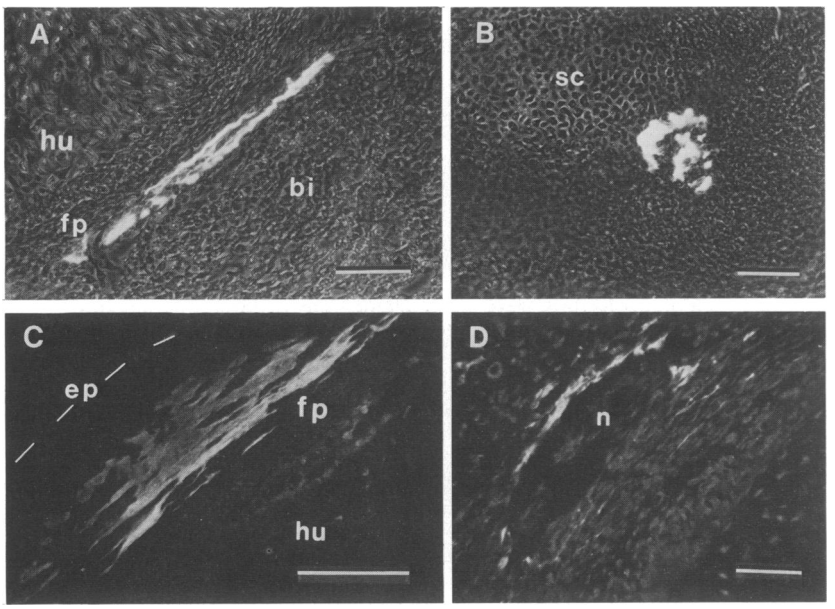

FIG. 3. (*A*) Injected collagen is located between the humerus (hu) and the biceps muscle (bi), partially in the perichondrium (fp), and partially in the perimysium. This is the same embryo shown in Fig. 2*C* viewed with combined fluorescence and phase-contrast optics. Embryo was fixed at stage 30. (Bar = 100 μm.) (*B*) Scapula (sc) is cut transversely and viewed with combined phase-contrast and fluorescence optics. In cartilage, which had undergone a small amount of growth, rearrangement of the labeled collagen was minimal with perhaps a slight expansion of this material. Embryo survived to stage 31 (7 days). (Bar = 50 μm.) (*C*) Perichondrium of the humerus (hu) and the loose mesenchyme surrounding it contains fluorescent collagen. Moving from the epithelium (ep) to the humerus, the injected collagen becomes progressively more rearranged as the humerus is approached. In the fibrous perichondrium (fp), the label is highly elongated, broken up into strands, and has a bright intensity. Viewed with fluorescence optics. Embryo survived to stage 35 (8–9 days). (Bar = 100 μm.) (*D*) Labeled collagen was distributed onto the surface of the perineurium. This fluorescence micrograph is from the same embryo shown in Fig. 2*A*. Rather than a network of strands, the label here has been incorporated into the connective tissue sheath wrapping the nerve (n). (Bar = 50 μm.)

the injection site was located just outside the shaft of a developing long bone. In these regions, the labeled collagen became highly elongated parallel to the long axis of the bone rudiment and compressed in the transverse direction (Figs. 2 *A–C* and 3 *A* and *C*). Fluorescent collagen was often found in a continuous distribution, which included the perichondrium, the connective tissue adjoining it, and sometimes the muscles or cartilage as well. The outer layer of the perichondrium or "fibrous perichondrium" (29) is made up of several layers of longitudinally oriented fibroblasts that form a ligament-like structure ensheathing the shaft of the long bones. In both the fibrous perichondrium and the connective tissue that grades into it, the labeled collagen was broken up into a series of long parallel strands and thicker elongate patches (Fig. 3 *A* and *C*). When viewed horizontally in a grazing section of the perichondrium, the labeled collagen was seen to be organized as a sheet (not shown). In one embryo, an unusually large amount of collagen was injected (≈3 μl). Label was found in a continuous mass between the humerus and the posterior margin of the limb, and both the perichondrium and the loose mesenchyme surrounding it contained labeled collagen (Fig. 3*C*). In the perichondrium, the fluorescent collagen was highly elongated and arranged as strands, while in the mesenchyme furthest away from the humerus (not shown) the label appeared to be unaltered, similar to what is shown in Fig. 1*C*. Similarly, in regions of prospective tendons and the fascia, which cover the muscles, the label was organized into elongate patches and thinner strands running parallel to the long axes of the cells (Fig. 2*B*). These strands of fluorescent collagen were not straight but were undulating and wavy, often extending in and out of the plane of focus.

In muscle, the labeled collagen was broken up into smaller irregular patches (Fig. 2*C*) or into finer strands that were scattered within the muscle tissue. The label did not form a regular pattern inside the muscle itself but seemed to be interdigitated within it; in the perimysium, the label was highly elongated as it graded into the perichondrium. Why so few embryos (only two) were found with label contained inside muscles is not clear.

Fluorescent strands of collagen were found incorporated within the walls of blood vessels. In two embryos, the label formed a complete circle around the outer vessel walls and appeared to be accumulated at the surface (Fig. 2*D*). Label also was rearranged onto the surface of the perineurium, which is the connective tissue sheath that wraps the nerves (Fig. 3*D*).

DISCUSSION

These observations indicate that, separate from the secretion or self-assembly of matrix components, forces exist within embryonic tissues whose effect is to rearrange extracellular matrices into anatomical patterns. In principle, the physical cause of these morphogenetic forces might be either simple growth (30, 31) or cellular traction or some combination of the two. Growth of the limb is predominantly in the long direction; however, it is not known what amount of stress, if any, this growth actually places on mesenchymal tissues. In general, the mechanical effect of growth will be opposite that of traction. Because of the increase in volume, either from cell proliferation or the secretion of extracellular components, the consequence of growth is to produce expansion. In contrast, the consequence of traction is compression as well as tension. In different mesenchymal tissues of the limb, the fluorescent collagen became arranged into patterns that were distinct from each other. These patterns were consistent with the predicted effects of cellular traction combined with expansion due to growth. Furthermore, the organization of the injected collagen corresponded well with the patterns of staining observed when antibodies to type I collagen are used to detect the distribution of collagen in the developing limb (27, 28, 32).

From our previous studies, several predictions can be made as to how cell traction may operate to help construct connective tissues during morphogenesis (4–7). Where cell traction is weak, the extracellular matrix will remain uncondensed, and loose connective tissues will form. This could explain the inability of cells in loose mesenchyme to remodel a cohesive mass of injected collagen. Chondrocytes *in vitro* are not able to contract collagen gels (17) so rearrangement of the injected collagen in cartilage should, primarily, be due to growth, as was observed. Inside the diaphysis of cartilage, the label became more diffuse as if being spread apart by growth, while in epiphyseal cartilage, which grows less, this expansion was less pronounced or absent (Figs. 2*A* and 3*B*). In this context, it is of interest that, although cartilage synthesizes type II collagen, the presence of the injected type I collagen did not interfere with normal morphogenesis.

Where strong traction is exerted by a population of cells and is not resisted by countervailing tension, compressive strains will predominate. Collagen and other extracellular fibers will be drawn toward the cells and compacted to form dense irregular connective tissues. Fluorescent collagen was wrapped as a dense sheet applied to the outer surfaces of perichondria, perineuria, and blood vessels, as predicted. Endothelial cells, which make up the walls of blood vessels, and fibroblasts, which make up the connective tissue sheath of the perineurium and perichondrium, are all capable of exerting strong tractional forces (5, 12).

Dense regular connective tissues, as in tendons and ligaments, will form where resistance to the forces exerted by cells causes tensile strains to develop in one direction (5). In the embryo, where cells become aligned parallel to one another, their traction will generate tension in the direction of cell elongation, causing fibers to be aligned parallel to one another. In tendons as well as in the fibrous perichondria, the fluorescent collagen either was organized into highly elongate patches or was broken up into more narrow parallel strands. A consistent feature of these strands was their wavy appearance (Fig. 2*B*). Later in development, collagen fibers in tendons develop sharp undulations with a characteristic period (33). The less extreme bending of the fluorescent collagen observed here may be antecedent to the development of the mature crimp morphology.

An unavoidable limitation of this technique is that alterations in the distribution and arrangement of the labeled collagen must be inferred from fixed tissue, raising the possibility that the collagen was injected in a form that was then mistaken for rearrangement. However, the pattern of labeling found later in development did not correspond to the patterns observed immediately after injection. In many embryos, more than one kind of tissue in the same embryo contained labeled collagen. But as development progressed, collagen from the same injection site became arranged appropriately for the tissue type where it happened to become localized. Since the collagen was injected before differentiated structures had yet emerged, this implies that the physical environment attending develoment is distinct from one tissue to another.

We suggest that the organization of collagen within tissues is due to the combined effect of short-range and long-range interactions. Previous studies on collagen morphogenesis have placed the greatest, if not sole, emphasis on the short-range ordering of collagen and, in particular, self-assembly (1, 2). Collagen molecules do possess a remarkable capacity for self-assembly into fibrils, and such findings have lent support to the expectation that collagen morphogenesis could be explained in terms of self-assembly and its modulation by the chemical environment. Another possibility is that motile cells secrete fibers into place by using specialized extracellular spaces to deposit the collagen in ordered arrays (34). However, by confronting the embryo with preformed collagen fibrils from another species, these experiments were designed to bypass these kinds of short-range interactions. We conclude that there exists within embryos mechanisms for the long-range ordering of collagen, apparently determined by the physical environment of mechanical stresses and strains, including those generated by cell traction.

We are grateful to our colleagues who have patiently listened and criticized while this work was in progress: Drs. R. Akers, T. Baskin, R. Grainger, G. Goldin, P. Green, C. Mason, D. Sinicropi, and J. Wrenn. This research was supported by American Cancer Society Grant PF-2251 to D.S., NIH Grant HD-04708 to N.K.W., and NIGMS Grant 24251 to A.K.H.

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