# An Instructive Role for the Interstitial Matrix in Tissue Patterning: Tissue Segregation and Intercellular Invasion

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*Abstract.* Intercellular invasion is the intrusion of the cells of one tissue into space occupied by a second tissue. The alternative situation to invasion, one characteristic of most coherent tissues, is segregation, with identifiable boundaries existing between contiguous tissues. The interfaces between mesenchymal and myocardial tissues in the developing avian heart show a profoundly different character in different regions of the heart: the interface between epicardial mesenchyme and heart wall myocardium is planar, without intermingling of the two cell types, whereas the interface between endocardial cushion mesenchyme and myocardium is diffuse, with extensive invasion of both tissue types across the border to produce intermingling of the two tissues. Thus, invasion and tissue segregation coexist in different regions of the mesenchyme–myocardium contact zone. Investigation of the involvement of the interstitial matrix in invasion and segregation has been conducted by maintaining the two tissues in mutual contact in organ culture. Investigation of the mechanisms by which the two cell types sort out in randomized chimeric tissue reaggregates has provided insight into the conditions for tissue segregation. We have modeled invasion in organ culture by fusing aggregates of myocardial cells with aggregates of cardiac mesenchymal cells. Cells of both tissues invaded the partner aggregate during a period of 1–3 d of coculture. Both invasion and segregation in the aggregates appear to depend on the presence or absence of a fibronectin-rich interstitial matrix elabo-

rated by the cardiac mesenchyme. During sorting, the matrix appears selectively in regions occupied by the mesenchyme. Under conditions of culture that are nonpermissive for matrix deposition, sorting fails to occur. Stimulation of matrix deposition by addition of serum, transforming growth factor $\beta$, or isolated matrix itself is accompanied by sorting out of the two tissues. Sorting out is blocked reversibly by inclusion of the fibronectin adhesion site peptide, GRGDSP. Invasion of fused aggregates is preceded by a redistribution of the fibronectin-containing matrix of the mesenchymal aggregate such that matrix-poor regions come to occupy the interface with the myocardial partner aggregate. The invasion that ensues involves mesenchymal cells emigrating from, and myocardial cells intruding into, matrix-poor regions of the mesenchymal aggregate. In the intact heart, invasion occurs in regions of the mesenchyme that are depleted of a fibronectin-containing matrix (e.g., endocardial cushion mesenchyme), and tissue segregation is maintained with regions of the mesenchyme that display a well-developed fibronectin-containing matrix (e.g., the epicardial mesenchyme). It is suggested that the composition of the interstitial extracellular matrix exerts an important influence on the mutual organization of mesenchymal tissues with respect to adjacent tissues: tissue segregation is promoted if the mesenchyme contains a well-developed adhesive interstitial matrix, whereas poor development of that matrix establishes conditions that favor tissue invasion.

I T has long been presumed that adhesive interactions of cell with cell and cell with extracellular matrix play important roles in tissue organization and cell recognition. One of the useful approaches to investigate this presumption developed from the discovery by J. Holtfreter that embryonic tissues could reestablish normal histotypic relationships when cohering tissue fragments or disorganized chimeric cell reaggregates were maintained in organ culture (Holtfreter, 1939, 1944; Townes and Holtfreter, 1955). These approaches have been applied to a variety of organs and have demonstrated that embryonic and adult tissues have remark-

able powers of self-assembly. Normal relations can be established when homogeneous tissue fragments are cultured in apposition or when random chimeric aggregates produced by reaggregation of mixed suspensions of dispersed cells are maintained in organ culture (for review see Armstrong, 1989).

The present study applies these procedures to the investigation of the determinants of tissue invasion and tissue segregation—the two basic modes of organization of the interfaces between apposed tissues. Intercellular invasion is the intrusion of one tissue into space occupied by a second tissue

(Abercrombie, 1970). The alternative situation to invasion, one characteristic of most coherent tissues, is segregation, with identifiable boundaries existing between contiguous tissues. The sorting out of disorganized mesenchyme—myocardium aggregates produces a restoration of the segregated organization. Invasion, which is shown by the cells of fused pairs of aggregates of mesenchyme and myocardium cells, results in cellular intermingling and a loss of tissue segregation.

## Materials and Methods

### Cell Culture

In all cases, the culture medium was DME (Gibco Laboratories, Grand Island, NY) with various additions, the gas phase was 95% air and 5% $CO_2$, and the culture temperature was 37°C. Both whole serum and serum depleted of fibronectin by passage over a gelatin-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) affinity column (Engvall and Ruoslahti, 1977) were used. 10-d chick embryo heart ventricle tissue was disaggregated in 0.5 mg/ml trypsin (1:250; Difco Laboratories Inc., Detroit, MI) (DeHaan, 1967) and freed of a majority of cardiac mesenchyme by culture of the resulting cell suspension in tissue culture-grade plastic Petri dishes (Falcon Labware, Oxnard, CA) for 1.5 h. During this period, the mesenchymal cells attach to the dish, whereas the myocytes do not, allowing the myocytes to be removed when the medium is decanted (Armstrong and Armstrong, 1978, 1979; Armstrong and Armstrong, 1984). Cardiac mesenchyme was prepared by incubating the myocyte-depleted cultures until the cells grew to confluence (3-4 d). The cultures contained predominantly cells of fibroblastic morphology, with <1% myocytes and unidentified epithelioid cells that may be endothelium. The mesenchymal cells were labeled with [³H]thymidine during growth of the cells to confluence in culture (1 μCi/ml and 2 Ci/mmol). Cardiac mesenchymal cells were removed from the culture dish by incubation of the monolayer with 0.5 mg/ml trypsin for 5 min (37°C). This was followed by exposure of the dissociated cells to 1 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.) to inactivate the trypsin. In situations where the attachment of freshly dissociated cells to fibronectin-coated surfaces was to be measured, the trypsinization was conducted using crystalline trypsin in the presence of calcium to prevent inactivation of the fibronectin receptors (Akiyama et al., 1986; Giancotti et al., 1985; Oppenheimer-Marks and Grinnell, 1984; Tarone et al., 1982).

### Cell Sorting

Disorganized chimeric reaggregates were prepared by centrifuging mixed cell suspensions containing unlabeled myocytes and [³H]thymidine-labeled mesenchymal cells in 16-ml screw-cap test tubes followed by stationary incubation at 37°C for 3-5 h. Centrifugation was performed from a small volume of culture medium (~100-200 μl) to prevent stratification of cells in the pellet as a function of specific gravity. After 3-5 h of incubation at 37°C, the pellets were chopped into pieces 1-2 mm square with knives made from sewing needles, and the pellet pieces were cultured further in shaker flask suspension culture (Armstrong, 1971). Unlabeled thymidine (0.1 mM) was included in the culture medium to reduce transfer of labeled thymidine from labeled cells to unlabeled cells.

### Tissue Invasion

The principal in vitro model for the investigation of invasion involved the pair-wise fusion of pure mesenchyme and pure myocardium aggregates. One each of a [³H]thymidine-labeled cardiac mesenchymal aggregate and

an unlabeled myocardial aggregate were placed in a 20-μl drop of culture medium on the surface of a bacteriological petri dish. When the dish was inverted, the standing drop of medium became a hanging drop, and the two aggregates were brought in contact at the concave bottom of the drop. Once the two aggregates adhered and established a common border (2-4 h of culture in the hanging drop), the aggregate pair was transferred to stirred suspension culture in a 25-ml Erlenmeyer flask maintained on a water bath gyratory shaker set at 37°C and 95 rpm. Invasion was monitored histologically after one or more days of culture by the presence of cells of one aggregate in the interior of its partner aggregate.

### Histology and Radioautography

Aggregates were washed thoroughly in several changes of PBS and fixed in freshly prepared 4% paraformaldehyde in PBS (0.5 h) or in ice-cold Carnoy's fixative (three parts chloroform, six parts absolute ethanol, and one part glacial acetic acid). Paraformaldehyde-fixed aggregates were subsequently washed in PBS, treated with PBS modified to contain 1 M NaCl and 0.1 M glycine, and then stored in PBS plus 1 mM NaN₃ at 4°C. Aggregates were cryosectioned or embedded in paraffin (exposure to melted paraffin was limited to 40 min) and sectioned at 6 μm. Radioautography was accomplished by coating deparaffinated sections with a 1:1 dilution of NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY) followed by incubation for 3 wk at 4°C in a light-tight box containing desiccant. Autoradiograms were developed in a 1:3 dilution of Dektol (2 min at 17°C) and were stained through the emulsion with Harris' hematoxylin and eosin Y (Kopriwa and Leblond, 1962).

### Immunocytochemistry

Immunocytochemical staining of tissue sections followed standard protocols. Anti-chick cellular fibronectin antiserum was obtained from Dr. K. Yamada (National Cancer Institute, National Institutes of Health, Bethesda, MD), and anti-chick plasma fibronectin antibodies were obtained from Drs. D. Garrod (Cancer Research Campaign Medical Oncology Unit, Southampton General Hospital, Southampton, United Kingdom) and E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) (Morgan and Garrod, 1984; Yamada, 1978). Polyclonal and monoclonal antibodies to chicken tenascin were obtained from Dr. D. Fambrough (Department of Biology, The Johns Hopkins University, Baltimore, MD) (Chiquet and Fambrough, 1984a,b). Polyclonal antibodies to human laminin were obtained from Dr. E. Engvall (La Jolla Cancer Research Foundation, La Jolla, CA) (Engvall et al., 1983). Affinity-purified polyclonal antibodies to chicken type I collagen were provided by Dr. J.-M. Chen (Department of Anatomy, Thomas Jefferson University, Philadelphia, PA) (Chen and Little, 1985). Controls included substitution for immune serum with nonimmune or preimmune serum and antisera inactivated by preincubation with antigen (fibronectin staining only). Double immunocytochemical staining was accomplished by staining initially with a first antibody produced in rabbit followed by FITC-conjugated sheep anti-rabbit IgG and then staining with goat anti-chicken fibronectin followed by rhodamine isothiocynate-conjugated rabbit anti-goat IgG. Coverslips were mounted with PBS/glycerol (1:9) containing 1 mM phenylenediamine (Johnson and Nogueira Araujo, 1981) to reduce photobleaching of the fluorochrome. Slides were viewed with a microscope (WL; Carl Zeiss, Inc., Thornwood, NY) equipped with a epifluorescence condenser (Carl Zeiss, Inc.), a 50 W high-pressure mercury lamp, and fluorescein and rhodamine filter sets. Photomicroscopy was performed on Ektachrome 400 film. Indirect immunoperoxidase staining used the Vector ABC biotin-avidin kit. Immunocytochemical staining for actin used Carnoy's-fixed, paraffin-embedded specimens and rabbit anti-actin (Miles Laboratories Inc., Elkhart, IN). Specimens stained with DAB by the immunoperoxidase technique were photographed with an FITC-495 filter to improve contrast (Gordon, 1988). Specimens destined for autoradi-

*Figure 1.* Frontal sections of the 10-d chick embryo heart. (*a*) Low magnification view of the heart. (*b*) Higher magnification view in the same tissue section of the epicardial mesenchyme–myocardium border, which is planar, with no apparent encroachment of either tissue into the other. (*c*) The inferior and superior right atrioventricular valves, in which the border between the endocardial cushion mesenchyme and the myocardium is diffuse, with extensive intermingling of the two tissues. Arrows indicate myocardial tissue that intrudes into the mesenchyme. (*d*) Transverse section at a more ventral level from that of *a* showing invasion of the mesenchyme by myocardium (*arrows*) in the vicinity of the aortic valve. The myocardium is selectively stained for glycogen by the periodic acid–Schiff stain and is darker than the mesenchyme. *A*, right atrium; *AV*, right atrioventricular valve; *C*, coronary artery; *E*, epicardial mesenchyme; *Mes*, mesenchyme; *My*, myocardium; *V*, right ventricle. Bars: (*a*) 400 μm; (*b*) 50 μm; (*c*) 50 μm; (*d*) 50 μm.
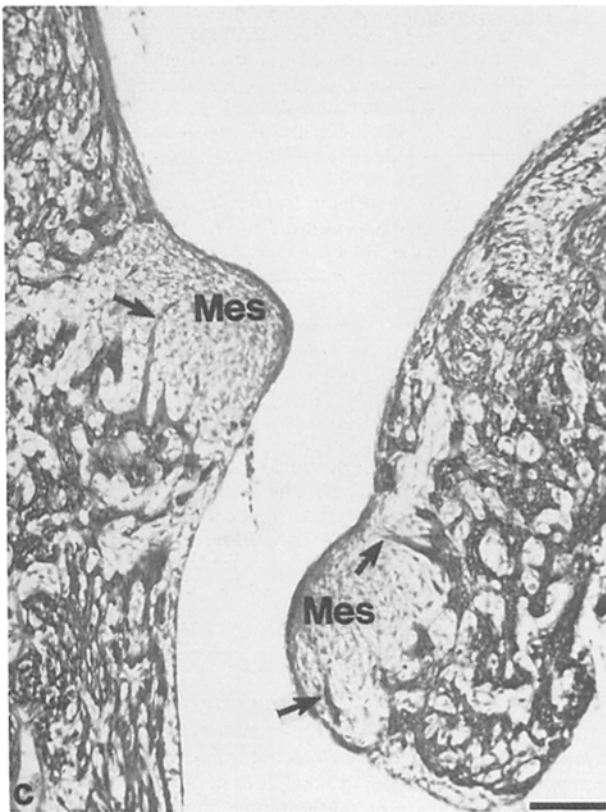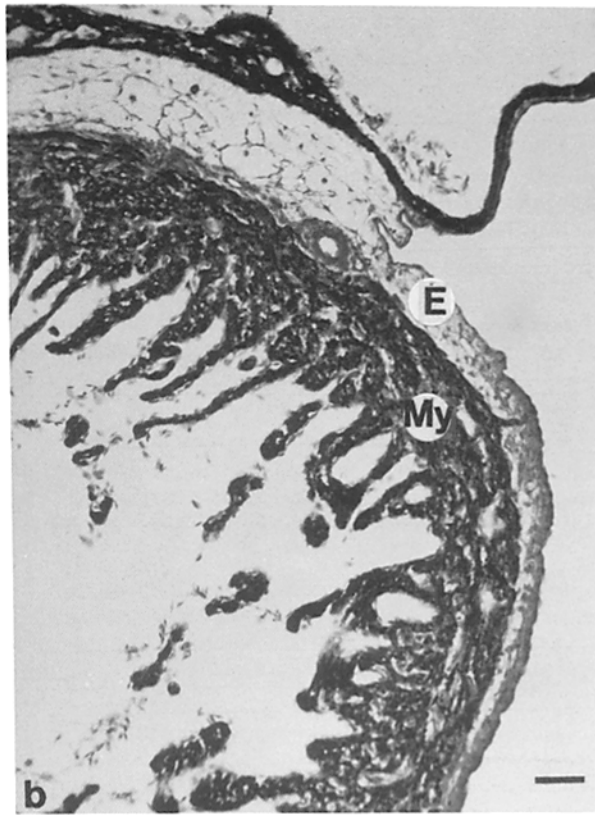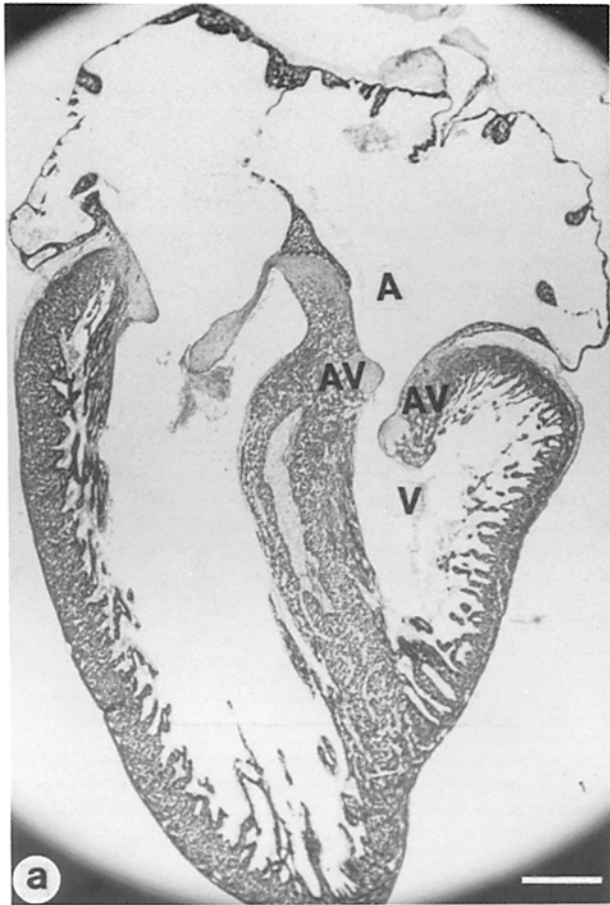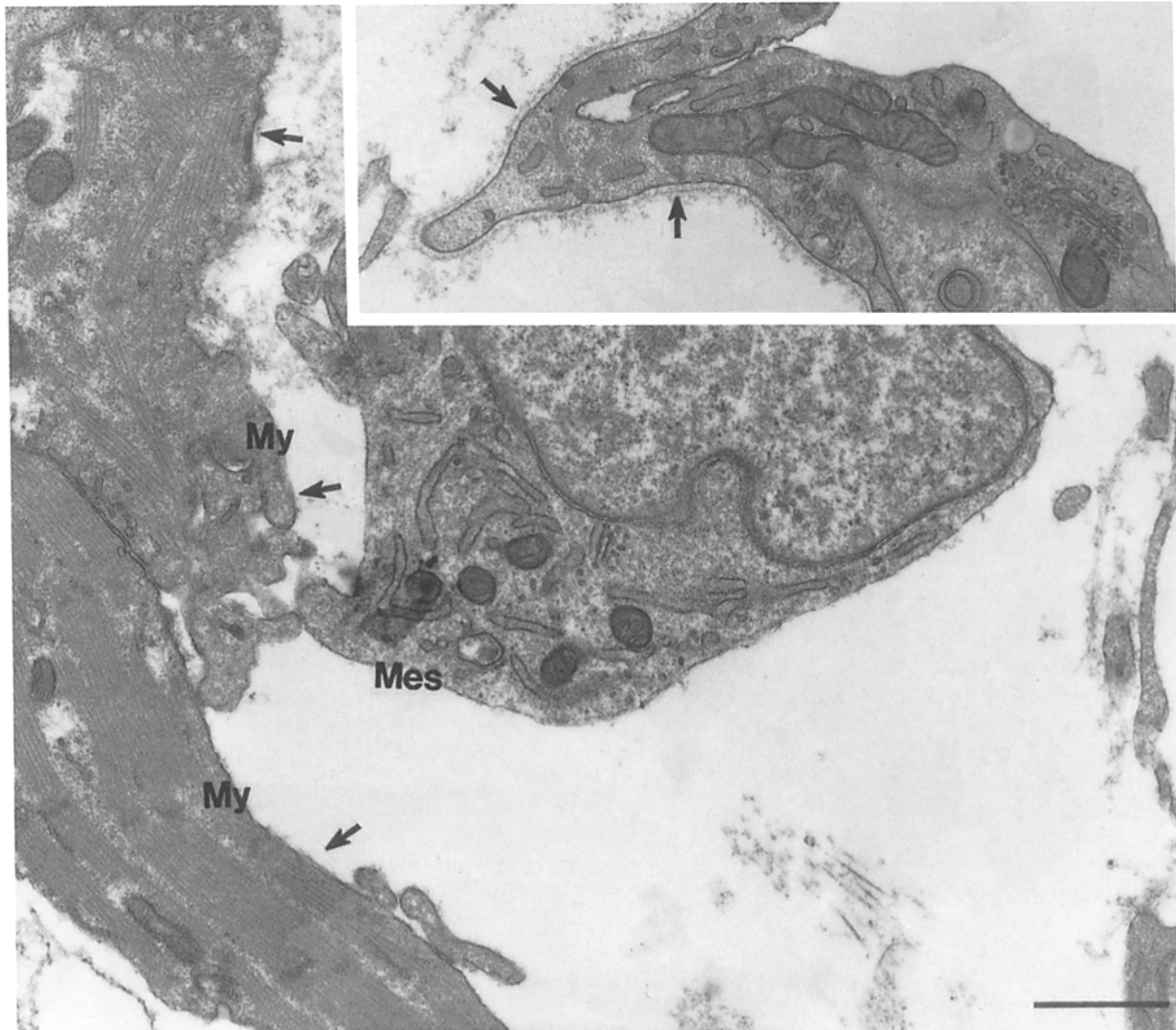
*Figure 2.* Transmission electron micrograph of the interface between epicardial mesenchyme (*Mes*) and ventricle wall myocardium (*My*) of the 10-d chick embryo heart (*arrows*). The myocardium is identifiable by bundles of thick and thin myofilaments. A basal lamina is absent at the interface between the two tissues. The basal lamina is distinct in micrographs taken at the same magnification of the boundary between the epicardium, a one-cell-thick mesothelial layer at the surface of the heart, and the epicardial mesenchyme (*inset; arrows*). The epicardial mesenchyme is sandwiched between the epicardium and the heart wall myocardium. The specimen was fixed with glutaraldehyde, postfixed with OsO₄, and treated with 1% tannic acid as recommended by Simionescu and Simionescu (1976). Bar, 1 μm.

ography and immunocytochemistry were first stained and then coated with radiographic emulsion.

## Synthetic Peptides

Peptides were synthesized using an automatic peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) with the chemistry provided by the manufacturer. After cleavage from the resin with hydrogen fluoride, the peptides were washed with cold ethyl ether, precipitated from solution in trifluoroacetic acid with ice-cold ethyl ether, redissolved in water, and further purified by HPLC using a Bio-Gel TSK SP-5-PW column (75 × 7.5 mm; Bio-Rad Laboratories, Richmond, CA). Lyophilized preparations were stored in a desiccator at −20°C. Solutions of peptides in culture medium were prepared from the lyophilized powder immediately before use.

## Results

### Tissue Organization in the Avian Heart

By 10 d of incubation, the chick embryo heart shows the ma-

jor features of the adult anatomy. The two principal tissues at this stage are the myocardium and the cardiac mesenchyme. The mesenchyme is prominent at two locations: at the surface of the heart wall (the epicardial mesenchyme) and in the outflow tract, septa, and the atrioventricular valves (the endocardial cushion mesenchyme) (Fig. 1 *a*). At 10 d of incubation, the interface between myocardium and mesenchyme shows a very different organization in these two regions, with strict segregation of the two tissues at the myocardium–epicardium border (Fig. 1 *b*) and an intermingling of the two cell types at the cushion mesenchyme–myocardium border that is especially prominent in the right atrioventricular valve (Fig. 1 *c*) and adjacent to the aortic valve (Fig. 1 *d*). There is limited invasion of the mesenchyme adjacent to the pulmonary and left atrioventricular valves, but no invasion of the actual valve leaflets. Both epicardial and endocardial cushion mesenchymes are in direct contact with myocardium, without an intervening basal lamina (Fig. 2).
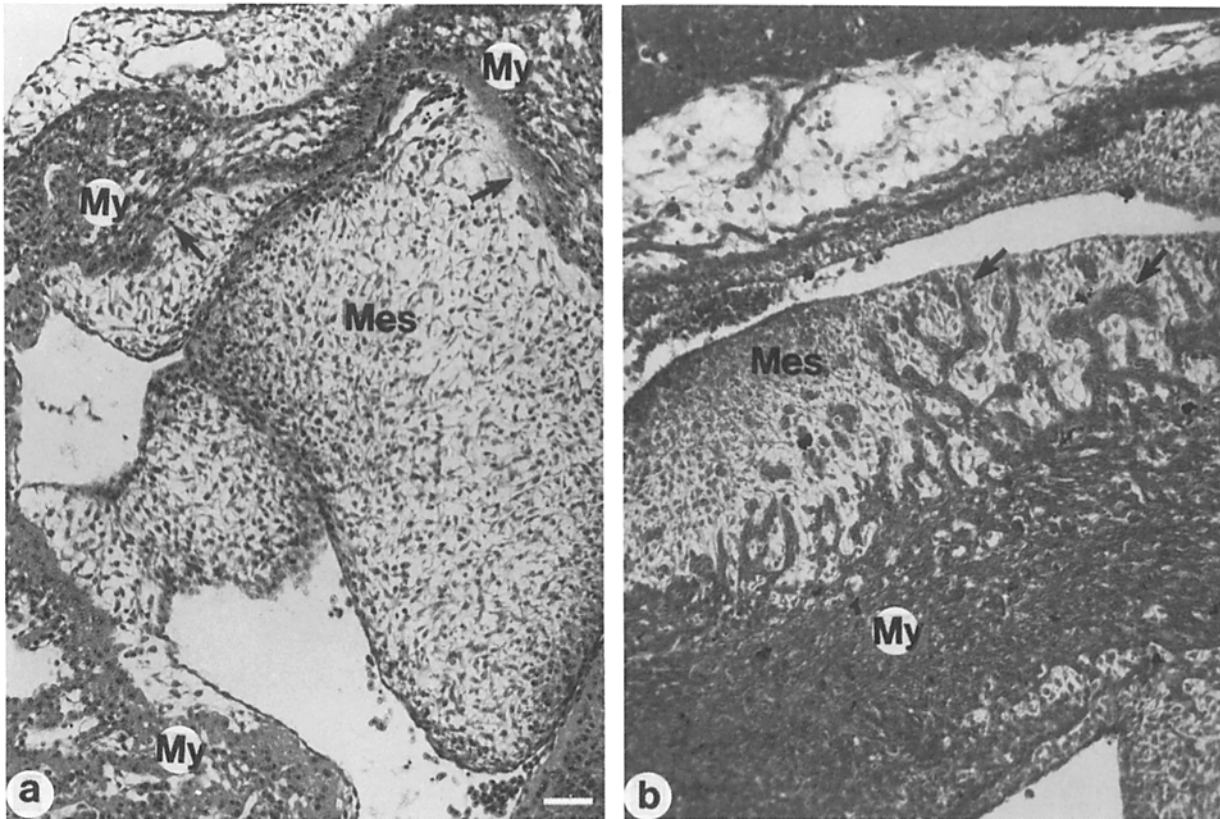
*Figure 3.* Transverse sections of the endocardial cushion mesenchyme–myocardium border of the 5- and 10-d heart. At 5 d of development, the endocardial cushion mesenchyme (*Mes*) is in contact with the myocardium (*My*), but neither tissue shows signs of invasion across the border (*arrows*). By 10 d, intermingling is extensive in the right atrioventricular valve (*b*, *arrows*). The progressive character of the process suggests that the intermingling of the two tissues is produced by active invasion. Bar, 50 μm.

The intermingled organization of cushion mesenchyme and myocardium appears to be a consequence of active invasion of the cells of each of the tissues into the other. At an early stage of heart development, the cushion mesenchyme–myocardium border is planar, without tissue intermingling (Fig. 3 a). Intrusion of myocardium into the domain of the cushion mesenchyme is first seen in the 6–7-d heart, and mutual intermingling of the two tissues is progressively more pronounced between then and 10 d (Fig. 3 b). Of interest are the mechanisms that preserve tissue segregation along the epicardial mesenchyme–myocardium border and that produce invasion along the cushion mesenchyme–myocardium border.
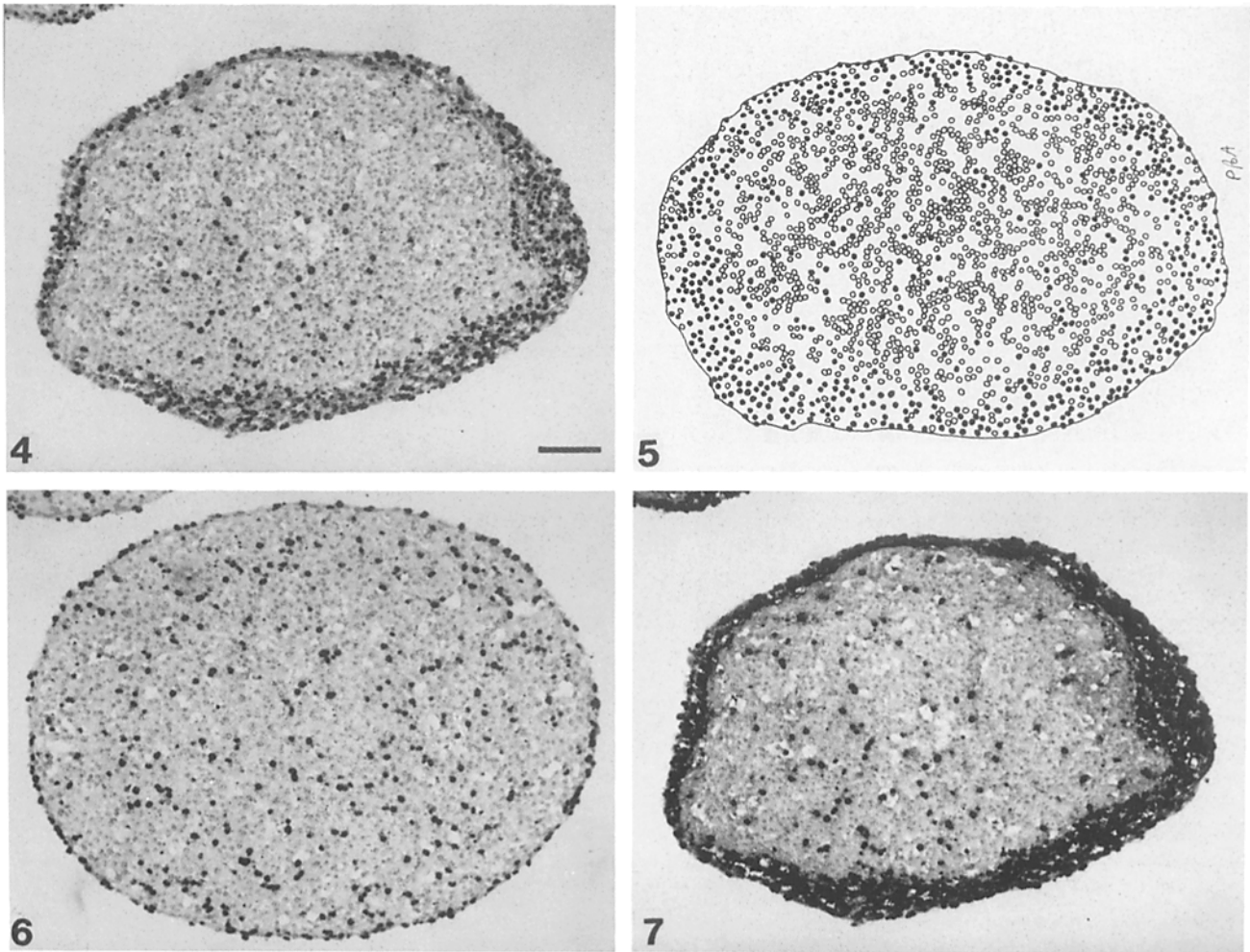
### Sorting Out of Mesenchyme–Myocardium Aggregates

To distinguish mesenchyme from myocardium in mixed aggregates, the mesenchymal cells were labeled with [³H]thymidine before coaggregation with myocardial cells and were subsequently identified in tissue sections by radioautography. Reaggregates contained either 75/25% or 80/20% mixtures of myocytes and mesenchymal cells. Aggregates cultured in serum-containing culture medium sorted out to establish a superficial layer of mesenchymal tissue surrounding a core of myocardium (Fig. 4). The accuracy of sorting was estimated by direct counts from camera lucida drawings of the fraction of labeled cells in the superficial and interior tissues (Fig. 5). The precision of sorting under standard conditions was ∼85%. In the aggregate pictured in Fig. 5, 82%

of the mesenchymal cells were in the periphery, and 87% of the myocytes were in the interior.

Sorting out was dependent on the inclusion of serum in the culture medium. Aggregates produced in serum-free medium (DME plus 1 mg/ml BSA) became strongly coherent with a smooth spherical or ovoid shape but failed to sort out (Fig. 6). The failure to sort was reversible; aggregates produced in serum-free medium sorted out within 6–8 h if transferred to serum-containing medium. In the experiments that follow, aggregates were prepared by culture overnight in serum-free medium, and the ability to sort out under a variety of experimental conditions was ascertained by determining the degree of sorting out after transfer to modified culture medium for 8–24 h of further culture. Since the aggregates produced in serum-free medium were fully compacted at the time of transfer, this procedure allowed us to separate sorting out from cell aggregation and the compaction of the aggregate that occurs during the first few hours of culture.

### Sorting Out Appears To Be Dependent on the Composition of the Interstitial Matrix

The sorting out of cultured aggregates composed of myocardium and cardiac mesenchyme appears to depend on the deposition by the cardiac mesenchyme of an adhesive interstitial matrix. The matrix is rich in fibronectin, which was used as a convenient marker for the presence of the matrix. Myocardium produces at best only scanty quantities of ma-

*Figures 4, 5, 6, and 7.* (Fig. 4) Sorting out of [³H]thymidine-labeled cardiac mesenchyme from unlabeled myocardium. The mesenchyme is present at the surface of the aggregate and surrounds completely the myocardium. (Fig. 5) Camera lucida drawing of a chimeric aggregate containing [³H]thymidine-labeled heart mesenchyme and unlabeled myocardium that has sorted out in serum-containing medium. The solid circles indicate the positions of the labeled nuclei; the open circles indicate the positions of the unlabeled nuclei. (Fig. 6) Aggregates maintained in serum-free medium were spherical or ovoid and compact, with satisfactory adhesion between the cells, but failed to sort out. (Fig. 7) Colocalization of fibronectin (stained by the immunoperoxidase-DAB procedure) and [³H]thymidine-labeled mesenchyme in the sorted out myocardium–mesenchyme aggregate. This is the same section as Fig. 4, with the DAB immunoperoxidase product emphasized in this figure by the presence of a FITC-495 filter in the light path. Bar, 80 µm.

trix fibronectin in aggregate culture (Armstrong and Armstrong, 1981). In aggregates that have sorted out, immunocytochemically detectable fibronectin colocalized with the mesenchymal tissue (Fig. 7). The aggregates maintained under serum-free conditions were compact, with satisfactory tissue cohesiveness, but lacked significant quantities of the matrix and, as noted above, failed to sort out (Fig. 6). Matrix deposition was stimulated if such aggregates were transferred to culture medium containing serum, human platelet transforming growth factor β (TGF-β)[1] (Cat. No. 619350; Calbiochem-Behring Corp., San Diego, CA), or preparations of the extracellular matrix itself. All of these agents stimulated sorting out of the chimeric aggregates. The pattern of sorting was similar in all cases, with the mesenchymal tissue occupying part or all of the surface of the aggregates and, thus, enveloping the myocardium. The extent of envel-

opment of the myocardial core by the mesenchyme was complete for aggregates cultured in serum- or TGF-β–containing medium, but was sometimes incomplete for aggregates cultured with isolated extracellular matrix. Mesenchyme and myocardium sorted out in these latter aggregates, but the mesenchyme was present in patches at the surface rather than as a coherent sheet.

TGF-β stimulated matrix deposition and sorting by 8 h of culture at the lowest concentration tested, 100 pM. Two different preparations of extracellular matrix were effective in promoting sorting: a 1 M urea extract of heart fibroblast monolayers (Yamada, 1982; Yamada et al., 1975) and the residual matrix that remains after lysis of the heart fibroblast monolayer in 0.025 M $NH_4OH$ (Fairbairn et al., 1985). In the first preparation, the protein of the urea extract was precipitated with 70% saturated $(NH_4)_2SO_4$, the precipitate was dialyzed into serum-free medium, and the insoluble material was dispersed by sonication in serum-free culture me-

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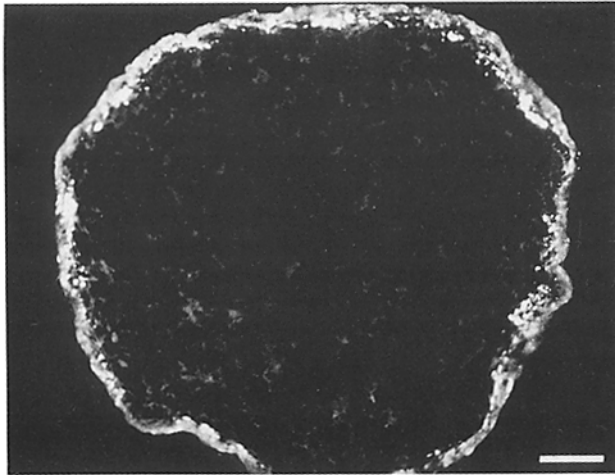1. *Abbreviation used in this paper:* TGF-β, transforming growth factor β.

Figure 8. Extracellular matrix isolated from cultured cardiac mesenchymal monolayers incorporates directly into chimeric myocyte–mesenchyme aggregates cultured in serum-free medium. After its isolation from the monolayer cultures, the isolated matrix was labeled with NHS-LC-biotin, dispersed by sonication in serum-free culture medium, and presented to preformed, unsorted aggregates prepared in serum-free medium. The biotinylated matrix was detected by fluorescence microscopy after exposure of tissue sections to FITC-avidin. Bar, 80 μm.

dium. The second preparation was prepared by treating confluent cell layers with NH₄OH until the cells lysed, leaving behind a coherent filmy matrix that released from the dish during the extraction process. This was transferred to PBS, treated with DNAase I in the presence of 5 mM MgCl₂ and 1 mM PMSF, and then sonicated in serum-free culture medium. The sonicated matrix was shown to incorporate directly into preformed, unsorted aggregates by demonstrating that exogenously supplied matrix that had been prelabeled with lissamine rhodamine B sulfonyl chloride (Cat. No. L-1908; Molecular Probes Inc., Junction City, OR) or NHS-LC-biotin (Cat. No. 21335; Pierce Chemical Co., Rockville, MD) was present in the aggregate after a few hours of incubation. The added matrix colocalized with the mesenchymal tissue as the latter sorted out to the surface of the aggregate (Fig. 8).

## A Differential Adhesion Mechanism for Sorting

The extracellular matrix may facilitate sorting out by a differential adhesion mechanism: if the mesenchymal cells are more adhesive than the myocardium for the interstitial matrix, this could enable them to monopolize the matrix and, in so doing, exclude the myocardial cells from regions rich in matrix. This explanation views tissue segregation as a byproduct of an adhesion-driven cosegregation of mesenchyme and matrix. Presumably, the restriction of matrix-synthetic capabilities to the mesenchymal cells further ensures the colocalization of matrix with mesenchyme. Supporting the suggestion for a preferential adhesive affinity of mesenchyme for matrix is the demonstration that heart mesenchyme attached more efficiently to fibronectin-coated surfaces than did the myocardium: fibronectin-coated latex spheres attached in significantly larger numbers to the surface of mesenchymal cells than to myocardial cells. These studies were conducted at 20°C to eliminate phagocytosis of the spheres. Two conditions of culture were used: (a) cells were dissociated under conditions that preserved the activity of cell surface integrin receptors (1 mg/ml crystallized trypsin in the presence of 1 mM CaCl₂ at 22°C for 10 min) (Akiyama and Yamada, 1985; Giancotti et al., 1985; Oppenheimer-Marks and Grinnell, 1984; Tarone et al., 1982) and plated onto BSA-coated culture dishes; and (b) myocardial or mesenchymal aggregates were prepared in serum-free medium (Fig. 9). The cells plated onto BSA-coated dishes attached but failed to spread. This condition was chosen to reduce the extent of redistribution of the fibronectin receptors to the ventral surface of the cells. In this situation, by 0.5 h, myocardial cells bound an average of 4.8 ± 0.53 fibronectin-coated beads per cell, and mesenchymal cells bound an average of 15.9 ± 1.02 beads per cell. In the situation of attachment to cell aggregates, the average number of beads per 1,000 μm² of aggregate surface (0.5-h incubation) was 3.6 ± 0.52 for myocardial aggregates and 7.6 ± 0.83 for mesenchymal aggregates. The isolated cells and aggregates of both cell types bound only 1–10% as many BSA-coated beads as fibronectin-coated beads. Thus, both cell types showed an adhesive affinity for fibronectin-coated surfaces, but this affinity was more pronounced for the mesenchymal cells than for the myocardial cells.
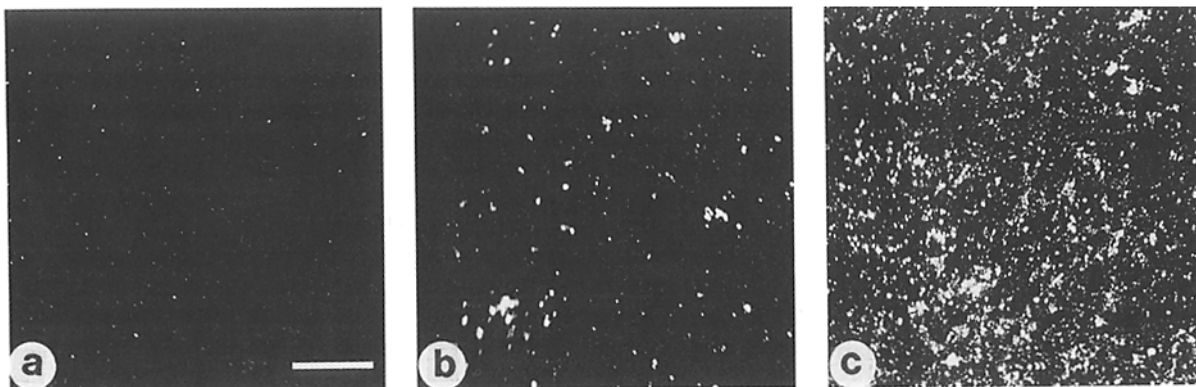


Figure 9. Binding of fluorescent 2-μm latex spheres to the surfaces of aggregates maintained in serum-free medium. Only low numbers of BSA-coated spheres attach to either type of aggregate (a; mesenchymal aggregate). Intermediate numbers of fibronectin-coated spheres attach to the surfaces of myocardial aggregates (b), while large numbers of fibronectin-coated spheres attach to mesenchymal aggregates (c). Bar, 100 μm.
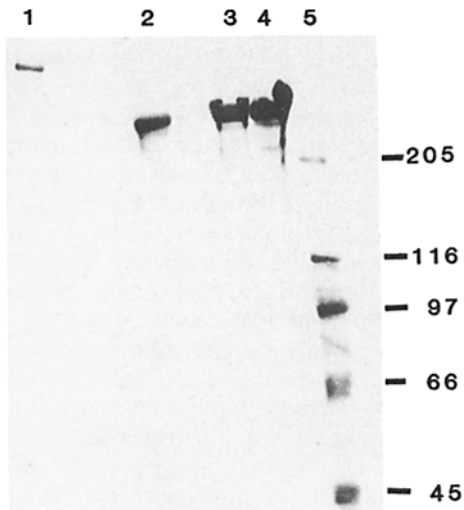
Figure 10. SDS-PAGE of the extracellular matrix material released from confluent chick heart mesenchyme monolayers by exposure to 1 M urea. (Lanes 1-5, respectively) Urea-extracted matrix under nonreducing conditions; urea-extracted matrix under reducing conditions; reduced bovine plasma fibronectin; reduced human plasma fibronectin; molecular weight standards. Coomassie blue staining. The origin of the gel is at the top of the photograph; the dye-front is at the bottom.

## Characterization of the Mesenchymal Interstitial Matrix

Preliminary characterization of the composition of the matrix preparations that promoted sorting of aggregates in serum-free medium and the histochemical characterization of the matrix of the sorted out aggregate have suggested several candidates for the matrix component(s) responsible for sorting.

*Fibronectin.* The most abundant protein(s) of the isolated matrix comigrated with fibronectin by SDS-PAGE (Fig. 10). This protein band stained with anti–fibronectin antibodies after electrophoretic transfer to nitrocellulose. Fibronectin was an abundant species in the interstitial matrix of aggregates that had sorted out in the presence of fibronectin-

depleted serum, TGF-$\beta$, or added extracellular matrix (Figs. 7 and 8) and served as a convenient indicator for the presence of the matrix. Both fibronectin-depleted serum and TGF-$\beta$ act to stimulate matrix production by the aggregate. Sonicated extracellular matrix added to the culture medium, in contrast, incorporates directly into the aggregate (Fig. 8). We have been unable to achieve sorting in serum-free medium by the addition of purified plasma fibronectin, so direct evidence for its involvement in the process is unavailable.

*Tenascin.* Tenascin (Bourdon et al., 1983; Chicquet and Fambrough, 1984a,b; Erickson and Inglesias, 1984) was also present as fibrillar elements in the interstitial matrix of the sorted out aggregate (Fig. 11 a) and, like fibronectin (Fig. 11 b), colocalized with the mesenchyme (Fig. 11 c). The different stimulators of sorting appeared to exert different degrees of stimulation of tenascin in the aggregates, with serum exerting a strongly stimulatory action and TGF-$\beta$ being only weakly stimulatory. At 100 and 300 pM TGF-$\beta$, the amounts of tenascin were below the limits of detectability, and, only at 1,000 pM, was tenascin detectable. Tenascin was present in the preparations of interstitial matrix that stimulated sorting of aggregates cultured under serum-free conditions (Fig. 12 a), where it localized on fibronectin-containing fibrils (Fig. 12 b).

*Laminin.* Laminin was present in the interstitial matrix of the mesenchymal tissue of the sorted out aggregate in a pattern reminiscent of the distribution of fibronectin and tenascin and was present in the isolated extracellular matrix preparations that stimulated sorting (Fig. 12 c). Laminin has previously been reported as a component of the interstitial matrix of mesenchymal tissue (Chakrabarty et al., 1987; Hayman et al., 1982; Woodley et al., 1988) and can serve as an adhesive substrate for mesenchymal cells (Codogno et al., 1987; Couchman et al., 1983; Horwitz et al., 1985). Sonicated preparations of polymerized collagen type IV- and laminin-containing matrices produced by Engelbreth-Holm-Swarm tumor cells and marketed as Matrigel (Collaborative Research, Lexington, MA) did not promote sorting of aggregates when used under the same conditions as the cardiac mesenchymal extracellular matrix, suggesting that cellular interactions with laminin are not sufficient to produce sorting.
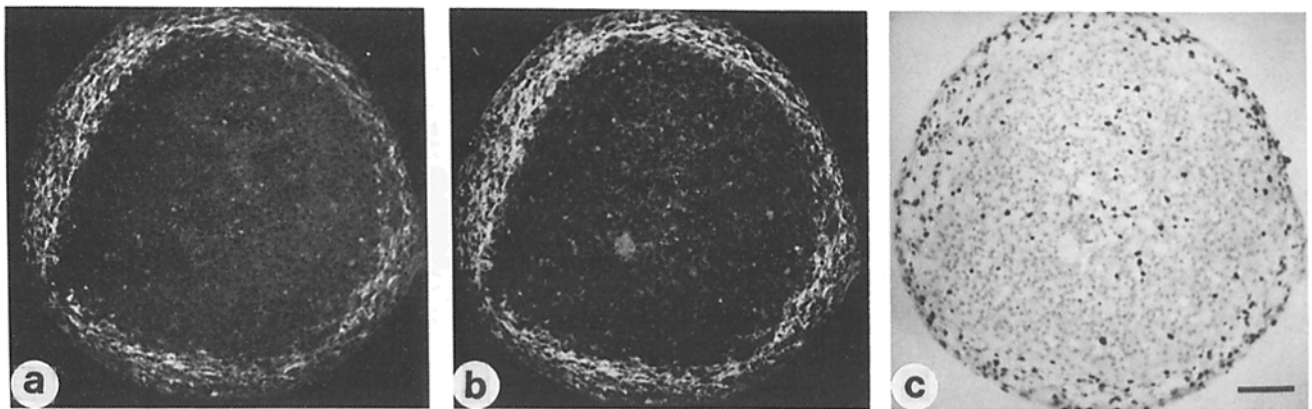


Figure 11. Double immunofluorescent staining for tenascin and fibronectin of a chimeric myocyte–mesenchyme aggregate. The tenascin (a) colocalizes with fibronectin (b) and with the mesenchymal tissue (c). Double immunocytochemical staining was accomplished by staining initially with rabbit anti–tenascin followed by FITC-conjugated sheep anti–rabbit IgG and then with goat anti–chicken fibronectin followed by rhodamine isothiocyanate–conjugated rabbit anti–goat IgG. The sections were then processed for autoradiography. Bar, 80 $\mu$m.
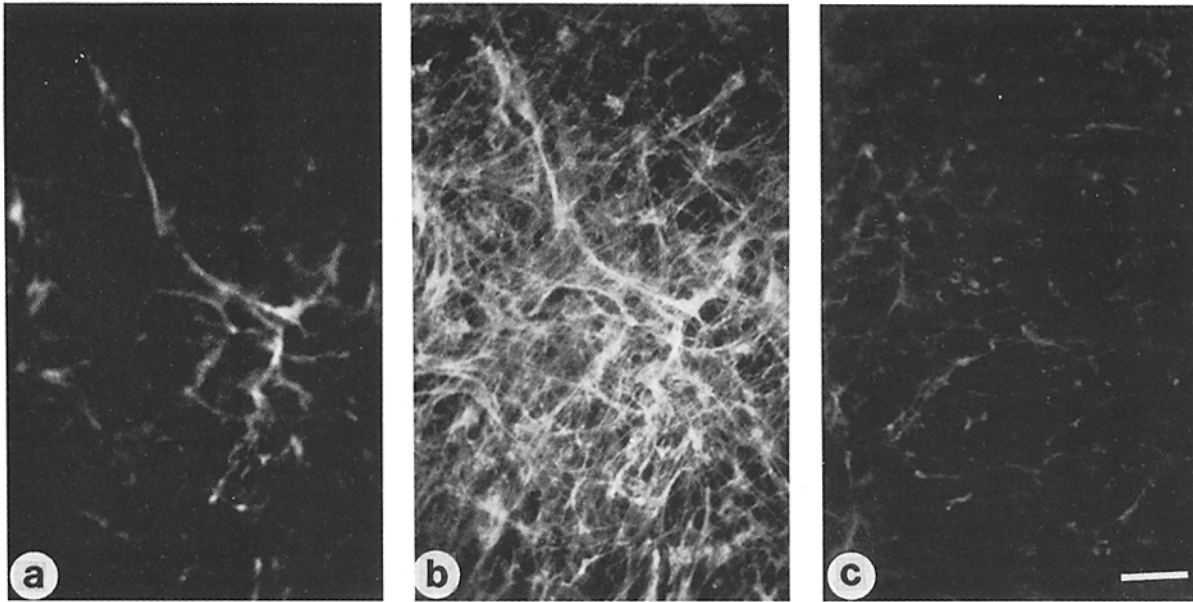
*Figure 12.* Extracellular matrix isolated by treatment of confluent heart mesenchyme monolayers with 0.025 M NH$_4$OH and immunocytochemically stained for tenascin (*a*), fibronectin (*b*), and laminin (*c*). Exposure to 0.025 M NH$_4$OH removes the cells, leaving the extracellular matrix attached to the dish. This is one of the matrix preparations that promotes sorting of chimeric aggregates when added as a sonicated suspension to the culture medium. *a* and *b* depict the same field of a preparation double stained for the two antigens as described in the caption of Fig. 11. *c* depicts a different preparation prepared from the same batch of monolayer cultures. Bar, 30 µm.

*Collagen Type I.* The amounts of collagen in the isolated matrix were relatively low under standard conditions because the culture medium did not contain ascorbate. In the sorted out aggregate, collagen type I was present in a fibrillar pattern and was, like fibronectin, laminin, and tenascin, restricted to the mesenchyme (not shown). If aggregates were cultured in the presence of serum and ascorbic acid, the amount of collagen type I was markedly increased, but without any alteration in the pattern of its localization (Fig. 13 *a*). The patterns of fibronectin (Fig. 13 *b*) and cell sorting were unaffected by the presence or absence of ascorbate in the culture medium (Fig. 13 *c*). Sonicated polymerized collagen matrix, reconstituted from Vitrogen collagen type I, did incorporate into aggregates maintained in serum-free
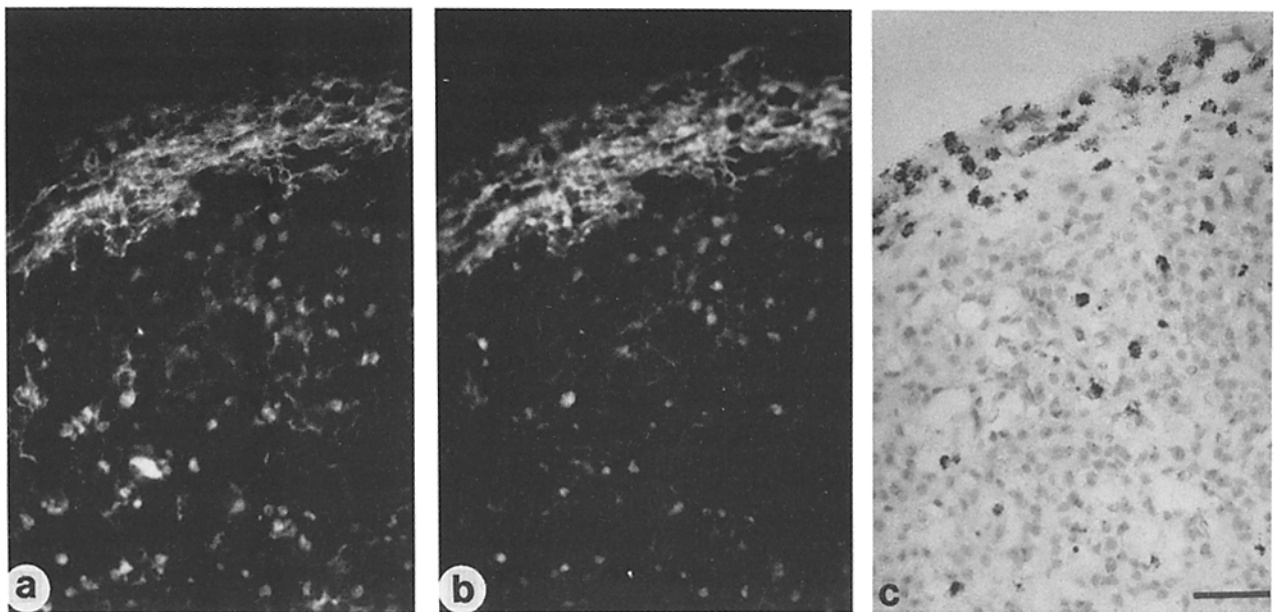


*Figure 13.* Sorted out heart mesenchyme–myocardium aggregate cultured in serum-containing medium supplemented with 50 µg/ml ascorbate and double-stained for collagen and fibronectin. The interstitial matrix of the mesenchymal layers contains collagen type I (*a*) and fibronectin (*b*). Antibodies to the two proteins stain the same individual fibrils, indicating extensive colocalization of fibronectin and collagen. Ascorbate increases the amount of collagen but fails to affect the pattern of sorting (*c*). Bar, 40 µm.
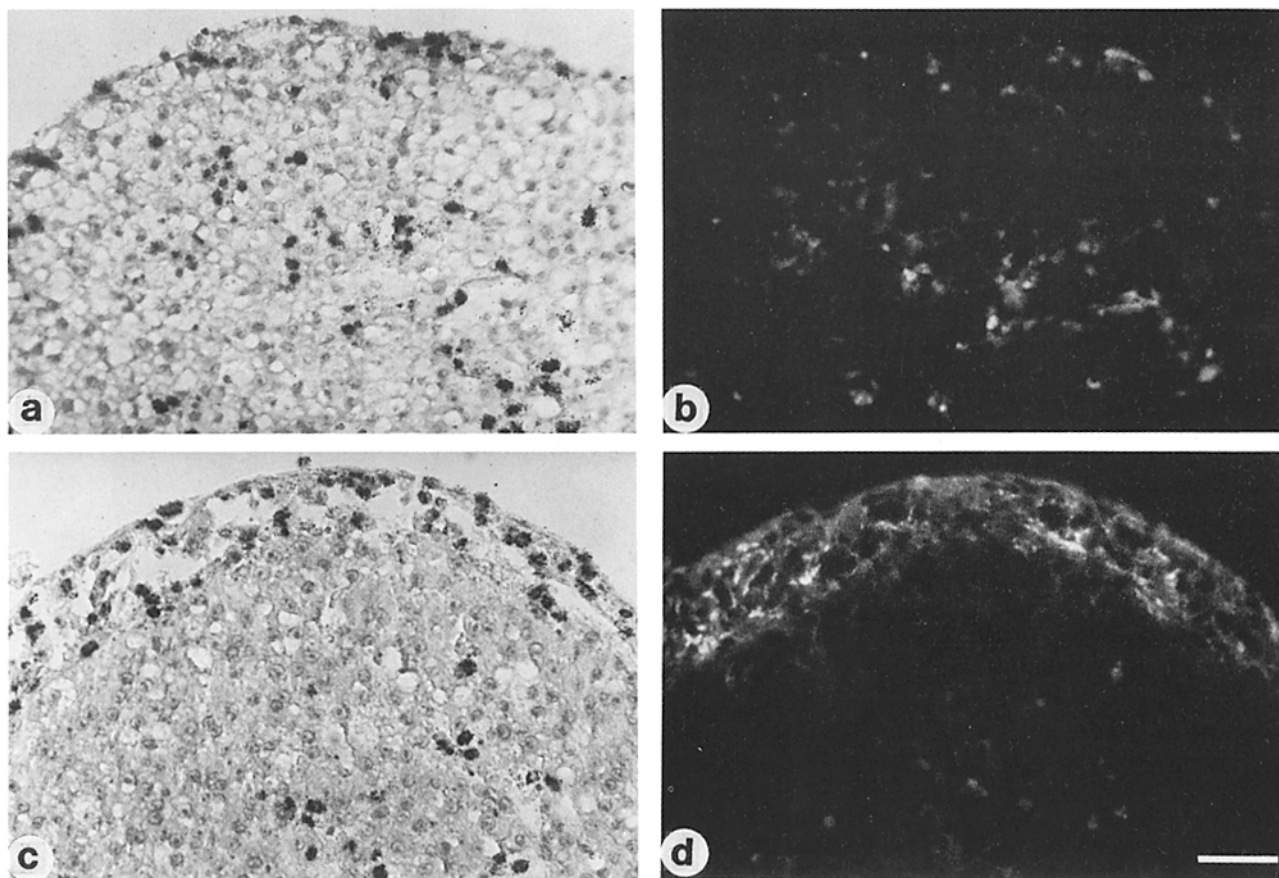
*Figure 14.* Effect of RGD-containing peptides on sorting out of myocyte–mesenchyme aggregates. Both sorting (*a*) and the deposition of a fibronectin-containing matrix (*b*) were strongly inhibited by 5 mM G*d*RGDSP. Both sorting (*c*) and deposition of the fibronectin matrix (*d*) were normal in the presence of 5 mM G*d*RGESP. 8-h culture in DME plus 3% chicken serum. Bar, 40 μm.

medium but did not provoke sorting out. In this trial, the collagen was allowed to polymerize in vitro and was then dispersed in serum-free medium by sonication.

### Effect of Arg-Gly-Asp-containing Peptides on Sorting

Fibronectin, interstitial collagen, and laminin interact with a class of integral membrane cell surface receptor proteins known as the integrins (Ruoslahti, 1988). The involvement of integrin-mediated adhesive interactions can be investigated by determining the effects of inhibitory peptides containing the sequence Arg-Gly-Asp (RGD) (Hayman et al., 1985; Pierschbacher and Ruoslahti, 1984; Ruoslahti, 1988; Yamada and Kennedy, 1984). Peptides with substitution at the aspartate residue are, typically, markedly less inhibitory and are used as negative controls. The hexapeptide, Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), retarded initial attachment and spreading of heart mesenchyme on fibronectin-derivatized dishes but, after a lag of several hours, attachment and spreading did occur. The medium from cultures that had spread was ineffective in preventing attachment of freshly dissociated cells. This suggested that the peptide may have been degraded during contact with the cell layer. For experiments of longer duration, the peptides G*d*RGDSP and G*d*RGESP were prepared with the D isomer of Arg substituted for the L isomer, with the hope that proteolysis by serine proteases would be reduced (Pierschbacher and Ruos-

lahti, 1987). G*d*RGDSP inhibited sorting in a dose-dependent fashion, with nearly complete inhibition at 5 mM (Fig. 14 *a*) and partial inhibition at 1.7 mM. At the latter concentration, some of the mesenchymal cells had segregated into patches of tissue at the surfaces of the aggregates. These patches of sorted out mesenchyme contained a fibronectin matrix. 5 mM G*d*RGDSP inhibited accumulation of the fibronectin matrix (Fig. 14 *b*). Inhibition of sorting by 5 mM G*d*RGDSP was reversible: removal of peptide-containing medium was followed by sorting out of the aggregates. The control peptide, G*d*RGESP, had no demonstrable effect on sorting at 1.7 and 5 mM (Fig. 14 *c*). G*d*RGESP-treated aggregates showed the normal accumulation of the fibronectin-containing matrix in the superficial layers of mesenchymal tissue (Fig. 14 *d*). These observations support the contention that sorting is dependent on integrin-mediated interactions of the cells with constituents of the extracellular matrix.

1.7 and 5 mM G*d*RGDSP are relatively high concentrations but are consistent with the efficacy of this form of the peptide in inhibiting the attachment of heart mesenchymal cells to fibronectin surfaces in monolayer culture. Cell attachment and spreading on fibronectin-coated bacteriological plastic surfaces were extensive in the absence of peptide by 1.5 h (Fig. 15 *a*). The control peptide, G*d*RGESP, failed to affect attachment or spreading at 5 mM (Fig. 15, *b* and *g*). The experimental peptide, G*d*RGDSP, suppressed at-
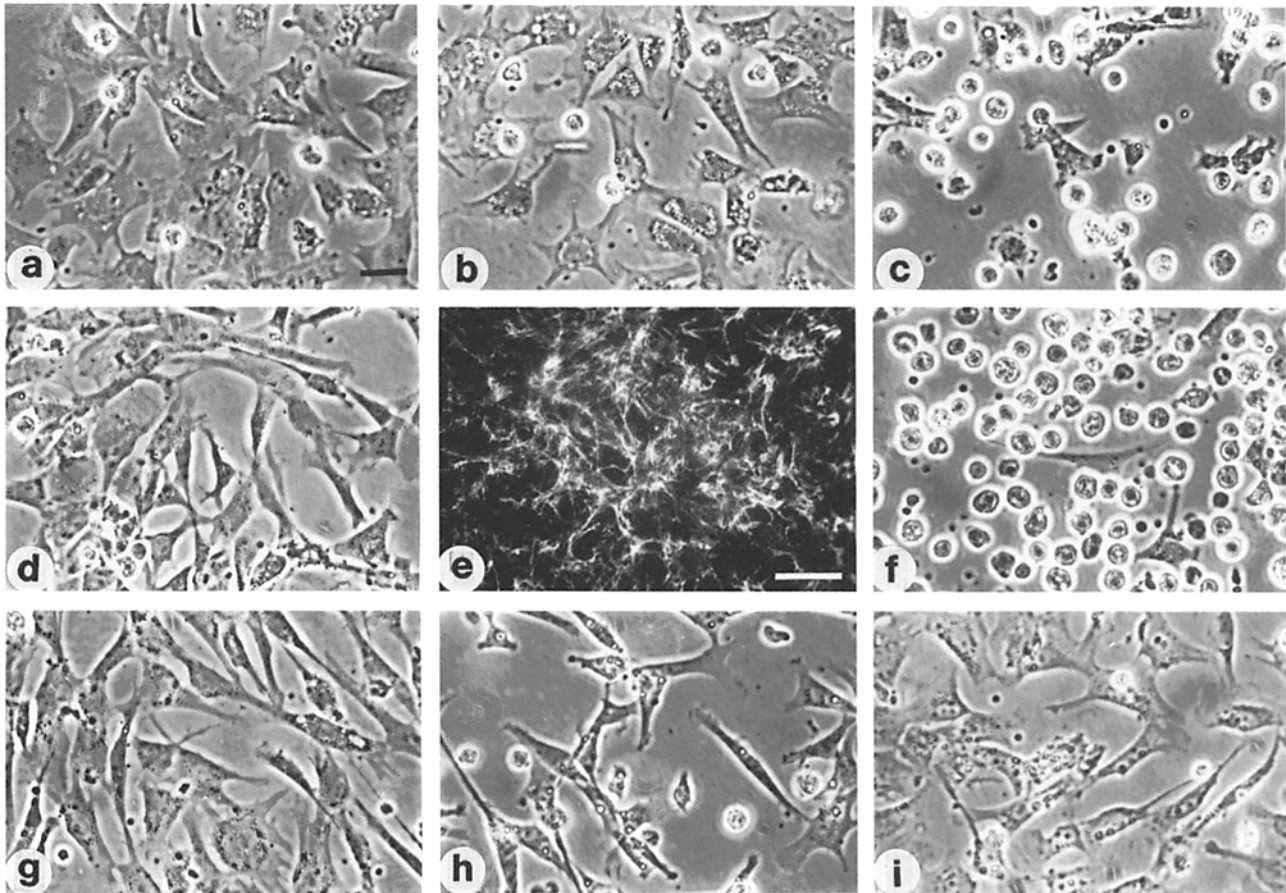
*Figure 15.* Effect of RGD-containing peptides on the attachment and spreading of cardiac mesenchymal cells on fibronectin-coated plastic in serum-free medium. The cells were prepared by exposure of primary heart mesenchyme cultures to 0.5 mg/ml crystallized trypsin dissolved in DME (e.g., in the presence of $Ca^{+2}$ and $Mg^{+2}$) for 10 min at 37°C. Trypsin was inactivated by exposure to soybean trypsin inhibitor, and the cells were suspended in serum-free DME and plated on fibronectin-coated bacteriological grade polystyrene surfaces. (*a*) Control culture, no peptide, 1.5 h; (*b*) 5 mM control peptide, G*d*RGESP, 1.5 h; (*c*) experimental peptide, G*d*RGDSP, 1.7 mM, 1.7 h; (*d*) G*d*RGDSP, 1.7 mM, 8 h; (*e*) fibronectin staining of culture shown in Fig. 15 *d*; (*f*) 5 mM G*d*RGDSP, 8 h; (*g*) 5 mM G*d*RGESP, 8 h; (*h*) 5 mM G*d*RGDSP, 21 h (cells have attached, but the extent of spreading is less than for control cultures); (*i*) reversal of inhibition of spreading using same culture as in Fig. 15 *h* 2.5 h after removal of G*d*RGDSP. Bars: (*a–d* and *f–i*) 50 μm; (*e*) 50 μm.

tachment and spreading only temporarily at 1.7, 0.5, and 0.2 mM, with the retardation of attachment and spreading being longer at the higher concentrations (Fig. 15 *c*). At 1.7 mM, a few cells had attached by 1.7 h (Fig. 15 *c*) and considerable spreading occurred between 4 and 8 h of exposure (Fig. 15 *d*). Spreading in the presence of 1.7 mM G*d*RGDSP was accompanied by the establishment of a fibrillar fibronectin matrix (Fig. 15 *e*). The eventual attachment and spreading were not due to breakdown of the peptide because the culture medium from peptide-treated, spread cultures was able to retard the spreading of freshly trypsinized cells (not shown). Attachment and spreading were low at 8 h (Fig. 15 *f*) and were somewhat reduced at 24 h (Fig. 15 *h*) by 5 mM peptide. This inhibition was reversed when peptide-containing medium was replaced by control medium (Fig. 15 *i*).

## Tissue Invasion in Fused Aggregates

Invasion is the intrusion of one tissue into space occupied by a second tissue (Abercrombie, 1970). It has been possible to model the invasion of cardiac mesenchyme and myocar-

dium that occurs in the atrioventricular and aortic valves of the avian heart by pairing aggregates of the two tissues in organ culture. When cardiac mesenchymal aggregates are cultured in contact with myocardial aggregates, the aggregates fuse to extend the border of mutual contact (Fig. 16 *a*), then mesenchymal cells infiltrate across that border into the myocardial aggregate (Fig. 16 *b*), and myocardial cells invade as short tongues of tissue into the mesenchymal aggregate (Fig. 16 *c*). Invasion and segregation are opposite modes of tissue behavior. The first produces an intermingled organization, obliterating a discrete border between two confronted tissues, whereas the second stabilizes that border. It is, then, puzzling that intermingled chimeric aggregates of myocardium and mesenchyme segregate into homogeneous tissue domains by sorting out, but the cells of aggregate pairs formed by fusing initially pure tissues intermingle by mutual tissue invasion.

A resolution of the apparent contradiction is suggested by studies of the distribution of the mesenchymal interstitial matrix, as monitored by the distribution of matrix fibronec-
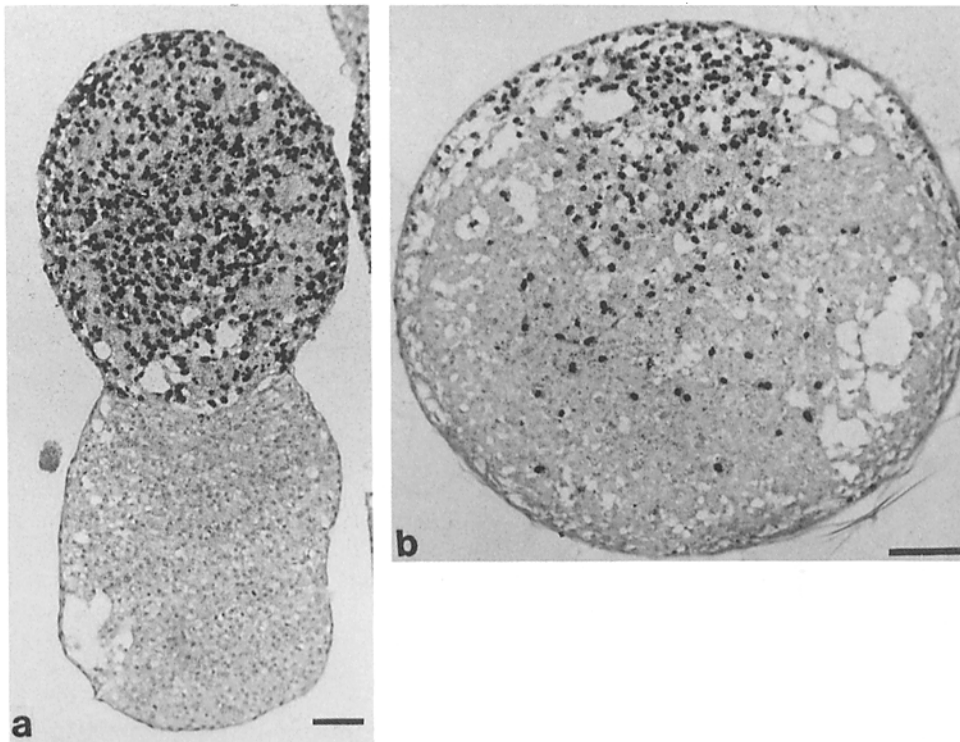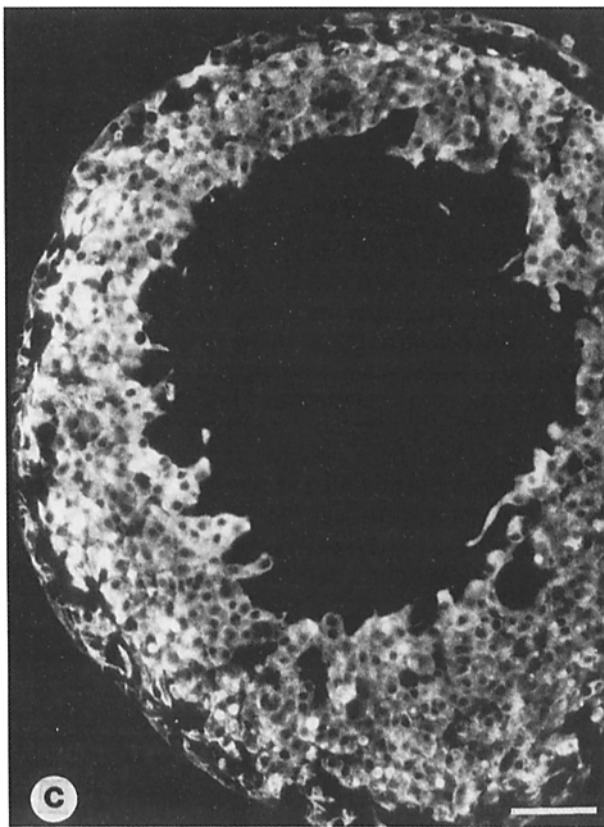
*Figure 16.* Fusion of homogeneous aggregates consisting of [³H]thymidine-labeled cardiac mesenchyme and unlabeled myocardium. The aggregates adhere to establish a common border within 3 h (*a*), and then the myocardium spreads over the surface of the mesenchymal aggregate (*b*). The mesenchyme invades into myocardial aggregates as individual cells (*b*), and myocardium intrudes into the mesenchyme as short tongues (*c*). In *c*, the myocardium is identified by immunofluorescent staining for actin; the mesenchyme is identified by immunofluorescent staining for actin; the mesenchyme is unstained. Bars: (*a*) 50 μm; (*b*) 50 μm; (*c*) 50 μm.



ready access experienced by the surface layers of the aggregate to elements of the culture medium, such as TGF-β, that stimulate matrix deposition. When cardiac mesenchyme aggregates were paired with aggregates of other tissues, the fibronectin matrix of the mesenchymal partner was lost from the region of mutual contact (Fig. 18). Thus, in mesenchyme–myocardium pairs, the myocardium–mesenchyme interface became depleted in fibronectin. It is suggested that it is just this loss of matrix that establishes the conditions for invasion, with the cells of the mesenchyme aggregate that invade the partner myocardial aggregate emigrating from the fibronectin-poor core rather than from the fibronectin-rich cortex.

## Distribution of the Fibronectin-rich Extracellular Matrix in the Developing Heart

Based on the tissue-organizing performance of myocardium and cardiac mesenchyme in organ-cultured tissue aggregates, it is suggested that the elaboration of a fibronectin-containing interstitial matrix by the mesenchyme promotes tissue segregation and organizational stability and that the absence of such a matrix establishes conditions in which invasion will occur. Both segregation and invasion are manifested in different regions of the mesenchyme–myocardium interface in the developing heart (Fig. 1). It is possible that an interstitial matrix similar to that developed in organ culture contributes to these forms of behavior. Consistent with this possibility is the observation that the abundance of immunocytochemically stained fibronectin in the 10-d heart is high in the epicardial mesenchyme, the region of tissue segregation, and low in the cushion mesenchyme, the region of tissue intermingling (Fig. 19). The staining pattern was unaltered in sections that were pretreated with collagenase or testicular hyaluronidase. The heart appears to lose fibronectin
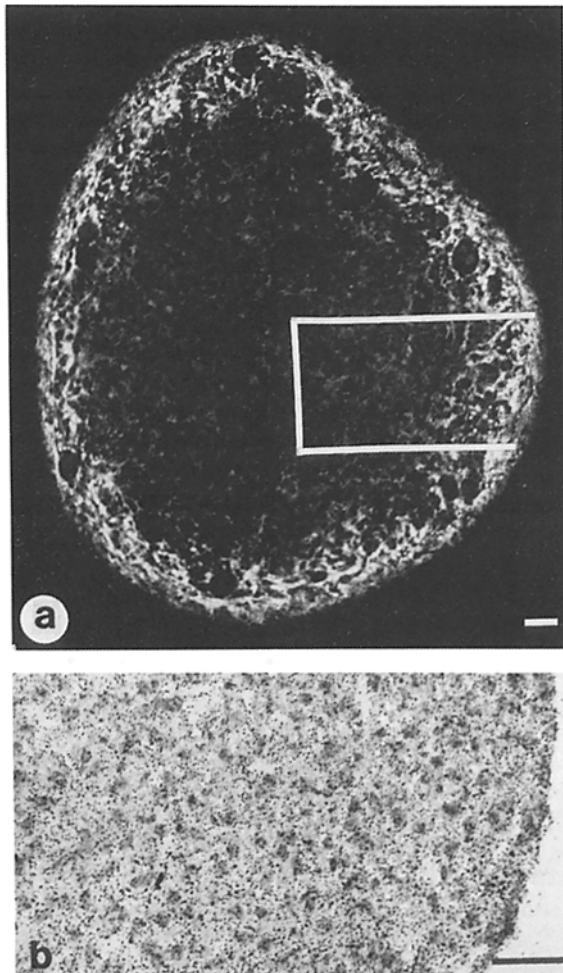
tin. Cultured aggregates of pure heart mesenchyme develop a dense fibronectin matrix only in a superficial zone three to five cell diameters in thickness (Fig. 17 *a*); the interior tissue is apparently healthy and metabolically active (Fig. 17 *b*) but contains relatively little fibronectin. The reason for the restricted distribution of the interstitial matrix in pure mesenchyme aggregates is not understood but may involve the

*Figure 17.* The fibronectin-containing matrix of heart mesenchyme aggregates cultured in serum-containing medium is abundant only in the surface layers of the aggregate (*a*). This aggregate had been exposed to [³H]leucine (10 μCi/ml for 1 d) before fixation. The interior tissues are apparently healthy and incorporate [³H]leucine into acid-insoluble material to approximately the same extent as cells in the surface layers (*b*). The field of *b* is outlined in *a*. Bars, 30 μm.

in the cushion mesenchyme at the developmental stage when invasion begins (Icardo and Manasek, 1984), consistent with the possibility that reduction in the interstitial matrix initiates invasion.

## Sorting Out of Myocardium and Mesenchyme in Cultured Atrioventricular Valves

Although endocardial cushion mesenchymal tissue contains only small amounts of the fibronectin matrix in situ, it can be stimulated to produce matrix when cultured in serum-containing medium. The patterns of sorting in serum-containing medium of chimeric reaggregates containing myocytes and either whole heart mesenchyme, valve mesenchyme, or epicardial mesenchyme are indistinguishable. The leaves of the right atrioventricular valve have an intermingled organization of mesenchyme and myocardium (Fig. 20 *c*). When

these were dissected out and placed in organ culture, the mesenchyme was likewise stimulated to reinitiate matrix deposition (Fig. 20 *a*) and the mesenchyme sorted out to the surface and the myocardium sorted to the interior (Fig. 20 *b*). Similar to the situation of cultured aggregates, fibronectin matrix production occurred in the presence of serum and failed in its absence. Sorting did not occur if valves were maintained in serum-free medium.

## Discussion

The sorting out of chick embryonic myocardial tissue from cardiac mesenchyme in organ-cultured chimeric aggregates appears to depend on the production by the mesenchyme of an interstitial matrix that is enriched in the adhesive protein, fibronectin. The fibronectin matrix colocalized with the mesenchyme as the aggregates sorted out and the conditions of culture that stimulated matrix deposition stimulated sorting. Under conditions where the quantity of this matrix was small, sorting failed to occur. This failure could be corrected by the addition of isolated mesenchymal extracellular matrix as a dispersed suspension, which incorporated into the aggregate and promoted sorting. Invasion of fused aggregates is preceded by a redistribution of the fibronectin-containing matrix of the mesenchymal aggregate such that matrix-poor regions come to occupy the interface with the myocardial partner aggregate. The invasion that ensues involves mesenchymal cells emigrating from, and myocardial cells intruding into, matrix-poor regions of the mesenchymal aggregate. In both situations, the establishment of a fibronectin-containing matrix correlates with tissue segregation and its absence with tissue intermingling. The proposed involvement of the matrix in segregation and invasion of heart tissues during development is reminiscent of the suggestion that a loss of the fibronectin matrix of mesenchymal cells after neoplastic transformation is responsible for acquisition of invasiveness by the cancerous tissue (Chen et al., 1976; Hynes and Wyke, 1975; Ruoslahti, 1984; Vaheri and Ruoslahti, 1974; Yamada et al., 1976).

This is one of only two reports describing a detailed biochemical explanation for a situation of cell sorting out. Recently, Nose et al. (1988) have demonstrated that expression of either of the adhesion systems, E-cadherin or P-cadherin, occasioned by transfection of the appropriate c-DNAs into L cells resulted in altered sorting out behavior in aggregate culture. In their system, the relevant adhesive components are integral membrane proteins that mediate direct cell–cell adhesion. In the heart mesenchyme–myocardium system described in the present report, adhesive interactions of cells with the interstitial matrix are proposed to be responsible for sorting. It may be that sorting systems involving epithelia, as exemplified by the report of Nose et al. (1988), are dependent on cell–cell adhesion molecules (the cadherins, cell adhesion molecules (CAMs), and intercellular junctional proteins), whereas systems, such as the heart system, that involve mesenchymal tissues use interactions between cell surface receptors and adhesive elements of the extracellular matrix for the adhesive recognition of tissues.

The results of both studies address a long-standing dispute about the necessity for tissue-specific adhesive ligands for sorting and indicate that sorting need not involve tissue-specific adhesive ligands. Instead, sorting can be a response
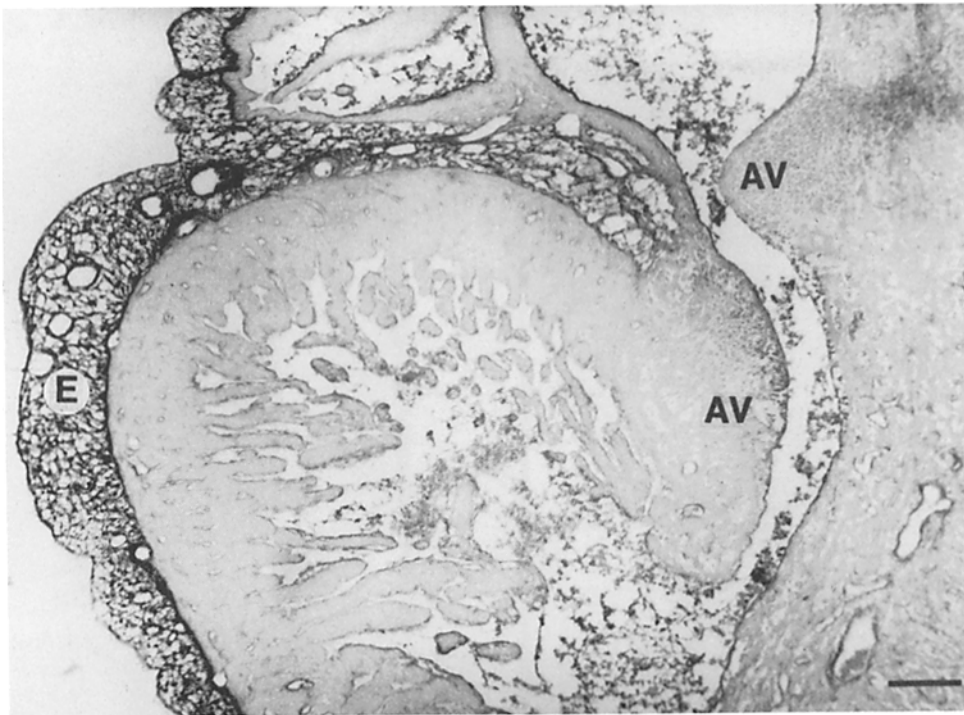
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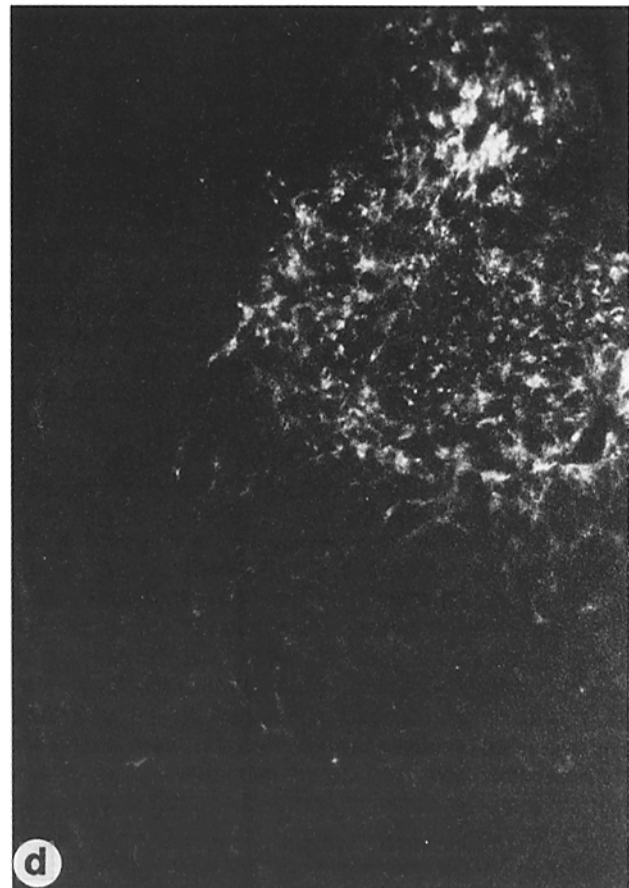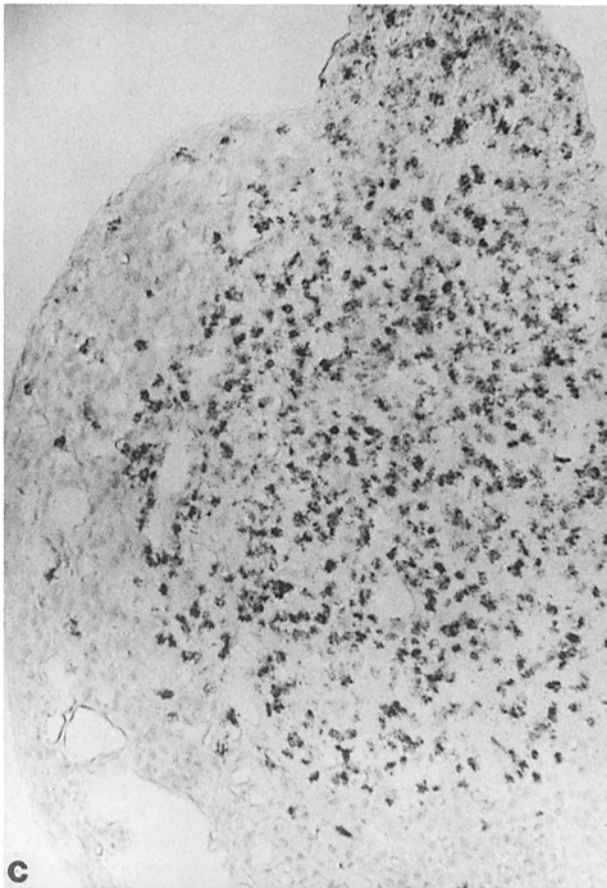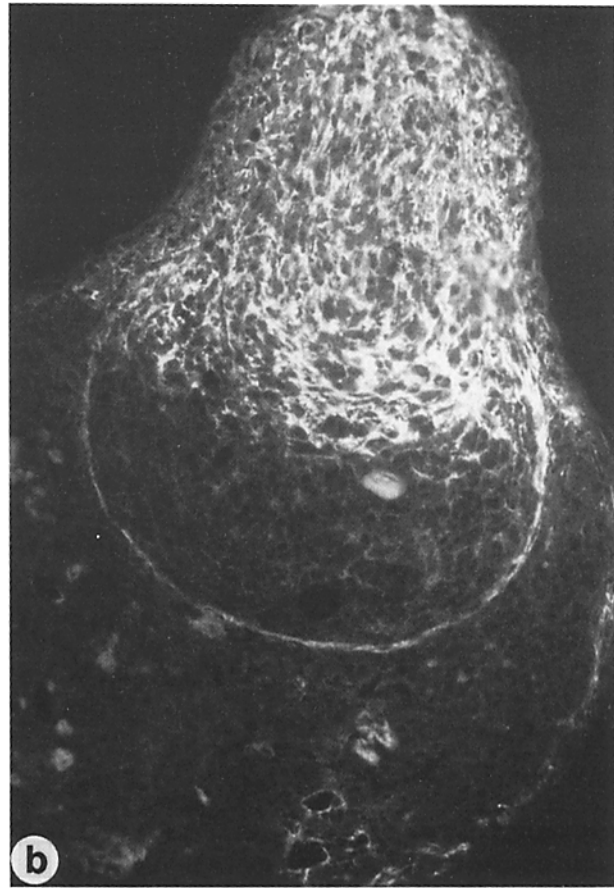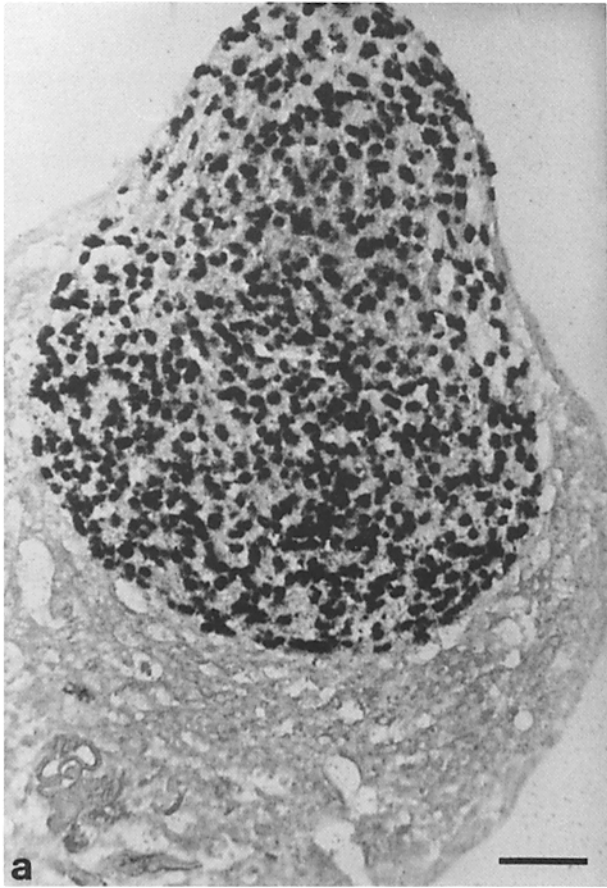
*Figure 19.* Immunoperoxidase staining of fibronectin in a frontal section of a 10-d chick embryo heart. The epicardial mesenchyme (*E*) stains intensely, whereas the staining of the right atrioventricular valve (*AV*) is much less intense. Bar, 50 μm.

to the activities of adhesive molecules of broad tissue distribution that are capable of mediating the adhesive interactions of a variety of different cell types. In the system studied by Nose et al. (1988), the relevant molecules were members of the cadherin family of adhesive proteins. In the heart system, the most prominent component of the matrix, fibronectin, is recognized by a number of epithelial and mesenchymal cell types (Ruoslahti, 1988; Yamada, 1983), including, as we have shown, both cardiac mesenchyme and myocardium. In the heart system, what is revealed are quantitative differences in the adhesiveness to fibronectin, with the mesenchyme being more adhesive than the myocardium. It is suggested that the differential affinity for the extracellular matrix provides the informational basis responsible for tissue segregation in this system with a preferential adhesion of mesenchyme for the matrix resulting in a colocalization of matrix and mesenchyme and the exclusion of the myocardium from regions containing abundant matrix. If this explanation is correct, these results are a demonstration at the biochemical level of the operation of quantitative adhesive differentials in cells sorting (Steinberg, 1963, 1970).

TGF-β is a pleiotropic mediator released by blood platelets and a variety of other cells that modulates tissue growth and differentiation (Cheifetz et al., 1987; Sporn et al., 1987) and stimulates wound repair (Mustoe et al., 1987). Several of the effects of TGF-β may be a consequence of a stimula-

tory effect on the deposition of fibronectin and several other constituents of the extracellular matrix (Ignotz and Massague, 1986; Ignotz et al., 1987; Roberts et al., 1986) and an increase in abundance of the fibronectin receptor at the cell surface (Ignotz and Massague, 1987; Roberts et al., 1988). In the present system, TGF-β has a profound effect on the organized state of the mixed heart tissue aggregates maintained in serum-free medium. The sorting out of mesenchyme and myocardium that is stimulated by TGF-β is in all probability a consequence of the concomitant stimulation of matrix deposition. TGF-β may be one of the active elements in serum that stimulate matrix deposition and sorting out.

Clearly one of the unsolved problems is the identification of the matrix component(s) that are responsible for sorting. The composition of the mesenchymal matrix is complex, with several candidates. Fibronectin is the most prominent element and is a prime candidate. Fibronectin served as a useful immunohistochemically identifiable marker for the presence of the interstitial matrix. Preliminary attempts to produce sorting with plasma fibronectin have been unsuccessful, but these efforts are continuing. In addition, polyclonal antisera directed against the isolated matrix preparations are being prepared, with the hope that these will block sorting. If a blocking antiserum can be produced, then it should be possible to identify the relevant element by deter-

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*Figure 18.* Loss of the fibronectin matrix from the myocardium–mesenchyme interface of cardiac mesenchyme aggregates concomitantly with the initiation of invasion. The mesenchyme has been labeled with [³H]thymidine and is shown by autoradiography (*a* and *c*); the fibronectin matrix has been identified by immunofluorescent staining for fibronectin (*b* and *d*). Loss of the fibronectin matrix from the myocardium–mesenchyme interface is incomplete, and invasion has not yet begun at 1 d of coculture (*a* and *b*; adjacent sections of the same aggregate pair). At 2 d of coculture, the fibronectin matrix is lost from the interface, and invasion has begun (*c* and *d*: same section). Bar, 50 μm.
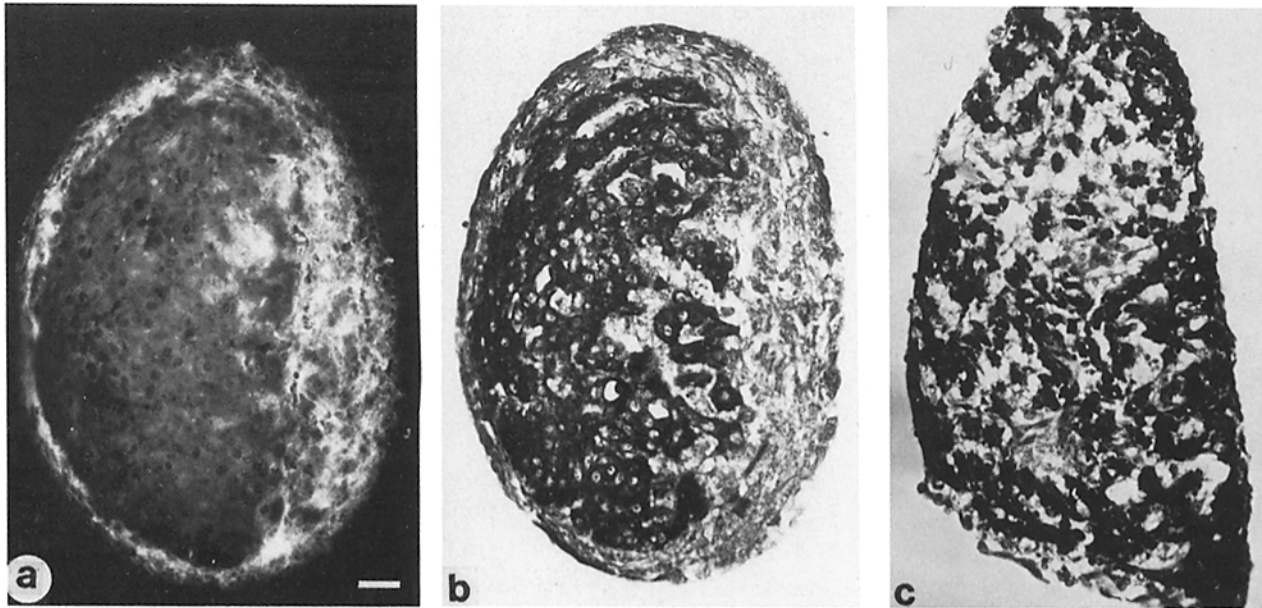
*Figure 20.* Dissected leaves of the right atrioventricular valve maintained for 2 d in organ culture reinitiate the deposition of an interstitial matrix in the mesenchymal tissue if the culture medium contains serum (immunofluorescence staining for fibronectin; *a*) and the mesenchyme sorts out to the surface and myocardium to the interior (immunoperoxidase staining of an adjacent section of the same specimen for actin; *b*). The two tissues are intermingled in the valve leaf before the initiation of culture (immunoperoxidase staining for actin; *c*). Bar, 10 $\mu$m.

mining the purified component(s) that will adsorb out the blocking activity.

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