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Fibroblast Behavior in the Embryonic Chick Heart

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ABSTRACT Intracardiac fibroblasts (mesenchymal cells) of Hamburger and Hamilton stage 36 chick heart reside in the epicardium and atrioventricular valves. The characteristics of the epicardial fibroblasts include segregation from the myocytes of the heart wall myocardium, voluminous extracellular matrix production, and some cell proliferation activity. The atrioventricular fibroblasts intermingle with myocytes at the mutual border between these tissues, produce smaller amounts of extracellular matrix, and show very active cell proliferation. Is the behavior of each population of fibroblasts predetermined or is each responding in a reversible fashion to local environment? A cell aggregate culture system, which permits 3-dimensional cell-cell and cell-matrix interactions, is used to study the behavior of each isolated population of fibroblasts *in vitro*. In the presence of serum-free medium, each population produces very little extracellular matrix, has relatively low mitotic activity, and does not segregate from myocytes when the aggregate is composed of randomly intermixed myocytes and fibroblasts. In the presence of chicken serum, each population increases matrix production, increases cell proliferation, and sorts from myocytes. Thus, we suggest that the two populations of fibroblasts in the developing heart are responding to local environments and the differences observed *in vivo* are not the consequence of irreversible states of cellular differentiation.

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Key words: Heart development, Mesenchymal cells, Epicardium, Extracellular matrix, Cell proliferation, Atrioventricular valve, Cell sorting

INTRODUCTION

The embryonic fibroblasts (mesenchymal cells) in the stage 36 (Hamburger and Hamilton, 1951) chick heart are found in two major locations—the cardiac valves and the epicardial surface. In the epicardium (Fig. 1a), a single layer of epithelial cells covers a sparsely cellular mesenchyme with a voluminous extracellular matrix rich in fibronectin. The layer of mesenchymal cells and matrix is cleanly segregated from the myocytes of the ventricular wall without an intervening basal lamina (Armstrong and Armstrong,

1990). The appearance of the epicardial fibroblasts is established at stage 18, but it is not clear whether the cells originate from the epithelial cells of the epicardium or migrate from the sinus wall (Hiruma and Hirakow, 1989). The mitral valve leaflets at stage 36, which are predominantly mesenchymal cells, are more densely cellular than the epicardium and contain significantly less fibronectin in the extracellular matrix. At the junction of the valve leaflets and the myocardium, the mesenchymal cells and myocytes intermingle without sharp demarcation between the two tissues (Fig. 1b). The tricuspid valve in the chicken is a band of tissue instead of actual valve leaflets (Vassall-Adams, 1982), and the myocytes and mesenchymal cells intermingle (Armstrong, 1985) with some surrounding matrix (Fig. 1c). The fibroblasts in the atrioventricular region originate from transformation of endothelial cells of the cardiac tube at stage 15 (Mjaatvedt et al., 1987; Mjaatvedt and Markwald, 1989; Potts and Runyan, 1989; Mjaatvedt et al., 1991; Potts et al., 1991). The mesenchyme of both the epicardium (personal observation) and the atrioventricular valves (Choy et al., 1990; Choy et al., 1991) demonstrate active cell proliferation, but the quantitative estimates of the actual rates of proliferation of each tissue have not been reported. Thus, the two different categories of cardiac mesenchymal cells behave differently in regard to matrix production and tissue segregation/intercellular invasion depending on their location in the embryonic heart. The epicardial fibroblasts and the atrioventricular fibroblasts are two populations of cells arising presumably from different sources and reveal different phenotypes. Is each population predestined to its phenotype *in vivo* or is each responding in a reversible manner to its local environment?

Although monolayer cell culture has been enormously important in advancing our understanding of cell behavior, this culture system does not accurately reflect the normal organization of mesenchymal tissues. Mesenchymal cells *in vivo* are not arranged as cell monolayers adherent to planar adhesive surfaces, but rather as 3-dimensional arrays of cells embedded in an extracellular matrix. The aggregate culture system employed in the present study affords the ability to

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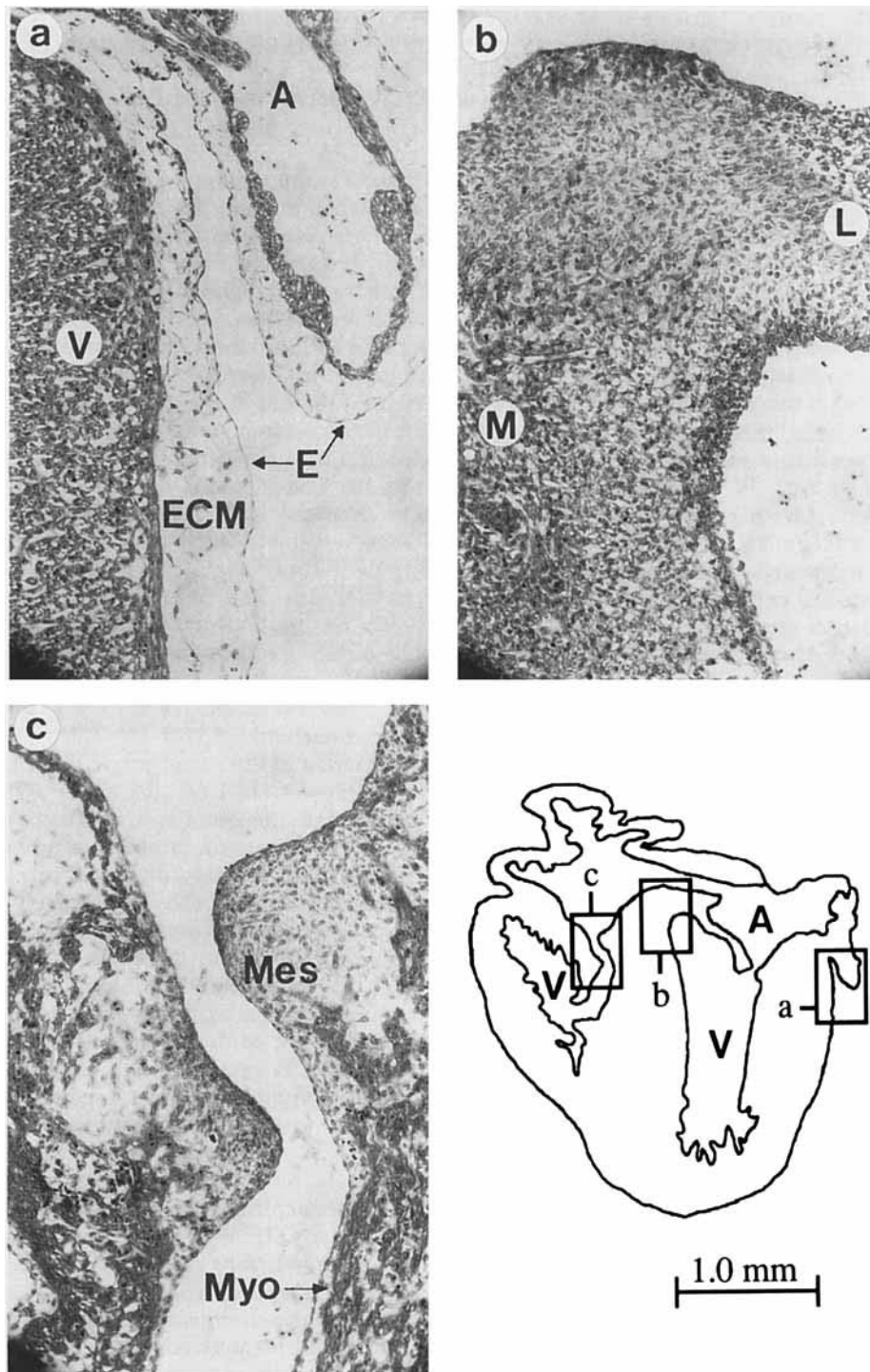


Fig. 1. Stage 36 chick heart stained with hematoxylin and eosin. **a:** The epicardium of the atrium (A) and ventricle (V) contains a single layer of epithelial cells (E) and sparse mesenchymal cells intertaced in a voluminous extracellular matrix (ECM) rich in fibronectin. **b:** The junction of the medial leaflet (L) of the mitral valve and the myocardium (M) contain

a mixture of mesenchymal cells and myocytes without sharp demarcation between the two cell types. **c:** The bands of the tricuspid valve contain both mesenchymal cells (Mes) and myocytes (Myo), which intermingle with each other. Both mitral and tricuspid valves contain some fibronectin-rich extracellular matrix, while the myocardium contains none.

study cell behavior in a model that allows cell-cell and cell-matrix interactions resembling the *in vivo* situation. The aggregate culture system has been used to study myocyte differentiation (Sarasa and Climent, 1991), basic electrophysiology of heart cells (Sachs and DeHaan, 1973; DeHaan and Fozzard, 1975; Zeng et al., 1991), desmosome formation between cells (Overton, 1974; Overton and Kapmarski, 1975; Nag and Cheng, 1980), and ionic coupling through gap junctions (Griep and Bernfield, 1978; Griep et al., 1978). Previous work has demonstrated that cell proliferation rate in embryonic myocytes in aggregate culture is much less than the rate in monolayer culture, but it more closely resembles that seen *in vivo* (Nag and Cheng, 1983). Normal myocyte morphology becomes very disrupted when the cells are dissociated with trypsin, but much of the structure returns to normal in aggregate culture (Fischman and Moscona, 1971). Using cells from the ventricles of chick or rat embryos, aggregates in serum-containing media reveal muscle cells making up the core, while non-muscle cells make up the surface of the aggregates—cell sorting (Shimada et al., 1974; Nag, 1978). Differences in cell cohesiveness may play a major role in cell segregation in the aggregate cultures and possibly *in vivo* (Wiseman et al., 1972; Phillips et al., 1977; Wiseman and Strickler, 1981; Armstrong and Armstrong, 1990).

This study addresses the question of whether two populations of mesenchymal cells in the developing heart are committed to a predetermined cell behavior or whether they simply are responding to local environments. The aggregate culture system provides an excellent model to study extracellular matrix production, proliferation, and cell sorting; and it allows us to manipulate the cell environment. Using aggregate cultures, we provide evidence suggesting that the mesenchymal cells of the epicardial surface and atrioventricular valve area are not predetermined in their differences and are, instead, responding to the local environments.

RESULTS AND DISCUSSION

Proliferation Rate of Cells in the Stage 36 Heart

We injected bromodeoxyuridine (BrdU) into the chorioallantoic or yolk sac vessel, allowed the agent to circulate in the intact embryo for 1.0 hour, and then isolated the heart for immunohistochemical identification of the cells that had incorporated the BrdU into the nuclei. The BrdU uptake revealed all the cells in S phase of their cell cycle, and thus represented an indirect measurement of the proliferation rate in each tissue (Fig. 2). The proliferation rate of the epicardial fibroblasts was not as high as the rate of the mitral valve fibroblasts or the myocytes in the apex (Table 1).

Matrix Production and Cell Proliferation

In previous work, mesenchymal cells were isolated from the whole heart in the stage 36 chick, which in-

cluded both populations of atrioventricular and epicardial fibroblasts (Armstrong and Armstrong, 1984). The mesenchymal cells cultured as 3-dimensional aggregates maintained in stirred suspension culture were exposed to growth factors such as those contained in chicken serum. The mixture of fibroblasts cultured under these conditions established aggregates with a distinctive bilayered organization of tissues (Fig. 3). The superficial 2 to 5 layers of cells (cortex) were flattened parallel to the surface of the aggregate, whereas the cells in the interior (medulla) were stellate. Cortical cells were embedded within a fibronectin-containing extracellular matrix and were actively dividing unlike the medullary cells, which were mitotically quiescent. We have shown that the fibronectin-rich extracellular matrix is a potent stimulus of cell proliferation (Choy et al., 1990). Two hypotheses can explain the organization of the aggregates. First, heart mesenchyme consists of two populations of cells with predetermined phenotypes capable of sorting out where one population migrates to the cortex and the other to the medulla to produce the bilayered organization. Factors in the medium are proposed to facilitate the expression of the predetermined phenotypic differences. In the second scenario, the bilayered organization is proposed to be due to a modulation of phenotype of an otherwise homogeneous population in response to the position in the aggregate. We presume larger molecules such as growth factors from the culture medium have limited penetration into the aggregates so that only the superficial cells experience a critical concentration of the factor. The factor induces the cortical cells to produce extracellular matrix and proliferate activity.

In the first experiment, mesenchymal cells from the atrioventricular site were grown in aggregate culture. The chicken serum (CS) provided unidentified factors that promoted the production of a fibronectin-rich extracellular matrix and cell proliferation in the cortical layer (Fig. 4a, Table 2). When we dissociated these primary aggregates into single cells and pelleted them into secondary aggregates, the cortical cells, which had actively incorporated the tritiated thymidine, scattered randomly throughout the newly formed aggregate. When these secondary aggregates were incubated in CS, the labeled cells remained distributed throughout the aggregates demonstrating that the original cortical cells were not migrating back to the cortex (Fig. 4c). Some of the secondary aggregates were incubated in CS with tritiated thymidine to establish that the new cortices were still responding to the CS in the same manner as the cortices of the primary aggregates, and, indeed, the CS induced cortical cell proliferation (Fig. 4e). The CS in any of these groups stimulated the production of a fibronectin-rich extracellular matrix in the cortices (Fig. 4b,d,f). Parallel procedures were carried out on mesenchymal cells isolated from the epicardium, and the behavior of the cells was similar to the atrioventricular cells (Fig. 4g-l).

The isolated epicardial mesenchyme population in

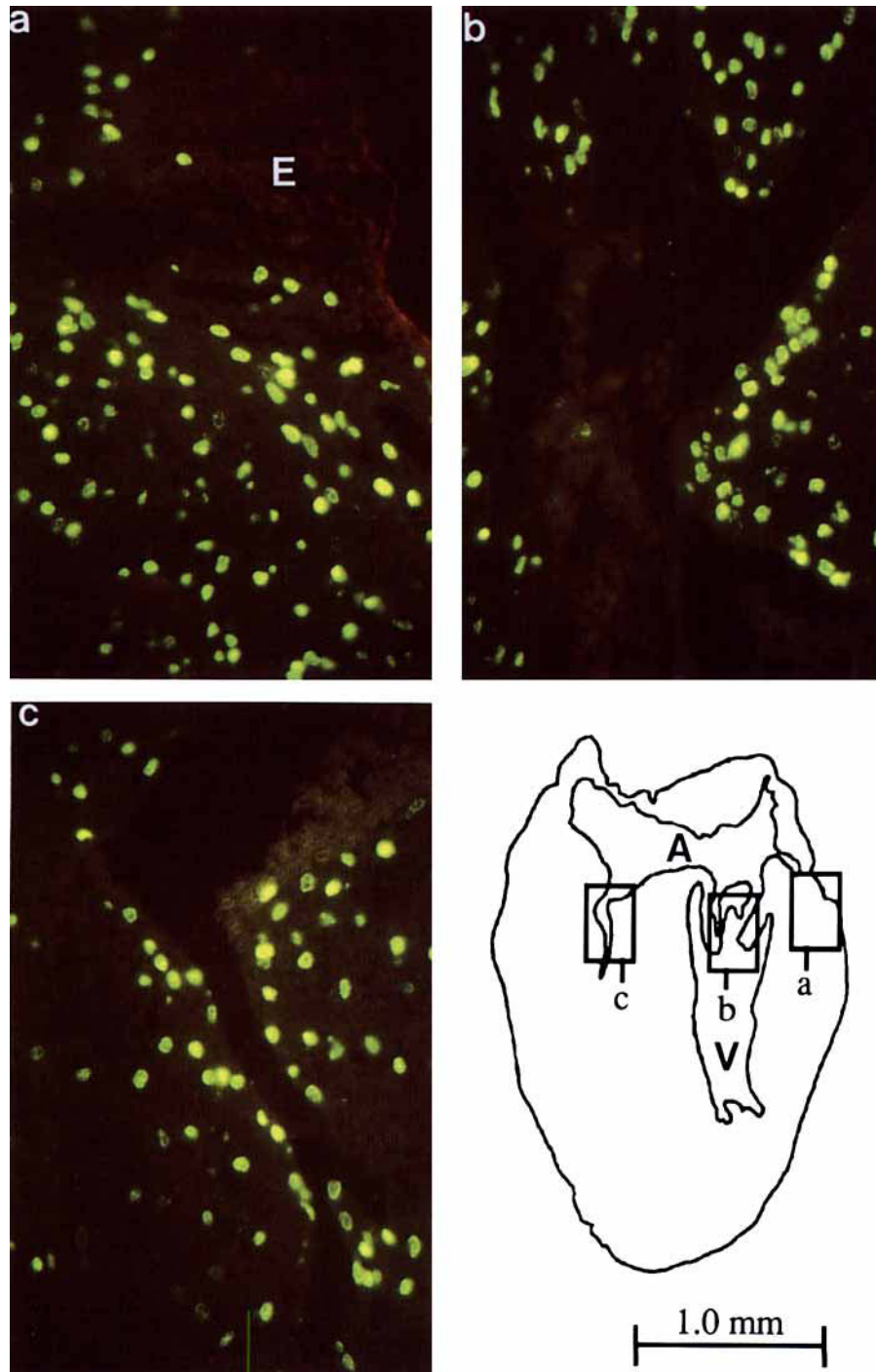


Fig. 2. Stage 36 chick heart exposed for 1 hr with bromodeoxyuridine to label cells in S phase and stained using a standard immunofluorescence procedure (labeled nuclei are green). The epicardium (E) that is shown (a) is located in the atrioventricular groove where the epicardial

layer has the greatest volume. The labeling index of the mesenchymal cells in the epicardium is significantly lower than the index of the mitral valve (b) or tricuspid valve (c).

the first experiment probably contained a small number of fibroblasts of non-epicardial origin, e.g., intramyocardial origin. To obtain a more pure population of epicardial mesenchymal cells, we isolated the cells by

dissociating the epicardial layer of the intact chick heart using a timed trypsin exposure. We confirmed that only epicardial cells had been dissociated by finding that only the epicardial layer had been disrupted

TABLE 1. Bromodeoxyuridine Labeling Index of Tissues in the Stage 36 Heart

Tissue type	Labeling index ^a
Fibroblast of the epicardium	5.3 ^b ± 1.3
Fibroblast of the mitral valve	12.3 ^c ± 1.1
Myocytes of the ventricular apex	12.2 ^c ± 0.4

^aThe labeling index was calculated by counting at least 250 cells in each region. The number of bromodeoxyuridine labeled cells was divided by the total number of cells counted and the quotient was multiplied by 100. The values are the means ± SEM of the 4 hearts.

^{b,c}Any 2 indices with different letters are significantly different, $P < 0.01$, Student's paired t-test.

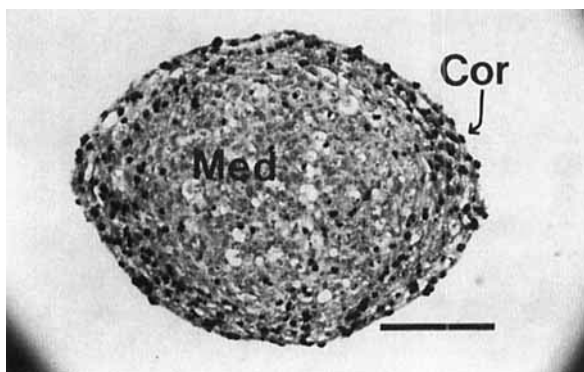


Fig. 3. Autoradiogram of an aggregate composed of mesenchymal cells from the whole chick heart at stage 36. The aggregate was incubated with tritiated thymidine to label mitotically active cells and stained with hematoxylin. After incubation in chicken serum the aggregate reveals a cortex (Cor) with actively dividing cells and a fibronectin-rich extracellular matrix (not shown). The cortex is approximately 2 to 5 cell layers deep. The cells in the medulla (Med) are relatively quiescent. Bar = 0.10 mm.

(hematoxylin and eosin staining of paraffin sections of hearts following the trypsin exposure). The atrioventricular population was isolated from the remaining hearts with the same technique described in the first experiment. Both atrioventricular and epicardial mesenchyme aggregates incubated in CS revealed a bilayered organization with a fibronectin-rich extracellular matrix and actively proliferating cells in the cortex (Table 3).

In the third experiment, reference aggregates were composed of a mixture of epicardial and atrioventricular fibroblasts. After incubating the reference aggregates in 10% CS to establish the bilayered organization, the cortical cells and the medullary cells were isolated as two separate populations by a timed trypsinization procedure. New aggregates were produced from each population and cultured in the presence of CS. Both populations of cells organized into bilayered aggregates with actively proliferating cortices and mitotically quiescent medulla (Table 4), and both sets of aggregates produced fibronectin-rich extracellular matrix.

The results of these experiments establish two points. First, the response of mesenchymal cells in aggregate culture to CS is determined by the location of the cell in the aggregate. Cortical cells that respond to the CS do not appear to be a special class of fibroblasts that migrate to the cortex. Instead, any cell in the aggregate can produce a fibronectin-rich extracellular matrix and divide when exposed to the appropriate factors. Second, the epicardial cells behave similarly to the atrioventricular cells. Thus, neither epicardial nor atrioventricular fibroblasts have committed phenotypes regarding extracellular matrix production or cell proliferation.

Viability of Cells in Aggregate Culture

Since aggregates incubated in CS always demonstrated a medulla consisting of mitotically quiescent cells, we questioned whether the medulla cells were receiving inadequate nutrients leading to dysfunction or death. Tritiated leucine was provided to aggregates cultured in bovine serum albumin (BSA) or CS medium in order to label newly synthesized protein. There was no significant difference in leucine incorporation between cortical cells and medullary cells of aggregates incubated in either BSA or CS (Table 5), suggesting that the cells of the medulla were viable and functionally intact. In addition, necrotic areas or significant numbers of pyknotic nuclei, indicated by hematoxylin and eosin staining, were not found in the medullary areas of aggregates incubated in either BSA or CS. Thus, the bilayered organization of aggregates incubated in the presence of serum does not appear to be due to nutrient starvation of medullary cells.

Cell Sorting

The epicardial fibroblasts form a tissue completely segregated from the myocardium of the heart wall, whereas the atrioventricular fibroblasts intermingle with the myocytes at the contact site between the two tissues. We conducted experiments investigating cell sorting in chimeric aggregates composed of cardiac fibroblasts labeled with tritiated thymidine and myocytes to examine the basis for the different organization of the myocardium-mesenchyme interface in the two regions of the heart. In chimeric aggregates composed of myocytes and fibroblasts from the whole chick heart that contained both epicardial and atrioventricular fibroblasts, the fibroblasts segregated to the cortex when the aggregates were cultured in the presence of CS (Fig. 5, Table 6, Experiment A). Two hypotheses can explain this behavior. First, the capacity to sort out from myocytes may be restricted to the epicardial fibroblasts, similar to the *in vivo* situation where epicardial mesenchyme is completely segregated from the myocardium. The minority of fibroblasts found in the medulla that failed to sort from the myocytes originated from the atrioventricular region. Second, we can suggest that fibroblasts from both regions of the heart have equal abilities to sort out from myocytes when the

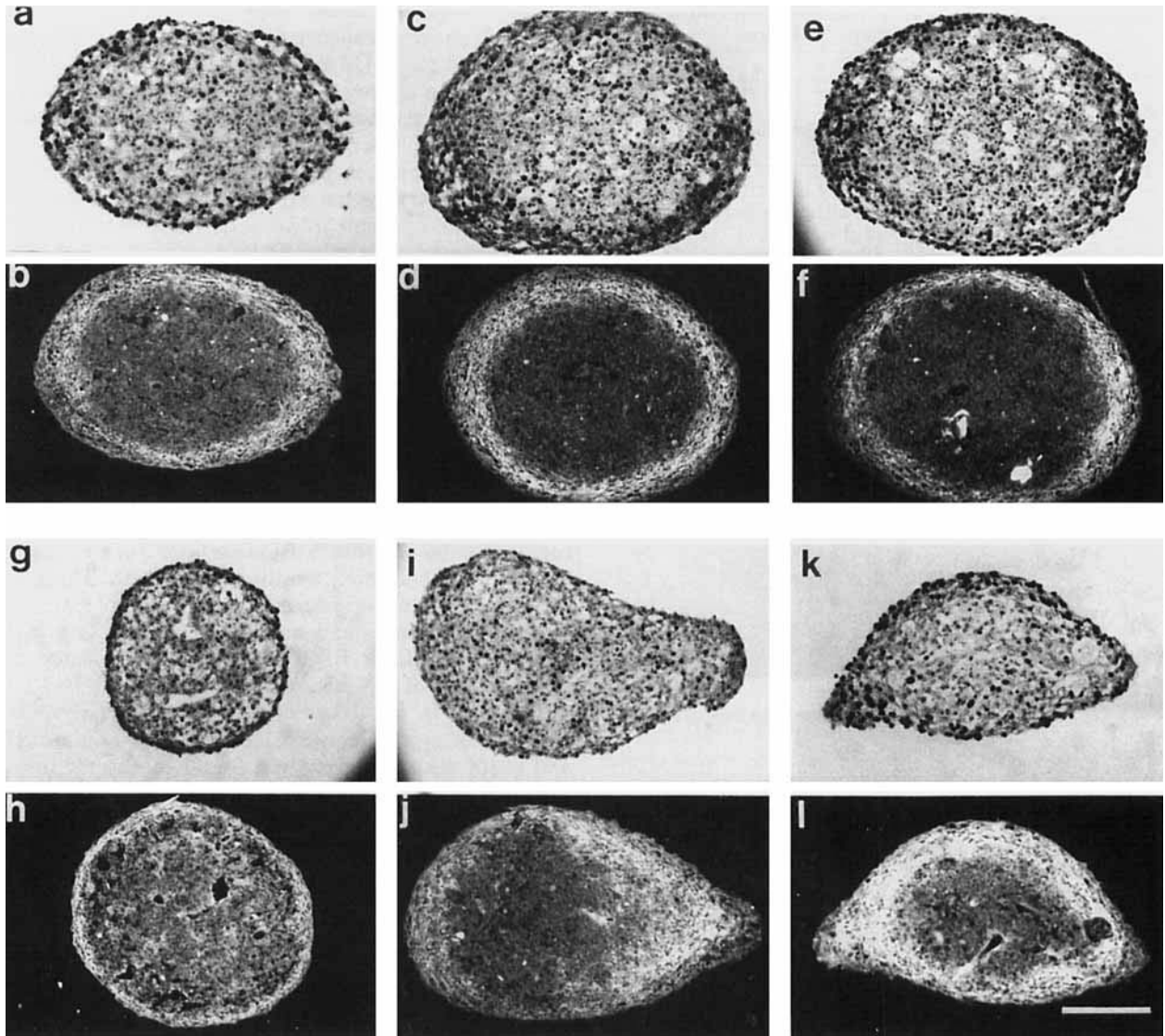


Fig. 4. Aggregates composed of atrioventricular (a-f) or epicardial (g-l) mesenchymal cells isolated from stage 36 chick heart. The first and third rows are autoradiograms showing the labeled cells that incorporated the tritiated thymidine, while the second and fourth rows are respective sections of aggregates stained for fibronectin with a standard immunofluorescence procedure. The primary aggregates made up of either atrioventricular or epicardial cells incubated in chicken serum reveal cortices with high mitotic activity (a,g) and an abundant extracellular matrix (b,h). If the aggregates are dissociated into single cells, pelleted back into secondary aggregates, and incubated in chicken serum without tritiated thymidine (c,i), the labeled cortical cells from the primary aggregates

become and remain more randomly distributed in the secondary aggregates. However, the chicken serum still stimulates the cortical cells of the secondary aggregates to produce a fibronectin-rich extracellular matrix (d,j). If primary aggregates are dissociated, pelleted, and incubated in chicken serum with tritiated thymidine, one finds that the new cortical cells of the secondary aggregates are actively dividing (e,k) and laying down the extracellular matrix (f,l). Thus, primary cortical cells that were distributed to the medullary areas of the secondary aggregates are not migrating to the cortices, but are remaining quiescent. The behavior of atrioventricular cells and epicardial cells are similar. Bar = 0.10 mm.

chimeric aggregates are cultured in CS. When we constructed chimeric aggregates containing mesenchyme from only the epicardium or the atrioventricular tissue, the sorting behavior was identical. In both cases, the mesenchymal cells sorted out to the surface leaving predominantly myocytes in the interior of the aggregates (Fig. 6, Table 6, Experiment B). Thus, both fibroblast types are equally able to segregate from myocytes

in aggregate culture maintained in serum-containing medium.

Summary

All of the mesenchymal cells from the atrioventricular region and the epicardium have the potential to produce a fibronectin-rich extracellular matrix, actively proliferate, and sort from myocytes in aggregate

TABLE 2. Labeling Index of Primary and Secondary Aggregates*

	Cortex	Medulla
Atrioventricular aggregates		
CS + tritiated thymidine	77.6 ^a ± 0.7	33.8 ^c ± 2.2
Secondary, CS	58.4 ^b ± 3.6	46.8 ^d ± 0.3
Secondary, CS + tritiated thymidine	83.4 ^a ± 1.7	49.6 ^d ± 2.2
Epicardial aggregates		
CS + tritiated thymidine	54.3 ^e ± 2.1	12.7 ^h ± 1.8
Secondary, CS	34.7 ^f ± 2.4	24.9 ⁱ ± 3.8
Secondary, CS + tritiated thymidine	80.6 ^g ± 3.9	34.2 ^f ± 2.0

*The labeling index of each region was calculated by dividing the number of tritiated thymidine labeled cells by the total number of cells in the particular region and multiplying by 100. Values are means ± SEM of five randomly chosen aggregates within the designated group.

^{a,b,c,d}Any 2 indices in the atrioventricular groups with different letters are significantly different, $P < 0.01$; split plot design where experimental unit is the aggregate and split plot is cortex versus medulla.

^{e,f,g,h,i}Any 2 indices in the epicardial groups with different letters are significantly different, $P < 0.01$; split plot design where experimental unit is the aggregate and split plot is cortex versus medulla.

TABLE 3. Labeling Index of Atrioventricular and Epicardial Aggregates*

	Cortex	Medulla
Atrioventricular aggregates		
BSA + tritiated thymidine	25.3 ^a ± 2.4	25.7 ^a ± 1.7
CS + tritiated thymidine	77.6 ^b ± 1.4	34.4 ^d ± 2.7
Epicardial aggregates		
BSA + tritiated thymidine	44.2 ^c ± 1.5	34.2 ^d ± 2.3
CS + tritiated thymidine	73.6 ^b ± 1.6	30.4 ^{a,d} ± 2.4

*The labeling index of each region was calculated by dividing the number of labeled cells by the total number of cells in the particular region and multiplying by 100. Values are means ± SEM of five randomly chosen aggregates within the designated group.

^{a,b,c,d}Any 2 indices with different letters are significantly different, $P < 0.01$; split plot design where experimental unit is the aggregate and split plot is the cortex versus medulla.

culture in the presence of an appropriate stimulus, e.g., serum-containing medium. Within a mesenchymal aggregate, an individual cell will produce an extracellular matrix and proliferate, or remain quiescent depending on its location.

Obviously, whenever a population of cells is isolated from intact tissue and placed in any culture system, the behavior of the cells exposed to certain growth factors may be modified by the cell culture system. In conventional culture systems, mesenchymal cells are arrayed as a monolayer attached to an adhesive glass or plastic surface. The aggregate culture system used in the present study allows 3-dimensional cell-cell and cell-matrix interactions that resemble the in vivo condition. We believe the behavior of cells in the aggregate

TABLE 4. Labeling Index of Reference, Cortex, and Medulla Aggregates*

	Cortex	Medulla
Reference aggregates in CS and BrdU	65.2 ^a ± 2.4	30.0 ^c ± 2.3
Cortex aggregates in CS and tritiated thymidine	82.7 ^b ± 2.6	27.4 ^c ± 2.9
Medulla aggregates in CS and tritiated thymidine	68.7 ^a ± 2.2	10.0 ^d ± 0.6

*The labeling index of each region was calculated by dividing the number of labeled cells by the total number of cells in the particular region and multiplying by 100. Values are means ± SEM of five randomly chosen aggregates within the designated group.

^{a,b,c,d}Any 2 indices with different letters are significantly different, $P < 0.01$; split plot design where experimental unit is the aggregate and split plot is cortex versus medulla.

culture system more closely represents their behavior in vivo. In summary, we suggest that the differences between epicardial and atrioventricular fibroblasts in vivo result from the modulation of the behavior of a phenotypically plastic cell in response to local environmental factors rather than from stable preestablished differences in the differentiated characters of the two cell populations.

EXPERIMENTAL PROCEDURES

Isolation of Cells and Cell Culture

Ten-day-old white Leghorn chicken eggs (stage 36) were harvested, and the hearts were isolated. We obtained atrioventricular mesenchymal cells by dissecting out the atrioventricular regions, including the atrioventricular valves, in a manner to exclude the epicardial mesenchyme. We obtained epicardial mesenchymal cells by isolating the lower two-thirds of the ventricles, which excluded atrioventricular mesenchymal cells.

Each tissue type was dissociated into individual cells by incubating (37°C, 1.0 hr) in calcium and magnesium-free phosphate buffered saline (PBS) containing 2.0 mM disodium ethylenediamine tetraacetic acid and 0.10% trypsin (Difco 1:250). The cells were rinsed and cultured in plastic tissue culture dishes at an initial density of 4.0×10^5 cells/cm² in Dulbecco-modified Eagle's medium (DMEM) plus 10% chicken serum (CS). The population of cells contained both mesenchymal cells and myocytes. After 1.5 hr of incubation at 37°C, the mesenchymal cells had attached to the plastic dish while the myocytes were still free floating (Polinger, 1970), and the culture medium, which contained the myocytes, was decanted. Fresh medium was poured back into the dish, and the attached cells were incubated until they grew to a confluent monolayer (approximately 2 days). The culture contained predominantly mesenchymal cells with less than 1% myocytes and unidentified epithelioid cells that may have been endothelium (Armstrong and Armstrong, 1978). The decanted medium was the source of myocytes for the cell sorting experiment described below.

TABLE 5. Tritiated Leucine Uptake^{a,*}

Aggregates incubated in	Grains/400 μm^2		Cells/400 μm^2		Grains/cell	
	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla
BSA + tritiated leucine	96 \pm 7.9	99 \pm 4.7	7.0 \pm 0.32	6.6 \pm 0.40	13.7 \pm 0.98	15.1 \pm 0.58
CS + tritiated leucine	125 \pm 6.8	126 \pm 3.4	6.4 \pm 0.40	6.2 \pm 0.36	19.9 \pm 1.70	20.5 \pm 1.21

^aThe number of grains and number of cells in an area of 400 μm^2 were counted in each region—cortex and medulla. Values are means \pm SEM of five randomly chosen aggregates within the designated group.

^{*}Student's paired t-test to compare cortex versus medulla, none of the pairs were significantly different at the $P < 0.05$ level.

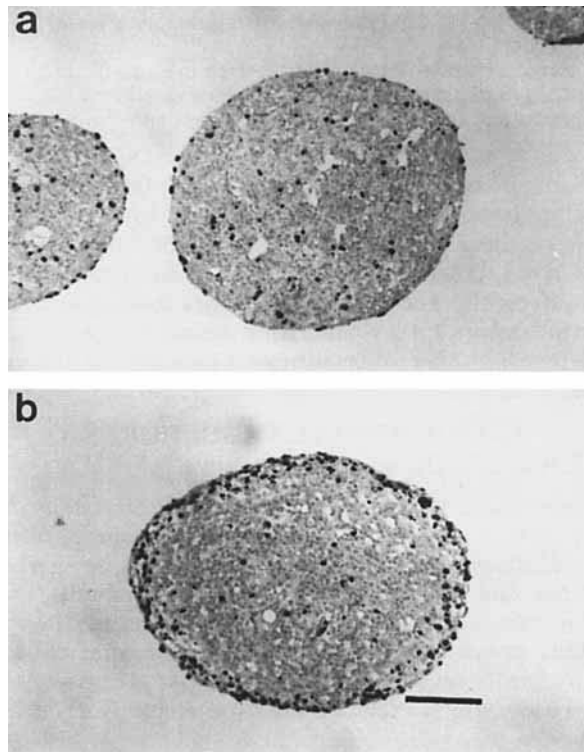


Fig. 5. Autoradiograms of aggregates composed of mesenchymal cells and myocytes from stage 36 chick heart. Cell suspensions of mesenchymal cells isolated from the whole heart, which were prelabeled with tritiated thymidine, were mixed with myocytes, pelleted, and incubated in medium without tritiated thymidine. **a**: In the presence of bovine serum albumin the two cell types remain mixed. **b**: In the presence of chicken serum many of the labeled mesenchymal cells migrate to the cortex. Bar = 0.10 mm.

Aggregate Culture

We produced the cell aggregates by either of two methods. In the first method, 5 \times 5 mm segments of confluent monolayers were scraped gently from the culture plates using a small rubber scraper. The sheets were transferred into a 25 ml Erlenmeyer flask containing 3.0 ml of DMEM plus 1.0 mg/ml bovine serum albumin (BSA), flushed with air containing 5.0% CO₂, and sealed with a silicon stopper. The medium did not contain growth factors, but the cells remained cohe-

sive. After incubating 4 days in an orbital shaker bath (37°C, 90 rpm), the sheets of cells had remodeled into smoothed-surface, spherical cell aggregates that ranged between 0.2 and 0.5 mm in diameter. Previous work revealed that the extracellular matrix present in the initial monolayers was lost in aggregate culture by day 2 of incubation (Choy et al., 1990).

In the second method, the cells of the monolayers were dissociated by incubating in DMEM plus 1.0 mg/ml BSA with 0.05% trypsin, which also removed the extracellular matrix. The cells were rinsed in PBS containing 1.0 mg/ml soybean trypsin inhibitor, pelleted by centrifugation, and incubated for 3 to 5 hr at 37°C allowing the cells to adhere to one another. The pellet was cut into aggregates, which were placed in a 25 ml Erlenmeyer flask system described above. In both methods the aggregates were left in serum-free medium long enough that the cells were mitotically quiescent before transfer to experimental media.

Immunohistochemistry and Autoradiography

After incubation in experimental media described in the experiments below, the aggregates were fixed in Carnoy's fixative (60% ethanol, 30% chloroform, and 10% acetic acid), embedded in paraffin, sectioned (6.0 μm), and mounted on gelatin-coated slides. Sections of aggregates were stained for fibronectin using anti-chicken fibronectin antibody obtained from Drs. K. Yamada (Yamada, 1978), D.R. Garrod, and E. Ruoslahti using the ABC indirect immunoperoxidase procedure (Vector Laboratories) or a standard immunofluorescence procedure. Control sections were stained with preimmune serum or fibronectin-adsorbed antiserum substituted for the first antibody. Slides used for autoradiography were coated with Kodak NTB-2 photographic emulsion and processed as described by Kopriwa and LeBlond (Kopriwa and Leblond, 1962).

We identified 2 regions in the aggregate, the cortex and the medulla. The cortex consisted of an outer layer of cells 2 to 5 cell layers deep, where extracellular fibronectin was abundant in the appropriate medium. The medulla included the remaining interior cells. The proliferation rates of the cortex and medulla were estimated by the tritiated thymidine labeling index. The labeling index of each region of the aggregate was calculated by dividing the number of cells labeled with tritiated thymidine by the total number of cells in the particular region and multiplying by 100.

TABLE 6. Cell Sorting With Labeled Fibroblasts*

	Cortex	Medulla
Experiment A		
Labeled whole heart fibroblast/myocyte aggregate in BSA	30.3 ^a ± 2.0	13.5 ^c ± 0.7
Labeled whole heart fibroblast/myocyte aggregate in CS	68.4 ^b ± 2.6	11.3 ^c ± 1.2
Experiment B		
Labeled atrioventricular fibroblast/myocyte aggregate in CS	75.6 ^d ± 2.8	18.2 ^e ± 2.8
Labeled epicardial fibroblast/myocyte aggregate in CS	78.5 ^d ± 2.0	25.1 ^e ± 2.7

*The percentage of fibroblasts in each region was calculated by dividing the number of labeled cells (fibroblasts) by the total number of cells in the particular region and multiplying by 100. Values are means ± SEM of five randomly chosen aggregates within the designated group.

^{a,b,c}Any 2 indices in experiment A with different letters are significantly different, $P < 0.01$; split plot design where experimental unit is the aggregate and split plot is cortex versus medulla.

^{d,e}Any 2 indices in experiment B with different letters are significantly different, $P < 0.01$; split plot design where experimental unit is the aggregate and split plot is cortex versus medulla.

Proliferation Rate of Cells in the Stage 36 Heart

Four white Leghorn chicken eggs were windowed at or before 7 days of incubation to allow enough time for healing of the chorioallantoic membrane. At 10 days, 0.05 ml of PBS containing 150 µg of bromodeoxyuridine (BrdU) was injected with a micropipette into either a chorioallantoic or yolk sac vessel. After 1.0 hr incubation at 37°C, the hearts were isolated, fixed in Carnoy's fixative, embedded in paraffin, sectioned (6.0 µm) in the frontal plane, and mounted on gelatin-coated slides. After deparaffinization, the DNA in the tissue sections was denatured with 2.0 M hydrochloric acid (37°C, 1.0 hr) and then neutralized with 0.1 M borate buffer (pH 8.5) for 10 min. Nonspecific protein binding sites were blocked with PBS containing 1.0 mg/ml BSA for 30 min. A standard ABC indirect immunoperoxidase procedure (Vector Laboratories) was performed using an anti-BrdU monoclonal antibody (Boehringer Mannheim Biochemicals) and the slides were used for calculation of the labeling index described in the next paragraph. With another set of slides, the staining for BrdU was performed using a standard immunofluorescence procedure. The anti-BrdU antibody from Boehringer was produced in a mouse and the second antibody was a fluorescein-tagged rabbit anti-mouse antibody.

Within each heart, we examined the cells in three regions—the fibroblasts of the mitral valves, the fibroblasts of the epicardium, and the myocytes of the ventricles near the apex. The labeling index was calculated by counting at least 250 cells in each region. The

number of BrdU labeled cells was divided by the total number of cells counted and the quotient was multiplied by 100.

Matrix Production and Cell Proliferation

We designed several experiments to compare the behavior of atrioventricular and epicardial mesenchymal cells, and compare the behavior of cortical and medullary cells within an aggregate. In the first experiment atrioventricular aggregates were transferred into DMEM containing 10% CS and 2.0 µCi/ml tritiated thymidine to determine the effects of CS on cell proliferation. After incubating 1 day, 10–15 aggregates were fixed and processed for labeling index and fibronectin localization as described above. To mix the cortical cells (found to have a high labeling index) in a random distribution with the medullary cells (found to have a low labeling index) within an aggregate, we dissociated the remaining primary aggregates into single cells with 0.10% trypsin. The cells were formed back into aggregates by the pellet method described above and incubated for 1 day in 1.0 mg/ml BSA medium. The aggregates were divided into two groups of 10–15 aggregates each and placed in 10% CS medium or 10% CS medium with tritiated thymidine for 1 day. The aggregates were fixed and processed for autoradiography and immunohistochemistry. Parallel procedures were done with epicardial aggregates.

In the second experiment we isolated the epicardial cells in a different manner to produce a more pure population of cells. Ten day chick hearts were isolated keeping the atria, ventricles, and proximal portions of the great vessels intact. The hearts were placed in the outer track of a 60 mm organ culture dish (Falcon #3037) with 3.0 ml of calcium and magnesium-free PBS containing 2.0 mM disodium ethylenediamine tetraacetic acid and 0.10% trypsin. The culture dish was placed on an orbital shaker at 100 rpm for 12 min at room temperature. The hearts were transferred to DMEM with 10% CS. Two hearts were fixed in Carnoy's fixative, embedded in paraffin, sectioned, mounted onto glass slides, and stained with hematoxylin and eosin to ensure that we had only stripped off epicardial cells. The atrioventricular tissue was dissected out of the remaining hearts and the atrioventricular cells were isolated as described above. The epicardial cells in the trypsin solution were pelleted and resuspended in 10% CS. We plated each population of cells in plastic tissue culture dishes, allowed them to form confluent monolayers, and made aggregates by the scraping method described above. After incubating 4 days in BSA culture medium, each population was split into 10% CS and BSA groups all containing 2.0 µCi/ml tritiated thymidine. The aggregates were left in medium for 1 day, fixed, and processed for fibronectin staining and labeling index as described above.

In a third experiment ventricles including the atrioventricular valves from 10 day chick hearts were isolated. Mesenchymal aggregates, which were composed

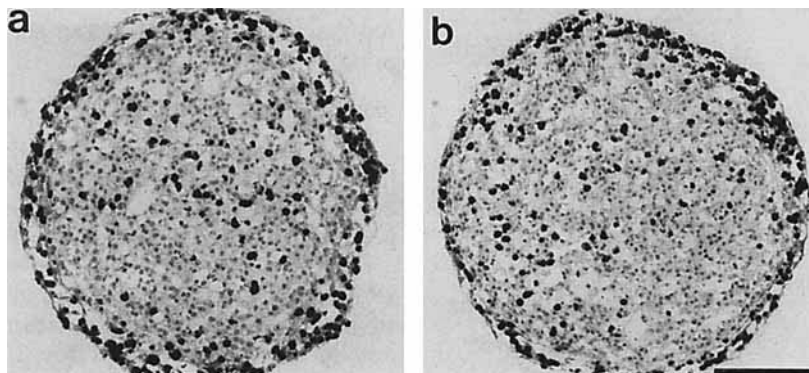


Fig. 6. Autoradiograms of aggregates composed of mesenchymal cells and myocytes from stage 36 chick heart. Cell suspensions of either atrioventricular (a) or epicardial (b) mesenchymal cells, which were pre-labeled with tritiated thymidine, were mixed with myocytes, pelleted, and

incubated in chicken serum without tritiated thymidine. The chicken serum stimulated the migration of the labeled mesenchymal cells to the cortical surfaces. Bar = 0.10 mm.

of both epicardial and atrioventricular fibroblasts were produced, and allowed to incubate in DMEM with 10% CS medium and 0.010 mM BrdU for 1 day. A few of the "reference" aggregates were fixed, embedded in paraffin, sectioned, mounted onto glass slides, and stained for BrdU as described above. The remaining aggregates were placed in the outer track of a 60 mm organ culture dish with 3.0 ml of calcium and magnesium-free PBS containing 2.0 mM disodium ethylenediamine tetraacetic acid and 0.05% trypsin. The culture dish was placed on an orbital shaker at 100 rpm for 25 min at room temperature. The cell suspension containing the cortex cells was poured off and saved. The aggregates were placed in fresh trypsin solution for 15 minutes to remove any remaining cortical layer. The cell suspension was discarded and fresh trypsin solution was added. The remaining medulla areas of the aggregates were completely dissociated. The cortex and the medulla cells were transferred to 10% CS. Each population was plated separately onto a plastic tissue culture dish and grown to confluence. "Cortex" and "medulla" aggregates were made by the scraping method and incubated for 1 day in 3.0 ml of 10% CS with 2.0 μ Ci/ml tritiated thymidine. The aggregates were fixed, processed for autoradiography, and stained for fibronectin as described above.

Each experiment was repeated at least once with another set of aggregates. Since all results were repeatable, the statistical analysis was carried out on one set of experiments. Five aggregates were randomly selected from each group, and the labeling indices of the cortex and medulla were determined and averaged. The statistical method is described in the tables.

Viability of Cells in Aggregate Culture

The cortical layer of aggregates incubated in CS contain an abundant extracellular matrix and many proliferating cells. It is possible that the medullary cells are not receiving enough nutrients by diffusion and are

dysfunctional or dying. To examine this possibility, mesenchymal aggregates were incubated for 1 day either in 1.0 mg/ml BSA or 10% CS medium. Both media included 10 μ Ci/ml of tritiated leucine to evaluate the amount of new protein synthesis. Some aggregates from each group were fixed and sectioned, and autoradiography was done. The remaining aggregates were sectioned and stained with hematoxylin and eosin for general histology.

Cell Sorting

Because mesenchymal cell and myocyte segregation is different in the epicardium and the atrioventricular valves, cell sorting experiments were carried out using the aggregate culture system. Initially, mesenchymal cells were labeled by growing the cells in tissue culture dishes to confluence in the presence of 1.0 μ Ci/ml tritiated thymidine. The cells were dissociated with 0.05% trypsin solution, and aggregates were prepared by mixing cell suspensions containing unlabeled myocytes and tritiated thymidine-labeled mesenchymal cells in the ratio of 4 to 1, respectively. Centrifugation was performed using a small volume of culture medium (~100–200 μ l) to prevent stratification of cells in the pellet as a function of specific gravity. After incubation (37°C, 3–5 hr) the pellets were chopped into smaller pieces, and the new aggregates were incubated in medium described in the next paragraph for 1 day. Unlabeled thymidine (0.1 mM) was included in the culture medium to reduce transfer of labeled thymidine from labeled cells to unlabeled cells. The aggregates were fixed and sectioned as described above. With autoradiography, the labeled cells in this experiment represent the mesenchymal cells rather than proliferating cells.

In experiment A the labeled mesenchymal cells were obtained from whole hearts, which included both epicardial and atrioventricular fibroblasts (Armstrong and Armstrong, 1990). The aggregates were divided into 2 groups and placed either in 1.0 mg/ml BSA or

10% CS medium for 1 day. In experiment B the labeled cells were either epicardial or atrioventricular mesenchymal cells, and both groups were incubated in 10% CS medium for 1 day.

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