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A role for fibronectin in the development of beat in chick embryo cardiogenesis

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Senior Biology Research Thesis

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Abstract

Fibronectin is believed to play a directional role in the migration of precardiac mesodermal cells and may be involved in other aspects of cardiogenesis. In this study we investigated the role of fibronectin in the development development of heart beat by employing a chick precardiac explant culture system. Fibronectin is recognized by an integrin receptor molecule via an RGD amino acid sequence. Using a synthetic RGD peptide we have blocked the ability of any existing receptor molecules to interact with fibronectin in an attempt to break communication of the mesodermal cells with the extracellular environment. Explanted tissues treated with this blocking agent failed to form contracting vesicles *in vitro* in a dose-dependent manner. This evidence suggests a role for fibronectin in precardiac cell differentiation and development.

Introduction

Cellular motility has long been known to be an important mechanism in embryogenesis, and cell migration also plays an important part in the development of the heart. In avian development, the precardiac mesoderm is formed from cells that migrate through the primitive groove and spread outward (Rawles, 1943). By 19 hours of development, two localized regions of mesodermal cells have migrated to the right and left lateral sides of the midline. These cells compose the precardiac mesoderm. Over the next seven to ten hours, these cells migrate in an arc-like, anterior direction to converge near the midline above the anterior intestinal portal (Rawles, 1943; DeHaan, 1963) as shown in Figure 1. By stage 9 (Hamburger and Hamilton, 1951), the migrating cells have converged and certain cells dissociate from the primary mesodermal layer and associate with each other to form endocardial tubes (DeHaan, 1963). These tubes then fuse while the remaining layer condenses around them as myocardium to form a contracting tubular heart (33 hours).

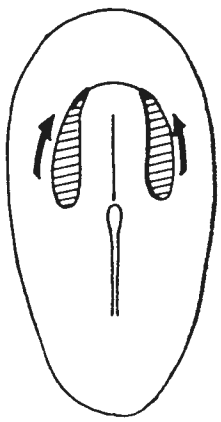

Figure 1. Migratory Crescent pattern of precardiac cell migration shown on a stage 6 embryo. Arrows indicate the direction of movement and cross-hatched areas represent the precardiac mesodermal cells.

This migratory process is probably mediated by active cell migration, rather than by a passive pulling into position (DeHaan, 1963). The constraints directing the migratory process are speculated to reside among the molecules of the extracellular matrix within the mesoderm-endoderm interface (Linask and Lash, 1988a). The molecules present in this matrix include collagens, glycoproteins, proteoglycans, and glycosaminoglycans (Borg, Raso, and Terracio, 1991). Prominent among these molecules is fibronectin, a glycoprotein, which has been implicated in several migratory processes, including the migration of neural crest cells (Bronner-Fraser, 1986; Riou *et al,* 1990) and Drosophila wing morphogenesis (Wilcox *et al,* 1989; Brower and Jaffe, 1989). In a study of *Xenopus* gastrulation, fibronectin was shown to be vital for directed active cell migration. Explants of the blastocoel roof were observed to move slowly and directionally over a fibronectin substrate (Winklbauer, 1990). When the fibronectin-integrin blocking agent RGD was added, only random movement occurred (Winklbauer, 1990).

Fibronectin is a cell-cell and cell-substratum adhesion molecule (Duband, Dufour, and

Theiry, 1991). As an extracellular matrix glycoprotein it plays a critical role in cell adhesion and in directing cell migration. Fibronectin connects the cells of the mesoderm to the underlying endoderm via a highly conserved (Albeda and Buck, 1990) transmembrane receptor complex of the integrin family (Buck *et al,* 1986). The integrin receptor recognizes an amino acid sequence of -arginine-glycine-aspartic acid- (RGD) in the ligand molecule. Integrins VLA-3, VLA-4, VLA-5, CD51, and CD41 have all been shown to bind fibronectin through the RGD site (Albeda and Buck, 1990). The VLA-3 integrin receptor appears to be the most common of these integrin complexes (Elices, Urry, and Hemler, 1991). These integrins are only expressed transiently in motile cells (Duband *et al,* 1986).

As an adhesion molecule, fibronectin has been implicated in directing the migration of chick precardiac cells. Linask and Lash (1986) have provided some evidence for an anterior to posterior gradient of fibronectin in the chick embryo which correlated with the general direction of migration of the precardiac cells. From this evidence, they concluded that precardiac cells follow the gradient of fibronectin by a haptotactic mechanism, ie. movement toward areas of greater cell adhesiveness (Linask and Lash, 1986). Later studies showed disruption of the directional orientation of precardiac cell migration in the presence of anti-fibronectin antibodies, while antibodies against other extracellular matrix molecules had no effect (Linask and Lash, 1988a). In a later experiment, Linask and Lash (1988b) removed the area of precardiac mesoderm and accompanying endoderm and reinserted the explants with the anterior to posterior polarity reversed. This resulted in precardiac cell migration in a posterior direction and the formation of two hearts (Linask and Lash, 1988b). Other authors have disputed this haptotactic migratory hypothesis by noting that the distribution of fibronectin is too widespread in the embryo to be directing this process (Winklbauer, 1990).

Other studies have reported interference in the development of beat in response to disruption by other extracellular matrix molecules. In one report, chick embryo precardiac

explants displayed an inability to develop contractions in response to a curtailing of collagen synthesis (Wiens *et al,* 1984). Fibronectin, as a molecule connecting the extracellular matrix to the cytoskeleton, may also play a role in the transduction of differentiation signals. If the blocking of fibronectin's binding to its receptor produced a result similar to that reported by Wiens *et al,* such a result could implicate fibronectin in the differentiation process.

We propose to observe the changes in chick embryo cardiac development in response to the blocking of fibronectin's binding site on the integrin molecule with the synthetic RGD peptide. At high concentrations, the RGD peptide binds all available integrin receptors to block any putative role fibronectin may play in heart development. In these studies we have observed a dose-dependent effect of the RGD peptide on early chick cardiac development in the form of an inhibition of development of heart beating. This may indicate fibronectin's involvement in cellular differentiation or in allowing the cells to initiate or perform the electrochemical depolarization required for contraction.

Methods

Chemicals

The RGD sequence used for the blocking step was added as a pentapeptide gly-arg-gly-asp-ser (GRGDS) suspended in Medium-199 (SIGMA, St. Louis, MO). The control pentapeptide, arg-lys-asp-val-tyr (RLDVT), was also suspended in Medium-199. Earl's Balanced Salt Solution (EBSS) was used as the dissection buffer and Medium-199 was used as an incubation buffer. Nile blue sulfate (HARLECO) was used as an aqueous solution for toxicity experiments. Fertilized eggs of white leghorn chickens were purchased from HyVac Labs, Gowrie, Iowa.

Explant Dissection

Eggs were pre-incubated at 38°C for approximately 32 hours prior to dissection to achieve the appropriate Hamburger-Hamilton stage. Eggs were removed from the incubator and

wiped with 70% ethanol to prevent contamination. The eggs were then windowed by cutting off the top circle of shell with sterile forceps. The thick albumen was decanted to leave the embryo exposed on the surface. Extra albumen was blotted with a Kim-wipe@ from the embryo's surface to enhance the membrane's adhesiveness to paper.

Once the position of the embryo had been identified, a paper ring (Whatman filter paper #3) was placed on the yolk surface encircling the embryo. The embryo was then removed from the yolk by cutting the yolk membranes with sterile scissors around the circumference of the paper ring. The entire unit (paper ring and adhering embryo) was then transferred, ventral side up, to a sterile dish containing prewarmed (37°C) EBSS for the dissection. At this time the embryos were staged by the Hamburger-Hamilton staging method. Embryos of stages six to eight were chosen for the experiments.

Fine glass needles were prepared for the microdissection. The right and left areas containing the precardiac mesoderm were removed by making straight cuts through the endodermal and mesodermal cell layers. Each explant, consisting of the precardiac mesoderm and the accompanying endoderm was then peeled away from the ectodermal layer and transferred to an individual well in Corning tissue culture plastic microtiter 96-well trays. Figure 2. shows an embryo immediately following explant dissection. The paired explants from each embryo were treated as experimental and control pairs.

Figure 2. H-H stage 8 embryo with precardiac mesoderm removed.

Treatment and Incubation

Each plastic microtiter well contained 45 ul of pre-warmed (37°C) Medium-199. In each microtiter well, additional Medium-199 containing either the RGD peptide or the non-RGD peptide was added to produce final concentrations of 1.0 mg/ml, 0.1 mg/ml, or 0.05 mg/ml. In a separate study, explants were also treated with Medium-199 alone in the plastic microtiter wells (Zars, unpublished results).

The microtiter trays were then placed in a high humidity incubator at 38°C and 5% CO_2 for a twenty-four hour incubation. Following the twenty-four hour period, the explants were removed and observed visually by stereo dissecting microscope. The explants were inspected for the presence or absence of vesicles, the ability to contract, and general morphology and cellular adhesiveness. Contraction was evaluated by observing either spontaneous beating or the ability to beat in response to mechanical stimulus (eg. prodding with a glass bulb). If the explants failed to contract in response to a mechanical stimulus, a negative result was scored.

The results were recorded by line diagrams and photographs.

Nile Blue Sulfate Evaluation of Cell Toxicity

An experiment was performed to determine the possible toxic effects of the peptides on the precardiac cells. Explants treated and incubated with 1.0 mg/ml of the RGD or with the control peptide were rinsed and treated with a Nile blue sulfate vital stain for 30 minutes to one hour. Following this incubation the explants were rinsed with PBS and placed in a wash solution of PBS to remove any unbound dye. The cells were then observed for any evidence of color.

Further Research

Ongoing research on this project is focusing on observing the explants in cross-section to determine the presence or absence of vesicles in RGD-treated explants. The effect of anti-fibronectin antibodies is also being studied to further observe the role of fibronectin in this migratory process.

Results

The results of this study were quantitated with respect to the presence or absence of contracting tissue. A table of results contains the total percent of explants which were inhibited from beating at each dosage (Table 1.). This table shows the large dose-dependent effect of the GRGDS treatment on explants Twelve to fourteen explants were observed at the two higher doses. Six explants were observed at the lower dose.

Table 1. Percent Inhibition (As Determined from Observable Contraction) in RGD-treated Explants.

Dose	PERCENT INHIBITION GRGDS Peptide	RLDVT Peptide
1.0 mg/ml	100%	42%
0.1 mg/ml	60%	25%
0.05 mg/ml	33%	16%

Figure 3. is a graphical depiction of the inhibition data showing the dose-dependent nature of the effect of the GRGDS peptide. The curve shows the minimum concentration of RGD required to produce 100% inhibition at 0.4 mg/ml. The inhibition of the control peptide was plotted on the same graph and shows a different characteristic slope. From this curve, it appears unlikely that 100% inhibition could ever be achieved by treatment with the RLDVT peptide.

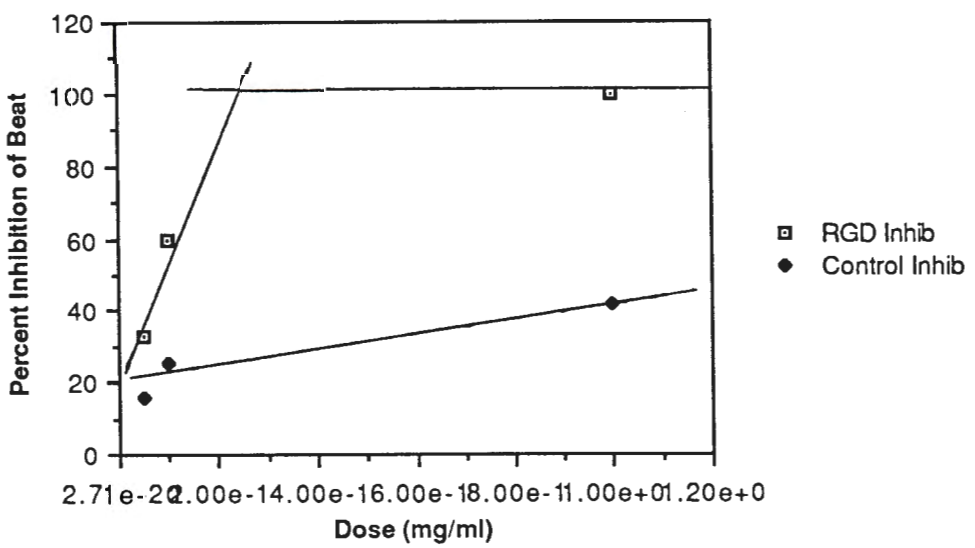

Figure 3. RGD and Control Peptide Dose-Response Curves. The RGD Curve Shows a Maximum Required Dose at 0.4 mg/ml. Notice the Shallow Slope of the Control Peptide Curve.

Figure 4. also depicts the effect of the GRGDS peptide on development. Shown in the

figure are a control explant with a fully functioning, contracting vesicle and a treated explant which has failed to develop a contracting vesicle. This figure shows the typical appearance of treated and control explants. Control explants often appear to have one or more bulbous lobes, one of which is the vesicle. An endocardium is usually visible through the transparent outer cell layer. Normally developed explants contain firm, healthy tissues. In the some explants treated with GRGDS at concentrations of 1.0 mg/ml or higher that failed to contract, cellular dissociation was reported around the borders of the explant. This was possibly caused by the RGD interaction with the integrin binding. The reported dissociation took the form of loose cells around the explants perimeter and an overall lack of integrity of the explant. This was observed when prodding the explant to stimulate contraction caused the cells to break contact easily allowing the probe to puncture or perforate the tissue.

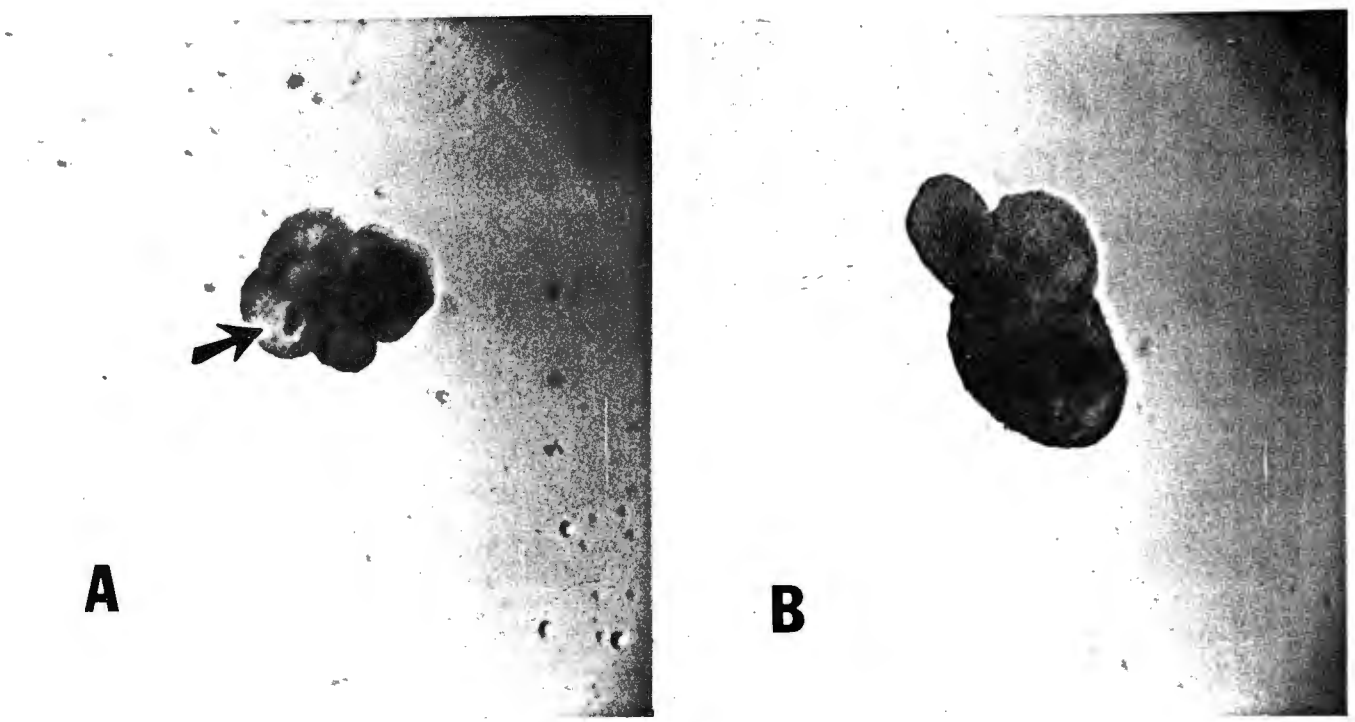

Figure 4. Explants following the 24 hour incubation. A) A fully functioning explant treated with the random control peptide, arrow indicated the contracting vesicle. B) A non-functioning RGD-treated explant with no observable vesicle formation.

Another finding is the apparent effect of the random control peptide, RLDVT, on heart development. Other investigators have used control peptides in analogous experiments and have observed normal development at much higher doses. Our observation of an apparent dose-dependent inhibition of physiological contraction suggests that there is a specific effect of RDLVT on some developmental process of the precardiac mesoderm. The effect is 41-48% less potent than that of GRGDS, but the mechanism of its effect is very likely mediated similarly at the cell surface. The results suggest the existence of an additional cellular recognition sequence within the extracellular matrix which may alter the development of cardiac function. The sequences of extracellular matrix molecules might give some indication of this interaction.

In order to explore the possibility that the inhibited development was caused by cell death, an experiment was performed using the vital stain, Nile blue sulfate. The test was

performed on explants treated with 1.0 mg/ml GRGDS or 1.0 mg/ml control peptide following the incubation process. The results showed no evidence that the cells had taken up the vital stain, thereby establishing that the treatment was not altering development of heart beat by inducing a toxic effect upon the cells.

Discussion

Using a pentapeptide with a recognition sequence for the integrin receptor site for fibronectin, the GRGDS peptide, we were able to break communication between the precardiac mesoderm and the fibronectin of the extracellular matrix. This created a condition in which the precardiac mesoderm was not only blinded to any specific distribution of fibronectin which might be playing a directional role in the cell's migratory process, but was also unable to adhere and respond to this important extracellular signal. The overall appearance of the RGD-treated explants in comparison to the controls and the inhibition of contraction would suggest that fibronectin and other extracellular matrix molecules have a more profound role in cardiogenesis than merely providing orientation to the migration process.

Other experiments of this type have been performed in whole embryo cultures. One such study reported partial cardiabifida in embryos treated with 20 ug/ml RGD (Linask and Lash, 1988a). The precardiac mesodermal cells appeared to have migrated to the lateral sides of the anterior intestinal portal and formed multiple heart vesicles. The fusion of the tissues to form a single heart, however, was prevented. While this data shows more organized movement and coherent cellular action than we report in response to RGD, the explant method which we utilized may be more effective at introducing the blocking peptide to the mesoderm-endoderm interface than by methods used in whole embryo culture. In addition, the RGD dose used in our experiments was significantly higher than the 20 ug/ml used by Linask and Lash (1988a).

The generally healthy appearance of RGD-treated explants and the results of the Nile

blue sulfate experiment suggest that these explants are viable. The morphogenic properties of the GRGDS pentapeptide are, therefore, mediated through an interference of mesodermal-endodermal communication via the fibronectin receptor and cell death is not responsible for the differences observed between the treated and control explants. The dose-dependent effect suggests that these receptor molecules are present and are important in early chick cardiac development. Furthermore, the absence of beating tissue suggests an important role for the fibronectin molecule as a stimulus or signal for the development. Fibronectin may indirectly mediate gene expression to elicit the contraction of the precardiac cells. Recent findings (J. Rathmell, Unpublished Results) have identified a localized region of fibronectin concentration during the migratory stages of the precardiac cells and shown a high degree of stage specificity in fibronectin distribution. These results suggest that fibronectin may invoke a stage-specific response in the precardiac cells, possibly in the realm of gene expression to regulate precardiac cell differentiation. In this way, fibronectin may be a molecular signal of the differentiation process. Cellular binding to extracellular matrix components like fibronectin may also promote differentiation by way of cytoskeletal changes communicated through receptor integrins, myofibril assembly, gap junction formation, or the synthesis of myofibrillar proteins (Wiens, Personal Communication).

Extracellular matrix proteins are likely candidates as signalling components in the precardiac cell differentiation process. The findings reported by Wiens (1984) implicate collagen in this precardiac cell differentiation process. Our findings suggest that fibronectin is also an integral component of the processes involved in the development of heart beat.

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