IMMUNOFLUORESCENT HISTOLOGICAL STUDIES OF THE ROLE OF FIBRONECTIN IN THE EXPRESSION OF THE ASSOCIATIVE PREFERENCES OF EMBRYONIC TISSUES

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SUMMARY

The identity of the chemical factors controlling the spreading behaviour of sheets of cells was examined in organ culture. When aggregates of two dissimilar tissues are apposed in organ culture, one tissue spreads reproducibly over the surface of the second. The present study employed indirect immunofluorescent localization techniques to evaluate the hypothesis that the spreading behaviour of chick embryonic heart tissue in culture is dominated by the presence or absence of the cell-surface and extracellular matrix protein fibronectin in the surface layers of the aggregates. Specifically, the hypothesis proposes that aggregates that display surface fibronectin earlier after culturing and/or in higher quantities segregate internally to aggregates that are slower to develop a surface layer of fibronectin or in which this layer contains reduced amounts of fibronectin. The hypothesis has been supported for 3 categories of behaviour of chick embryo heart tissue: (1) myocyte aggregates spread over myocyte aggregates containing a 20% admixture of heart fibroblasts, which in turn spread over heart fibroblast aggregates; (2) 5-day embryonic ventricle-tissue fragments maintained in culture for 0-5 days spread over ventricle fragments cultured for 2-5 days; and (3) 2-day embryonic ventricle spreads over 5-day ventricle. In all these situations, the aggregate type that segregates to an internal position displays more fibronectin at its surface than aggregate types that spread to occupy an external position. Evidence is presented that the fibronectin in heart tissue aggregates is elaborated by heart fibroblasts.

INTRODUCTION


The in vitro system is a useful model for the study of cell movement because it provides the opportunity to study the effects of alterations of the cellular composition of the tissues and the composition of the extracellular matrix. In previous studies employing chick embryo ventricle tissue, it was observed that mesenchymal cells (fibroblasts), which constitute a minority of the cells of the ventricle (myocytes constitute 70-80% of the 10-day embryonic ventricle; DeHaan, 1967), can influence the behaviour of heart tissue in organ culture such that tissues containing fibroblasts...
tend to be spread over by tissues lacking fibroblasts (Armstrong, 1978, 1980a). Based on the observation that myocyte aggregates treated with a preparation whose principal component is fibronectin behave in essentially the same way as myocyte + fibroblast aggregates, it has been suggested that the ability of fibroblasts to modify the associative preferences of ventricle tissue is produced by the release of fibronectin into the external milieu (Armstrong, 1980b). One prediction of this hypothesis is that the amounts and distribution of fibronectin in tissue aggregates should correlate with their spreading behaviour, aggregate types that adopt superficial positions should contain less fibronectin at their surfaces than do aggregate types on which spreading occurs. The present study reports an investigation of this prediction using indirect immunofluorescent localization techniques on tissue sections for 3 categories of altered spreading behaviour displayed by chick embryo heart tissue. In all cases, the predictions of the hypothesis are validated.

**MATERIALS AND METHODS**

In all cases the culture medium was 90% Dulbecco-modified Eagles medium + 10% chicken serum. The gas phase was 95% air/5%CO₂. In some cases the medium was depleted in plasma fibronectin by passing it over a gelatin-Sepharose 4B affinity column (Engvall & Ruoslahti, 1977). Pigmented retina was prepared as described previously (Armstrong, 1970). Whole 2-day chick embryo heart ventricles were prepared as described by Lesseps (1973) and fragments of 5-day ventricle prepared by chopping heart tissue with fine knives as described by Phillips, Wiseman & Steinberg (1977) were maintained in shaker flask culture. Ten-day chick embryo heart ventricles were disaggregated in 0.1% trypsin (Difco, 1:250) and freed of a majority of fibroblasts by culture of the resulting cell suspension in tissue-culture-grade plastic Petri dishes (Falcon Plastics) for 2 h. During this period the fibroblasts attach to the dish whereas the myocytes do not and the myocytes can be removed when the medium is decanted (Armstrong & Armstrong, 1978, 1979). Myocyte aggregates were prepared by centrifuging the cell suspension in 16-ml screw-cap test tubes followed by stationary incubation at 37°C for 3-6 h. The coherent pellets were chopped into smaller pieces, which were cultured further in shaker-flask culture.

**Immunofluorescence microscopy**

Aggregates were washed exhaustively in phosphate-buffered saline (PBS) to remove loosely associated plasma fibronectin and were fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min at room temperature. Following washing in PBS, the aggregates were exposed to PBS with an elevated concentration of NaCl (1 M) and with 0.1 M-glycine, 0.5 M-histamine (G-H). Aggregates were embedded in paraffin with a 40-min embedding time and sectioned at 6 µm. Sections were deparaffinized and hydrated, treated with G-H, then treated in sequence with 1:5 normal rabbit serum (0.5 h, 37°C), 1:50 monospecific goat anti-chick embryo fibroblast cellular fibronectin antiserum (Yamada, 1978) and 1:16, fluorescein (FITC)-labelled rabbit anti-goat immunoglobulin G (Miles). The anti-fibronectin antiserum was affinity-purified and has been extensively characterized (Yamada, 1978). Control sections were run on each slide in which PBS or preimmune serum replaced the anti-fibronectin antiserum. Coverslips were mounted with PBS/glycerol (1:1) and the slides were viewed with a Zeiss WL microscope equipped with a Zeiss epifluorescence condenser, a 50 W high-pressure mercury lamp and a fluorescein filter set. Photomicroscopy was performed with a Wild automatic exposure camera on Ektachrome 400 film. Standardization was achieved by control of section thickness, rigorous control of the staining protocol and restricting the time of exposure of the section to the illuminating beam to 5 s prior to photography (to minimize bleaching of the fluorescein). Constant exposure times of 90 s with a Plan 16/0-32 objective and 12 s with a Planapo 40/1-0 objective were employed throughout. A control section of each aggregate was also photographed to estimate the intensity of autofluorescence.
RESULTS

Fibronectin content in aggregates with differing fibroblast contents

The principal cell types that can be obtained from trypsin-dissociated 10-day chick embryo heart ventricle are myocytes (70–80%) and fibroblasts (20–30%). The effects of fibroblasts on spreading behaviour are displayed in various situations: (1) chick embryo pigmented retinal tissue spreads weakly over myocyte (M) aggregates but strongly over 80% myocyte + 20% fibroblast (M + F) and pure fibroblast (F).

![Autoradiogram of [H]leucine-labelled M + F aggregate maintained in shaker-flask culture for 2 days in contact with an unlabelled F aggregate. Labelled cells are distinguished by the black silver grains lying over their cytoplasm. During the period of culture, the M + F aggregate has spread to envelop the F aggregate. Arrows designate the limit of spreading. × 200.](image)

Fig. 1. Autoradiogram of [H]leucine-labelled M + F aggregate maintained in shaker-flask culture for 2 days in contact with an unlabelled F aggregate. Labelled cells are distinguished by the black silver grains lying over their cytoplasm. During the period of culture, the M + F aggregate has spread to envelop the F aggregate. Arrows designate the limit of spreading. × 200.

In these experiments, aggregates were cultured alone for 1 day and then pairs were cultured in apposition for 2 days. This behaviour constitutes a hierarchy of tissue positioning with pigmented retina occupying the most superficial position in the hierarchy, followed progressively by M aggregates, M + F aggregates and F aggregates.
The hypothesis under examination predicts that the fibronectin content of these aggregate types should be lowest in pigmented retina and progressively higher in M, M + F and F aggregates.

Indirect immunofluorescent localization employing a well-characterized, affinity-purified antiserum to chick embryo fibroblast cellular fibronectin (Yamada, 1978) confirms these predictions. Even after 3 days in organ culture, pigmented retina tissue fragments showed no fluorescence above background and so are presumably devoid of fibronectin. When the dissection of pigmented retina from the eye was performed such that choroid mesenchymal tissue was left on the fragments of pigmented retina, the choroid was fluorescent but the pigmented retina was not. Myocyte aggregates contained very little fibronectin after 1 day in culture and progressively more at 2 and 3 days. At 1 day (e.g. the age when fragment pairs would be prepared), the fibronectin was present as strands distributed sparsely in the interior of the aggregate. At 2 days, fluorescent strands were present homogeneously in the aggregates with no higher density in the surface layers of cells (Fig. 2A). By 3 days, fluorescent strands were present in the interior and were, in some preparations, somewhat more abundant in a layer of cells 10–30 \mu m thick at the surface. In those trials in which the surface fibronectin-rich layer was present it was patchy and discontinuous. The cytoplasm of the cultured myocytes was more fluorescent in sections treated with...
anti-fibronectin antiserum (Fig. 2A) than in control sections in which pre-immune serum was substituted (Fig. 2D), suggesting that cultured myocytes may contain small amounts of cytoplasmic fibronectin. At 1, 2 and 3 days in culture, M + F aggregates have more fibronectin than M aggregates of the same age. Fibronectin was present abundantly in the surface cell layers and more sparsely in the interior (Fig. 2B). The amount of fibronectin in both regions increased between 1 and 3 days of culture. Most of the fibronectin of M + F aggregates is apparently of cellular origin rather than being adsorbed plasma fibronectin from the culture medium, since the fibronectin content was not decreased if aggregates were cultured in fibronectin-free culture medium. F aggregates had more fibronectin than M + F aggregates at 1, 2 and 3 days of culture. As in the M + F aggregates, the fibronectin was present as fibrillar strands and was much more abundant in the surface cell layers than in the interior (Fig. 2C). The amount of fibronectin showed a progressive increase between 1 and 3 days in culture. In conclusion, the hierarchy of tissue positioning is reflected in progressively higher contents of fibronectin in those aggregate types that segregate progressively to the internal position in aggregate pairs. The site of highest abundance of fibronectin in heart aggregates is in a layer of cells 10-30 \( \mu \text{m} \) thick at the surface of the aggregates (e.g. the site at which partner aggregates are in contact).

**Effects of prolonged culture of ventricle tissue fragments**

The time of residence in organ culture has been reported to affect the spreading behaviour of fragments of tissue excised from 5-day chick embryo heart ventricle. Fragments that had been cultured for 0-5 days spread preferentially over fragments cultured for 2-5 days (Phillips, Wiseman & Steinberg, 1977). Based on the discussion presented above, it can be suggested that this behaviour results from an accumulation of fibronectin at the surface of the tissue fragments during prolonged culture. This prediction is supported by immunofluorescent localization of fibronectin. Fibronectin was present in the fragments cultured for 0-5 days as thin layers bordering the trabeculae carneae (Fig. 2E) and at the border between the epicardium and the myocardium (Fig. 2F). The epicardial cells themselves did not have a layer of fibronectin at the surface of the tissue fragment itself (Fig. 2F). The cut edges of the fragments lacked the epicardial layer with its fibronectin-rich basement membrane (Fig. 2E,F). The surface of the fragment is, thus, largely free of fibronectin. The pattern of fibronectin distribution after 2-5 days in culture is much altered. Not all trabeculae are lined by a fibronectin layer (Fig. 2G) and, of more interest, the surface of the fragment is now composed of a thick layer of cells rich in fibronectin (Fig. 2G,H). This fibronectin-rich layer is exposed at the very surface of the aggregate over large areas (Fig. 2H). Elsewhere, a surface layer of flattened cells 1 cell thick is present above the fibronectin-rich layer. Culture in fibronectin-depleted medium does not affect the accumulation of fibronectin in the tissue. In summary, the tissue that adopts the internal position (ventricle tissue-cultured for 2-5 days) displays more fibronectin at the surface of tissue fragments than the tissue that adopts a superficial position (ventricle cultured for 0-5 days).
Effects of embryonic age

Prior to 3 days of embryonic development, the ventricle wall is a pure population of myocytes (Manasek, 1970, 1976, 1979). Subsequent to this, fibroblasts migrate to the inner margin of the heart wall from sites of origin in the endocardium and pharyngeal arches (Patten, Kramer & Barry, 1948; Markwald, Fitzharris & Manasek, 1977; Thompson & Fitzharris, 1979). Based on the observations reported above, it might be suspected that the behaviour of heart tissue would change following fibroblastic colonization. Consistent with this, Lesseps (Lesseps, 1973; Lesseps & Brown, 1974) has reported that 2-day heart spreads over a variety of heterologous tissues (neural tube and pigmented retina) but 5-day heart segregates internal to these. Two-day heart spreads over 5-day heart. Of present interest is the possibility that fibronectin at the surfaces of heart fragments may mediate this effect. Consistent with this suggestion is the observation that ventricles excised from 2-day hearts show no fibronectin at their surfaces even after 2 or 3 days in organ culture (Fig. 21) whereas, as reported in the previous section, 5-day ventricle fragments develop a sheath of fibronectin-rich tissue at their surfaces (Fig. 2G, H). In the cultured 2-day ventricle, fibronectin is relatively sparse and is confined to remnants of the endocardium in the interior of the tissue (Fig. 21). This represents, thus, a third situation in which internal segregation correlates with the presence of fibronectin at the surface of tissue fragments.

Histology of the aggregates

The factors that cause the distinct zonation of fibronectin into a surface fibronectin-rich layer surrounding an interior fibronectin-poor zone in F and M + F aggregates

Fig. 3. The superficial fibronectin-rich domains of M + F (A) and F (B) aggregates are occupied by cells flattened in a plane parallel to the surface of the aggregate whereas the cells of the interior of the aggregates are unflattened. M aggregates (C) do not show a zonation into superficial and interior domains of morphologically distinguishable cells. ×75.
and in 5-day ventricle fragments cultured for 2.5 days are not known. The cells occupying the fibronectin-rich zone are morphologically distinct from the cells of the interior. In M + F and F aggregates, the cells of the surface zone are flattened in a plane parallel to the surface of the aggregate whereas the interior cells are unflattened (Fig. 3A, B). The surface cells are more basophilic in sections stained with haematoxylin and eosin than the interior cells, especially in the M + F aggregate. In 5-day ventricle fragments cultured for 2.5 days, the surface zone is composed of loosely-packed basophilic cells whereas the interior is composed of more tightly-packed eosinophilic cells (Fig. 4A). In these 3 aggregate classes, the boundary between the 2 zones is sharp. The aggregate types that lack the fibronectin-rich surface layer (namely, M aggregates (Fig. 3C) and 2-day ventricle cultured for 2.5 days (Fig. 4B)) lack the

![Image](image_url)

**Fig. 4.** The surface layers of 5-day ventricle fragments cultured for 2.5 days (A) are mesenchymal in character while the deeper layers are composed of more tightly-packed cells. The superficial cells have a basophilic cytoplasm in preparations stained with haematoxylin and eosin; the interior cells have an eosinophilic cytoplasm. 2-day ventricle cultured for 2.5 days (B) lacks the surface mesenchymal layer. ×75.

surface domain of cells that are morphologically distinguishable from the cells of the interior.

**DISCUSSION**

Fibronectin is a cell-surface and extracellular matrix glycoprotein that is produced by mesenchymal and endothelial cells and has been implicated in cell–cell adhesion (Hynes et al. 1978; Yamada, Olden & Pastan, 1978) and cell–collagen attachment (Klebe, 1974; Kleinman, Martin & Fishman, 1979; Pearlstein, 1976). The hypothesis under examination in the present report is the notion that fibronectin can influence the associative behaviour of embryonic heart tissue such that tissue aggregates displaying abundant fibronectin at their surfaces are spread over by aggregates showing smaller amounts of fibronectin. Fibronectin levels were estimated by indirect
immunofluorescent localization procedures on tissue sections employing a well-characterized monospecific antiserum to cellular fibronectin (Yamada, 1978). In 3 separate categories of behaviour of heart tissue, the fibronectin content at the surfaces of aggregates is consistent with the predictions of the hypothesis. (1) With the positioning hierarchy: pigmented retina fragments envelop M aggregates, M aggregates envelop M + F aggregates, and M + F aggregates envelop F aggregates, the fibronectin content is lowest in the pigmented retina aggregate and is progressively higher in M, M + F, and F aggregates. (2) Five-day ventricle fragments that have been cultured for 0-5 days envelop 5-day ventricle fragments cultured for 2-5 days. The former show little fibronectin at their surfaces, with the superficial fibronectin being restricted to the space between epicardium and myocardium, whereas the latter show abundant fibronectin in a continuous layer at their surfaces. (3) Two-day embryonic ventricle envelops 5-day ventricle. Even after 2 or 3 days in culture, 2-day ventricles never develop a surface layer of fibronectin whereas, as reported above, 5-day tissue develops a continuous layer rich in fibronectin at the surface of the tissue fragment.

The immunofluorescent histological observation of fibronectin distribution in different types of heart aggregates, coupled with the demonstration that myocyte aggregates exposed to a partially purified preparation of fibronectin segregated internally to aggregates not exposed to fibronectin (Armstrong, 1980a,b), supports the suggestion that fibronectin dominates the associative behaviour of ventricle tissue. The observation that culture of M + F aggregates and 5-day ventricle fragments in fibronectin-depleted medium does not reduce fibronectin content in the tissue suggests that cellular fibronectin rather than adsorbed plasma fibronectin is the principal molecular species present. The observation that the fibronectin content of M, M + F and F aggregates correlates with the abundance of fibroblasts suggests that this cell type is the principal source of this molecule in heart tissue. Indeed, the minor contaminant of fibroblasts, known to be present in M aggregates (Armstrong, 1978), may be the principal source of the low levels of extracellular fibrils of fibronectin that accumulate during prolonged culture. The myocytes may contain fibronectin in the cytoplasm (compare Fig. 2A and D) but myocyte aggregates accumulate fibronectin fibrils only after prolonged culture.

Correlated with the accumulation of fibronectin in the surface layers of M + F and F aggregates, and 5-day ventricle fragments cultured for 2-5 days is the presence of a superficial zone of flattened or mesenchymal cells. Two possibilities can be entertained to explain the presence of 2 distinct cellular zones in these aggregate types: (1) the surface zone is composed of a cell type distinct from that in the interior; or (2) the superficial and interior tissue domains have the same cellular composition with the differences in cellular morphology being a consequence of the zonation of components of the extracellular matrix such as fibronectin. If the first possibility is valid, then the zonation of fibronectin would presumably be a consequence of the sorting-out of a fibronectin-secreting cell type (presumably the heart fibroblast) from a non-secreting cell (presumably the myocyte). This explanation does not account for the presence of 2 layers of cells (Fig. 3B) and the inhomogeneous distribution of fibronectin (Fig. 2C)
in heart fibroblast aggregates, or the observation that \(^{3}H\)thymidine-labelled fibroblasts are not present in large numbers at the surfaces of M + F aggregates that have been enveloped by pigmented retina tissue (Armstrong, 1978). In the latter situation, however, the \(^{3}H\)thymidine-labelled fibroblasts may indeed have been present at the surface of the M + F aggregate prior to its envelopment by pigmented retina, only to migrate from the surface following envelopment.

An interesting extension of the present series of experiments will be to explore the possible role of fibronectin in the spreading movements of cells \textit{in vivo}. The immuno-fluorescence localization procedures can be used to determine whether during morphogenesis of the embryo tissue spreading is carried out over carpets of fibronectin. Critchley, England, Wakely & Hynes (1979) and Sanders (1980) have provided evidence for the existence of a fibronectin carpet during spreading of the mesodermal mantle over the hypoblast in gastrulating chick embryos. In the heart, this is being studied in regard to the spreading of the epicardium over the surface of the ventricle. Epicardial spreading begins at 3.5 days of development (Challice & Virágh, 1973; Ho & Shimada, 1978; Manasek, 1969), shortly after colonization of the heart wall by fibroblasts. Of interest will be the presence or absence of fibronectin at the surface of the ventricle wall in advance of the migrating epicardium and its source (epicardium or newly arrived ventricle fibroblasts).

One characteristic of cells that is important for the stable organization of coherent tissues is that they stay put (e.g. remain within the confines of the parent tissue) once the cell migrations of embryonic morphogenesis have been completed. The converse to the stability of positioning of tissue cells is intercellular invasion, which is the migration of cells into adjacent tissues (cf. Armstrong, 1977, 1980c). An interesting group of related problems concerns the nature of the factors that stabilize the organization of coherent tissues on the one hand, and on the other hand those that cause certain cell types to display invasive motility. Since embryonic neural crest (Loring, Erickson & Weston, 1977; Sieber-Blum, Sieber, Yamada & Cohen, 1978), which is invasive, and transformed fibroblasts (Hynes \textit{et al}. 1978; Vaheri \textit{et al}. 1978), which have been presumed to be invasive, usually display low levels of fibronectin, it has been suggested that, for mesenchymal tissues at least, high fibronectin levels have a stabilizing effect on cellular positioning and that low levels of fibronectin result in invasive activity (Chen \textit{et al}. 1978; Erickson, Tosney & Weston, 1980; Weber, Hale & Losasso, 1977). The first aspect of this hypothesis appears not to be valid for chick heart fibroblasts maintained in tissue-like aggregates in organ cultures. Cell motility in such aggregates is appreciable (Armstrong & Armstrong, 1978, 1979, 1980), even though the aggregates contain high levels of fibronectin (cf. Fig. 2c). In fact, the surface layers, which are the regions with the highest amounts of fibronectin (Fig. 2c), are also the regions in which the most rapid and extensive cell motility occurs (Armstrong & Armstrong, 1978). Motility in the interior, where lower quantities of fibronectin are present, is very low. Not only are heart fibroblasts motile within fibroblast aggregates, they are also invasive to heart myocyte aggregates (Armstrong & Armstrong, 1978). It is interesting that in this situation fibroblasts desert a fibronectin-rich tissue (Fig. 2c) to invade a fibronectin-poor tissue (Fig. 2a). In addition to being invasive
itself, heart fibroblast tissue is readily invaded by leukocytes (Armstrong, 1977, 1980c; Armstrong & Lackie, 1975).

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REFERENCES


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