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Changes in the transferrin requirement of cultured chick embryo mesoderm cells during early differentiation

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SUMMARY

Mesodermal tissue from the chick embryo at various stages of early differentiation was cultured in hydrated gels of type I collagen in the presence and absence of transferrin. Primary mesoderm explants from primitive-streak-stage embryos responded to the presence of avian transferrin by significantly improved outgrowth which appeared to be related to the ability of the cells to attach to, and migrate in, the collagen. No evidence was obtained which suggested that this observation was dependent on increased cell proliferation. This outgrowth enhancement was not duplicated by transferrin of human origin. The avian transferrin did not produce this effect on cells cultured on plastic substrata, suggesting that the species-specific effect involves modulation by the extracellular matrix. Mesoderm explants from somite stages of development showed no increase in outgrowth in the presence of either avian or human transferrin as judged by counting the number of outwandering cells. Ultrastructural immunocytochemistry indicated surface binding of transferrin by cells in the gels, and the presence of endogenous transferrin on the surfaces of mesoderm cells *in situ* and in their extracellular environment. It is suggested that by binding to cell surface receptors, transferrin may be able to influence the strength of cellular adhesion to collagen and hence the capacity for cell locomotion.

INTRODUCTION

At the time of gastrulation in the chick embryo, primary mesoderm cells emerge from the primitive streak and migrate laterad where they differentiate into segmental plate and lateral plate mesoderm, which themselves give rise to a variety of other tissues (Sanders, 1986). In order to investigate the migratory capacity and behavioural characteristics of these mesodermally derived cells, they have been explanted into culture using conventional tissue culture substrata (Bellairs *et al.* 1980; Sanders, 1980) and also three-dimensional collagen gel culture (Sanders & Prasad, 1983). Using the conventional technique, several of the early mesoderm populations have been explanted, including primary mesoderm, segmental plate (unsegmented) mesoderm, early somitic mesoderm, dermomyotome and sclerotome. All of these tissues produce successful outgrowths of fibroblast-like cells which show characteristic differences in details of behaviour and, or, morphology. The primary mesoderm cells respond to the presence of exogenous

Key words: chick embryo, mesoderm, transferrin, insulin, collagen gel culture, cell migration.

fibronectin on the substratum by showing increased spreading and outgrowth (Sanders, 1980), a characteristic which may reflect the behaviour of these cells *in vivo*, where they contact fibronectin-rich substrata (Sanders, 1982, 1983, 1984*a*).

In contrast to these results on tissue culture plastic and glass, collagen gel culture of these tissues results in successful outgrowths of segmental plate and somitic mesoderm, but very poor outgrowth of the early primary mesoderm (Sanders & Prasad, 1983). In this culture environment, the primary mesoderm cells do not respond favourably to the addition of exogenous fibronectin. The question arises, therefore, as to why the primary mesoderm is unable to produce as profuse an outgrowth in collagen as on conventional substrata, and why is it different in this regard to the later, more differentiated, tissues to which it ultimately gives rise?

It is well known that cells in culture require transferrin for optimum growth (Aisen & Listowsky, 1980), and primary cultures of chick embryo cells are no exception (Coll & Ingram, 1981; Beach *et al.* 1983, 1985). In addition to this, however, there is a species specificity such that chick cells are best grown in transferrin of avian origin (Imbenotte & Verger, 1980; Ozawa & Hagiwara, 1981; Hasegawa & Ozawa, 1982). In particular, myogenesis by cultured avian myoblasts is dependent on the presence of avian transferrin (Ii *et al.* 1982; Kimura *et al.* 1982). Since primary mesenchyme cells give rise to myoblasts, and since other evidence is accumulating to suggest a role for transferrin in embryogenesis (Ekblom *et al.* 1981, 1983; Reynolds *et al.* 1983; Partanen *et al.* 1984; Thesleff & Ekblom, 1984), the present study was designed to investigate the effects of this glycoprotein on chick embryo mesoderm cells cultured in collagen gel. Particular attention was paid to the possibility of species specificity and to differences in the response of cells at various stages of early differentiation.

MATERIALS AND METHODS

Chick embryos, staged according to Hamburger & Hamilton (1951), were removed from their yolk and handled in sterile Tyrode's saline. The tissues dissected out were, mesoderm adjacent to the primitive streak of stage-5 embryos, both unsegmented axial mesoderm and newly segmented somites from stage-12 embryos, and sclerotome and dermomyotome from stage-18 embryos. The stage-5 tissue was dissected without any prior enzyme treatment, but the stage-12 and -18 embryos were immersed in 0·1% trypsin (Becton, Dickinson Co.) in calcium- and magnesium-free Tyrode's saline for 1 h at 37°C. This treatment was designed merely to loosen the tissues and facilitate dissection, which was performed in normal Tyrode's saline, and not to dissociate the cells. The tissue pieces dissected were matched to the size of an individual somite, so that all explants were approximately 0·15 mm in diameter.

Sterile collagen gels were prepared as previously described (Sanders & Prasad, 1983). Briefly, eight parts of type I collagen solution (Vitrogen 100, Collagen Corp.) were mixed with one part of medium 199 (×10 concentrated) and one part of 0·1 M-NaOH in 200 mM-Hepes buffer. Gentamycin and 10% foetal bovine serum (GIBCO) were added, and the pH adjusted with one or two drops of 1 M-NaOH. This was made up freshly, and remained fluid during the course of dissection if maintained at 4°C. A thin layer of collagen was poured into a sterile Sykes–Moore chamber (Bellco Glass Inc.) and gelled at 37°C. Fluid collagen, containing the tissue, was used to top up the chamber which was then fully assembled and placed in the incubator for final gelling. Cell outgrowth in collagen gels was measured by counting the number of outwandering fibroblast-like cells from each explant using phase-contrast microscopy (Sanders & Prasad, 1983). Rounded cells were excluded from the count. Tissues cultured on a planar substratum

were explanted onto Falcon dishes (No. 3301) in medium 199 plus 10% foetal bovine serum and gentamycin.

Avian transferrin purified from chick serum (a gift from Professor E. Ozawa) and human transferrin (Collaborative Research Inc.) were added to the culture medium at a final concentration of 30 $\mu g\,ml^{-1}$. ITS® supplement (Insulin/human Transferrin/Selenium; Collaborative Research Inc.) was added to the culture medium such that the final transferrin concentration was also 30 $\mu g\,ml^{-1}$. Insulin (Sigma Chemical Co.) and selenium (selenous acid, sodium salt, Collaborative Research Inc.) were used at concentrations of 5 $\mu g\,ml^{-1}$ and 5 $ng\,ml^{-1}$ respectively. In some gels, chicken serum (GIBCO) was substituted for foetal bovine serum.

The extent of cell division and cell movement was assessed using time-lapse cinemicrography with a Bolex camera attached to a Nikon model M inverted phase-contrast microscope, or time-lapse video using a JVC camera and Panasonic 6010 and 8950 recorders with the same microscope.

Immunocytochemistry

For ultrastructural immunocytochemistry of transferrin bound to the surfaces of cells in collagen gel culture, small pieces of gel containing cells that had been cultured for 3 days were washed in Tyrode's saline and fixed in 0·5% glutaraldehyde in 0·1 M-phosphate buffer, pH 7·4, for 10 min. Cells cultured both in the presence and absence of exogenously supplied chick transferrin were examined. All steps were carried out at room temperature. After washing overnight in phosphate-buffered saline (PBS) containing 1% goat serum (gs) and 0·1 M-lysine, the pieces were incubated in rabbit anti-human transferrin (Calbiochem-Behring Corp.) diluted 1:100 with PBS+gs for 4 h. This antibody cross-reacted with the chick transferrin as shown by enzyme-linked immunosorbant assay (ELISA), using Nunc curved-bottom microtitre plates, antigen concentrations down to 0·2 $\mu g\,ml^{-1}$ and antibody dilutions to 1:1000. ELISA controls were run using normal rabbit serum in place of antiserum. For cytochemical controls, material was incubated in PBS or non-immune serum instead of antiserum. After incubation in primary antibody, the tissue was washed overnight with three changes of PBS+gs followed by incubation in goat anti-rabbit IgG (Calbiochem-Behring Corp.) diluted 1:10 with PBS+gs for 1 h. Specimens were rinsed with two changes of buffer for 1 h and incubated in peroxidase–antiperoxidase reagent (Miles-Yeda Ltd), diluted 1:50, for 30 min. The pieces were rinsed with PBS (two changes, 10 min each), and immersed in diaminobenzidine (DAB) reagent for 20 min. The latter was freshly prepared in clean glassware by dissolving in 0·05% DAB in PBS and adding 0·01% hydrogen peroxide. After washing (three changes, 10 min each), postfixation was carried out using 1% osmium tetroxide in 0·1 M-phosphate buffer for 1 h, and this was followed by washing and dehydrating in a graded ethanol series and propylene oxide. Specimens were embedded in Araldite, sectioned and examined without staining in a Philips 300 electron microscope. Thorough washing between incubations was essential to ensure low background staining.

Whole embryos to be examined for endogenous transferrin localization were washed with Tyrode's saline, fixed and processed in the same way as described for the gel pieces. The stage-12 and -18 embryos were cut open in the region of the most mature somites to allow penetration of reagents, and sectioned near the opened surface.

RESULTS

Stage-5 mesoderm

The outgrowth of gastrulation-stage mesoderm (stage-5 embryos) in the standard collagen gel was very poor (Fig. 1, Table 1) and duplicated precisely the previously published results for this tissue (Sanders & Prasad, 1983). Addition of avian transferrin (30 $\mu g\,ml^{-1}$) to the gel resulted in a significantly increased outgrowth of cells (Fig. 1, Table 1) which was sustained for at least 10 days, in

contrast to the poor control growth. In morphology, cells in the presence of avian transferrin showed a healthy appearance, being well spread and fibroblast-like with many processes that time-lapse observation showed to be ruffling lamellipodia, filopodia and retraction fibres. This appearance (Fig. 2A,B) was maintained beyond the 5-day observation period, by which time the sparse control outgrowth had deteriorated and showed many rounded cells.

Time-lapse observations were made on five experimental and five control cultures, for a total 'real time' period of 30 h for each. During this time, no mitotic figures were observed in either situation, even though the optical qualities of the gels were amply good enough to detect them. Clearly this does not rule out the possibility of a certain low base level of proliferation in both experimental and control cultures. In several experiments the region of the embryo from which the mesoderm was dissected was noted; there was no correlation between the source of the mesoderm relative to the primitive streak and outgrowth efficiency.

The addition of human transferrin alone or in combination with insulin and selenium failed to stimulate outgrowth over control levels, despite indications at 48 h that they would prove efficacious (Table 1). Insulin alone, but not selenium alone, appeared to have a beneficial effect on outgrowth after 4 days (Table 1). The substitution of chicken serum for foetal bovine serum in the controls resulted in no outgrowth whatever, and the addition of fibronectin to these chicken serum cultures produced no detectable increase in these outgrowths.

These mesoderm cells were also explanted on tissue culture plastic, where they produced extensive outgrowths as previously reported (Sanders, 1980). The

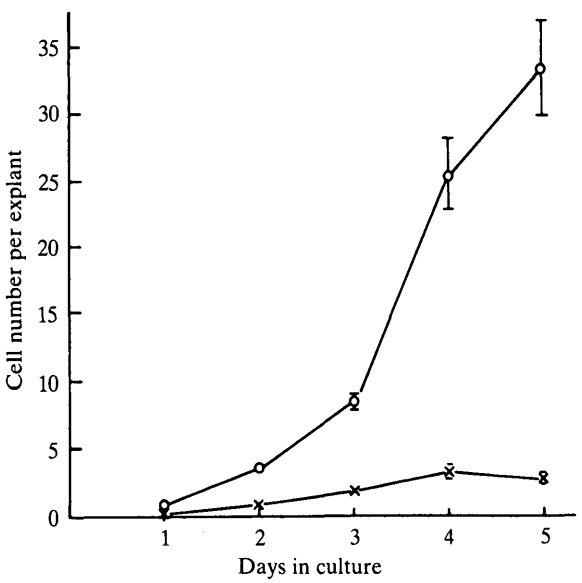


Fig. 1. The effect of adding avian transferrin (30 $\mu g\,ml^{-1}$) to collagen gel cultures of primitive-streak-stage mesoderm. The number of spread cells leaving the explant were counted over a 5-day observation period. O——O, with transferrin; ×——×, without transferrin.

Table 1. *Stage-5 mesoderm outgrowth*

	n′	n	Number of cells growing out of each explant (±S.E.)				
			1 Day	2 Days	3 Days	4 Days	5 Days
Standard gel	63	10	0·27 ± 0·10	0·83 ± 0·13	1·84 ± 0·30	3·16 ± 0·69	2·76 ± 0·56
Avian transferrin	146	18	0·33 ± 0·07	3·52 ± 0·27*	8·35 ± 0·69*	25·38 ± 2·75*	33·53 ± 3·52*
Human transferrin	42	5	0·32 ± 0·14	2·16 ± 0·53*	2·98 ± 0·99	5·14 ± 2·30	3·66 ± 1·52
ITS®	44	5	0·36 ± 0·15	1·70 ± 0·10*	3·28 ± 0·63	6·20 ± 1·78	8·58 ± 2·42
Bovine insulin	35	3	0·30 ± 0·10	1·40 ± 0·10	4·43 ± 0·72	9·17 ± 2·52*	15·65 ± 4·81*
Selenium	35	3	0·10 ± 0·08	0·20 ± 0·10	1·22 ± 0·43	2·27 ± 0·80	2·60 ± 0·66

n′, number of explants examined.
n, number of separate experiments.
Asterisks indicate values significantly different from the control ($P < 0·05$).

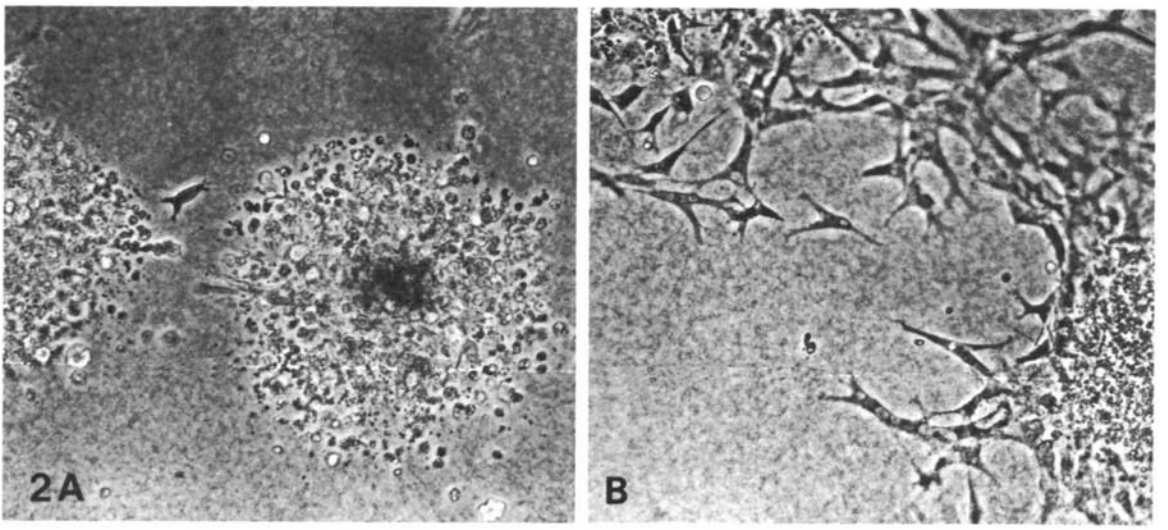


Fig. 2. Primitive-streak-stage mesoderm (stage-5) cells cultured in collagen gel for 5 days: (A) in the absence of avian transferrin; (B) in the presence of avian transferrin. Since the culture is three-dimensional, not all cells are in the plane of focus. ×95.

presence of avian transferrin had no appreciable effect on these explants as judged by the size of the outgrowth and the frequency of mitoses.

Stage-12 mesoderm

In contrast to the above results, the outgrowth of stage-12 unsegmented axial mesoderm and somitic mesoderm in collagen gel was unaffected by the addition of either avian transferrin or of human transferrin alone or in combination with insulin and selenium (Tables 2, 3). Inspection of Table 3 indicates some influence of the ITS® supplement in increasing the outgrowth of somitic tissue, but the high variability of the results precluded any statistical significance at the 5% level. Insulin or selenium alone did not produce this enhancement.

Stage-18 mesoderm

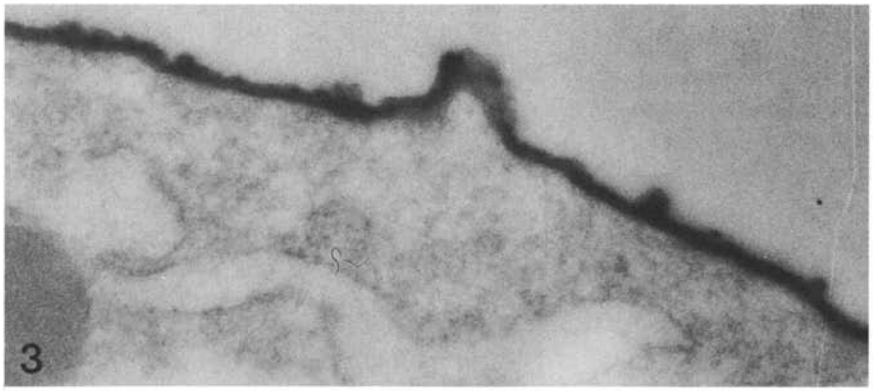
Sclerotome cells are extremely efficient in their attachment and locomotion in collagen gel (Sanders & Prasad, 1983), and the addition of transferrin to these cultures made no detectable difference to their outgrowth. Dermomyotomes were occasionally affected by the addition of avian transferrin. This tissue, which is of an epithelial nature, always maintained its integrity in the absence of transferrin, allowing very few individual cells to escape the epithelial mass which itself spreads very little in the confining environment of the gel. Myoblasts eventually appear to grow out as chains of elongated cells. In the presence of avian transferrin, however, individual cells sometimes left the explant and attached to, and migrated in, the gel. The inconsistency in this result may have been due to the varying degrees of disruption suffered by the dermomyotome during dissection.

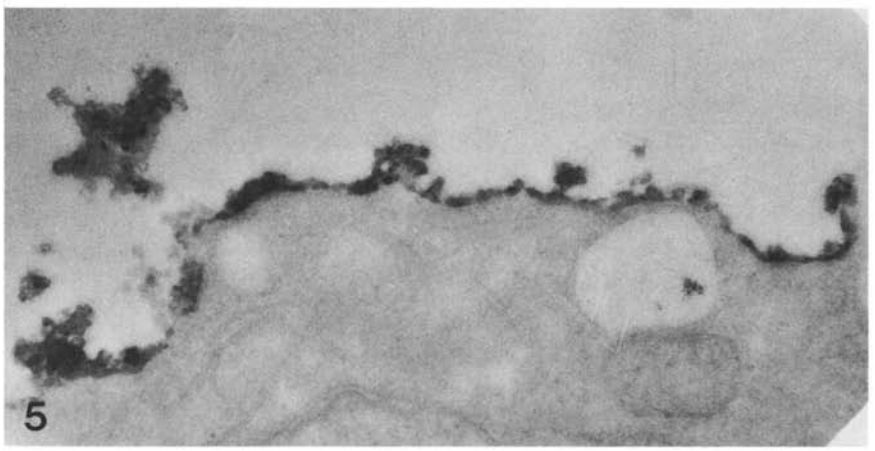
Table 2. *Unsegmented mesoderm outgrowth*

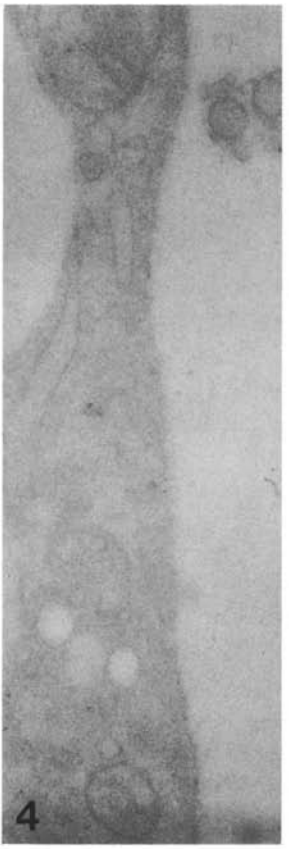
	n′	n	Number of cells growing out of each explant (±S.E.)				
			1 Day	2 Days	3 Days	4 Days	5 Days
Standard gel	44	7	0·75 ± 0·12	6·81 ± 0·87	7·71 ± 1·27	5·91 ± 1·31	4·59 ± 1·43
Avian transferrin	29	5	1·72 ± 0·32	3·82 ± 0·56	4·40 ± 1·02	2·50 ± 0·79	0·86 ± 0·24
Human transferrin	23	3	0·86 ± 0·36	7·83 ± 1·90	12·60 ± 2·37	10·83 ± 2·15	8·20 ± 2·38
ITS®	21	3	1·76 ± 0·90	12·56 ± 10·45	21·60 ± 8·99	15·60 ± 5·79	12·50 ± 4·42
Bovine insulin	22	3	1·30 ± 0·10	4·32 ± 0·80	8·13 ± 1·66	7·85 ± 1·57	5·55 ± 1·31
Selenium	20	3	1·51 ± 0·44	6·66 ± 1·12	6·91 ± 2·20	8·12 ± 2·41	7·63 ± 1·84

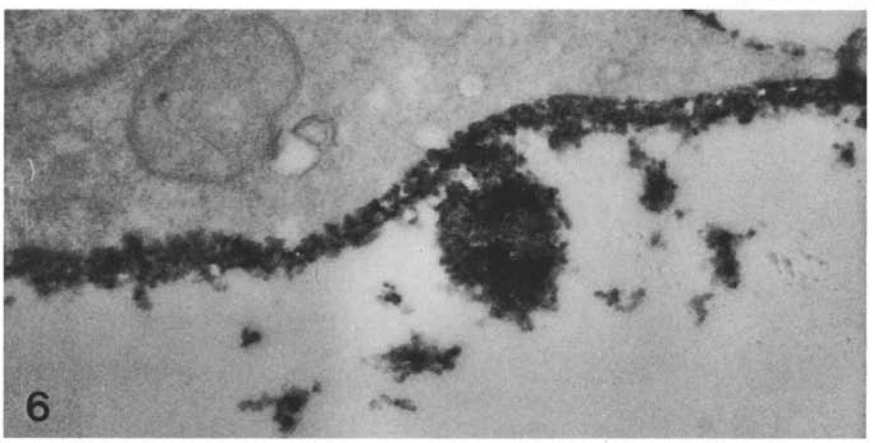
Table 3. *Segmented mesoderm outgrowth*

	n′	n	Number of cells growing out of each explant (±S.E.)				
			1 Day	2 Days	3 Days	4 Days	5 Days
Standard gel	43	7	14·28 ± 2·06	40·14 ± 7·11	46·45 ± 9·38	41·81 ± 14·67	36·83 ± 14·99
Avian transferrin	48	6	10·05 ± 1·83	27·48 ± 4·70	25·31 ± 4·75	14·80 ± 3·73	10·61 ± 3·28
Human transferrin	17	3	13·73 ± 5·04	56·03 ± 10·92	49·40 ± 10·38	32·34 ± 3·52	23·33 ± 6·21
ITS®	17	3	26·86 ± 6·51	48·20 ± 5·96	61·90 ± 13·48	88·26 ± 24·77	81·83 ± 26·56
Bovine insulin	26	3	10·10 ± 2·02	36·66 ± 4·71	50·32 ± 8·33	55·53 ± 9·06	48·68 ± 9·46
Selenium	14	3	15·21 ± 3·31	25·23 ± 4·42	37·67 ± 3·92	40·44 ± 5·51	35·41 ± 10·27









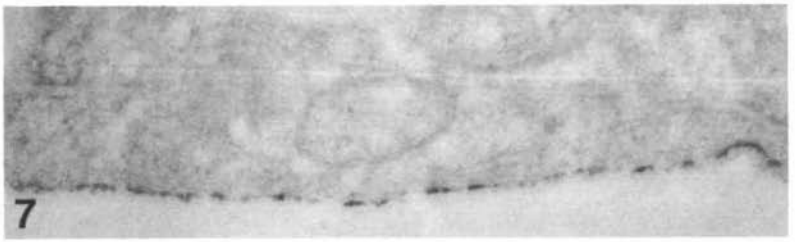
Figs 3–6. Transmission electron micrographs showing cell surface localization of transferrin.

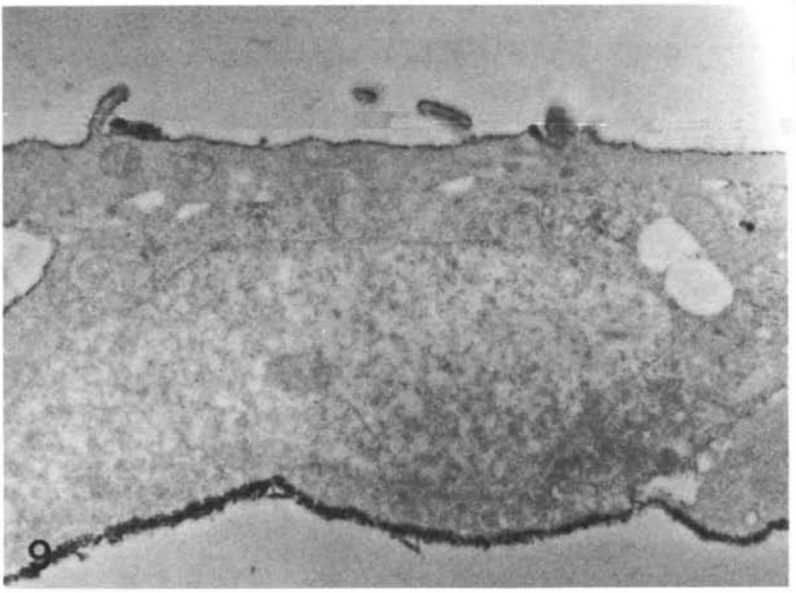
Fig. 3. Stage-5 mesoderm cell cultured in collagen gel. ×47900.

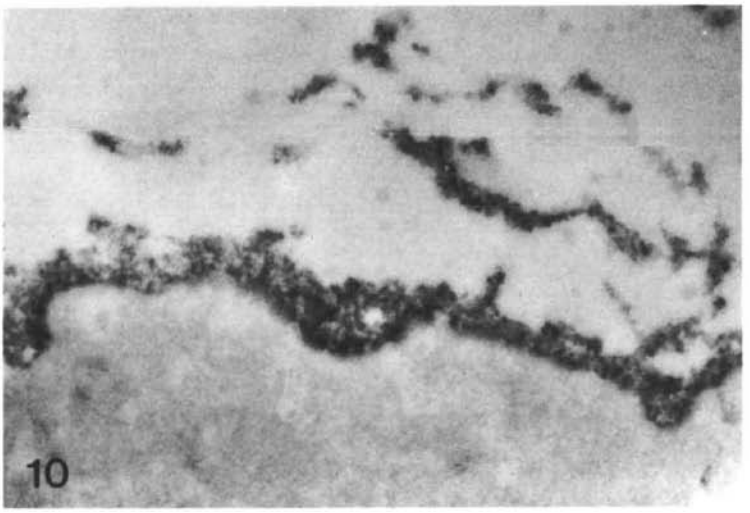
Fig. 4. Control for Fig. 3 in which transferrin antiserum was replaced by non-immune serum. ×28900.

Fig. 5. Endogenous transferrin associated with the surface of a mesoderm cell in a stage-5 embryo. ×31900.

Fig. 6. Endogenous transferrin localized on the basal surface of the epiblast of a stage-5 embryo. ×31160.







Figs 7–10. Transmission electron micrographs showing the localization of endogenous transferrin.

Fig. 7. A negative reaction on the ventral surface of the endoblast of a stage-5 embryo. ×31900.

Fig. 8. Control for Fig. 7 showing a typical level of non-specific stain. ×31900.

Fig. 9. Ectoderm from a stage-18 embryo showing a weak reaction on the apical surface but a strong one basally. ×14250.

Fig. 10. Deposit on and around a dermomyotome cell from a stage-18 embryo. ×37100.

Immunocytochemistry

All tissues cultured in gels showed a positive reaction product for cell surface-bound transferrin (Fig. 3). Using the current technique, no difference was discernible between the various tissues, nor between tissues cultured in the presence or absence of added transferrin. The latter result was probably due to the presence of transferrin in the serum that was added to all cultures. Control gels processed using PBS or non-immune serum in place of transferrin antiserum showed little or no non-specific background reaction (Fig. 4).

Whole embryos, fixed and processed to reveal endogenous transferrin, reacted as follows:

Stage 5

Transferrin was found associated with the surfaces of the mesoderm cells (Fig. 5), and on the basement membrane of the epiblast (Fig. 6). The outer surfaces of the embryo, i.e. dorsal surface of the epiblast and ventral surface of the endoblast, were negative (Fig. 7). The controls at this and other stages, using PBS or non-immune serum (Fig. 8), showed little deposit.

Stage 12

Deposit was found on the basement membrane of the ectoderm and dorsal surface of the endoderm, but little or none of the outer surfaces of the embryo. No deposit was associated with somite or segmental plate cells, despite their close proximity to stained surfaces, but a reaction in the lateral plate was occasionally detectable.

Stage 18

Similar patterns of deposit were found to stage 12, with the ectoderm showing a polarized distribution (Fig. 9). In addition, reaction product was encountered deep within the dermomyotome (Fig. 10) and sclerotome but with a discontinuous and erratic distribution.

DISCUSSION

One of the most interesting results demonstrated in this study is that avian transferrin selectively increases the outgrowth of the very early mesoderm of the chick embryo only when these cells are grown in gels of type-one collagen. This transferrin does not have this effect on mesoderm at the later stages of development when somites are being formed, and further, the effect on primitive-streak-stage mesoderm is not observed when these cells are cultured on tissue culture plastic. The latter observation emphasizes the importance of the extracellular matrix in modulating the responsiveness of cells to growth factors (Gospodarowicz, 1984).

Time-lapse observation, however, provided no evidence for increased cell proliferation among the cells migrating out of the stage-5 explants, although such

an effect has been reported for chick embryo cells at later stages of development (Imbenotte & Verger, 1980; Ii *et al.* 1982). There is a possibility that in the present case the species-specific stimulatory effect of transferrin on outgrowth may be a cell-surface phenomenon. Earlier results (Sanders & Prasad, 1983) showed that as the mesoderm differentiates during early development, it progressively acquires the ability to adhere to, and locomote in, type-one collagen. This parallels the general increase in adhesiveness among these cells as differentiation proceeds (Bellairs *et al.* 1978).

Since it is specific to the collagen substratum, it is possible that this effect may be related to the recent demonstration that the cell surface receptor for transferrin in fibroblasts is the core protein of heparan sulphate proteoglycan, HSPG (Fransson *et al.* 1984). The cell surface HSPG has binding sites for fibronectin (Laterra *et al.* 1983) which in turn bind to collagen (Engvall & Ruoslahti, 1977), providing at least one mechanism for the attachment between the cell and collagen substrata. These relationships permit the speculation that the addition of transferrin to the culture medium supporting the early mesoderm cells (which are fibroblastic) competes away, or at least hinders, the HSPG–fibronectin link, thereby releasing the cells from tight binding to the collagen and allowing increased locomotion. Increased locomotion would be a natural consequence of the decreased adhesion to the substratum (Gail & Boone, 1972; Sanders, 1984*b*).

In addition to transferrin, the present results indicate the desirability of supplementing the growth media for these tissues with ITS® or insulin alone in future work.

The immunocytochemical results described here show that the cells growing in the gels do, in fact, bind transferrin at their surfaces. This is in agreement with similar conclusions reached with other cells indicating surface binding of transferrin (Sullivan *et al.* 1976; Parmley *et al.* 1983; Willingham *et al.* 1984; McArdle *et al.* 1985) and the surface localization of transferrin receptors (Sutherland *et al.* 1981; Hopkins, 1985; Willingham & Pastan, 1985).

The immunocytochemical results for primitive-streak embryos (stage 5) show that endogenous transferrin appears to be present among the mesoderm cells (see also Adamson, 1982) and in the overlying basement membrane on which many of them move. It is therefore present in locations which might enable it to modulate cell attachment. Although transferrin appears to be retained in the ectodermal basement membrane at the somite stage (stage 12), it is apparently absent from the somite cells themselves. This may be correlated with the fact that these cells are not motile at this stage *in situ*, although in collagen gels they show considerable migratory capacity. On the other hand, the present results are in agreement with the observations of Reynolds *et al.* (1983) that transferrin was detectable among the dermomyotome cells, which are also not motile *in situ*.

Endogenous transferrin has previously been localized at the cell surfaces of a variety of embryonic or differentiating tissues (King, 1976; Adamson, 1982; Parmley *et al.* 1983; Reynolds *et al.* 1983; Meek & Adamson, 1985) and has been implicated in a number of developmental events in both kidney (Ekblom *et al.*

1981, 1983; Thesleff & Ekblom, 1984) and tooth (Partanen *et al.* 1984) morphogenesis. These events involve mesenchymal differentiation, and the results have been interpreted as depending on the proliferative effects of transferrin resulting from its iron-carrying function (Landschultz *et al.* 1984). It is possible that some of these interactions may also involve a cell surface component similar to that suggested by the present results. Further work is in progress in this laboratory in order to substantiate the speculations raised here.

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