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Inhibitory and stimulatory influences on mesodermal erythropoiesis in the early chick blastoderm

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Summary

We use a standing-drop culturing method to investigate the effect on mesodermal erythropoiesis of ectoderm and endoderm from the area opaca vasculosa (AOV) and area pellucida (AP) of stage-4 chick blastoderms. We find that ectoderm from the AOV and ectoderm and endoderm from the AP exert an inhibitory influence on mesodermal erythropoiesis. This inhibitory influence is coupled with the tendency of the explants to spread out and become flattened in culture. In contrast, endoderm from the AOV is found to be stimulatory, in agreement with previous studies. We correlate these *in vitro* inhibitory and stimulatory influences with the morphogenetic patterns that occur during normal development.

Key words: chick embryo, inhibition, stimulation, erythropoiesis, ectoderm, mesoderm, endoderm.

Introduction

We have been interested in the influence of environmental factors in the chick area opaca vasculosa (AOV) and area pellucida (AP) on the erythropoietic differentiation of the extraembryonic mesodermal cells (Kessel & Fabian, 1986). Wilt (1965) and Miura & Wilt (1969) have previously shown that stage-4 AOV endoderm has a salutary influence on the erythropoietic differentiation of stage-4 AOV ectoderm-plus-mesoderm explants cultured on egg–agar medium (Wilt, 1965) or on plasma clots on the same or on opposite sides of Millipore filters (Miura & Wilt, 1969). Using a standing-drop culturing method, we have subsequently demonstrated (Kessel & Fabian, 1986) that stage-4 AOV endoderm also stimulates the erythropoietic differentiation of the mesodermal population that is normally non-erythropoietic, i.e. stage-8 to -9 AP extraembryonic mesoderm, and which does not normally come into contact with AOV endoderm.

Using the standing-drop culturing method, our present study indicates that in contrast to the favourable influence of stage-4 AOV endoderm on mesodermal erythropoiesis, stage-4 AOV ectoderm and stage-4 AP ectoderm and endoderm exert an inhibitory influence on mesodermal erythropoiesis *in vitro*. The inhibitory influence is coupled with the tendency of the explants to spread out and become flattened in culture. We discuss these observations in terms of the morphogenetic patterns observed during normal development.

Materials and methods

In vitro *culture on coverslips*

Fertilized hens' eggs of the Ross breed (Cornish females × White Rock males) were obtained from Farm Fare (Pty) Ltd, Wynberg 2090 and incubated at 38°C until they had reached stage 4. The blastoderms were dissected from their yolks and vitelline membranes under chick embryo saline (0·7% NaCl, 0·018% $CaCl_2$, 0·037% KCl). Subsequent manipulations were carried out using hairloops (Rugh, 1962), stainless steel needles (D. F. Tayler and Co., UK, size 0) and a microsyringe (Burroughs Wellcome, UK). Blastoderm fragments approx. 0·5 mm in diameter, comprising one or more germinal layers (see Results), were excised from the extraembryonic AP or AOV and transferred to 0·03 ml drops of liquid culture medium (Hagopian & Ingram, 1971) on the surfaces of siliconized coverslips (Kessel & Fabian, 1986). The fragments were explanted either separately or sandwiched together to constitute given combinations of the germinal layers (see Table 2). The explants were cultured for 72 h as standing-drop cultures according to the method previously described (Kessel & Fabian, 1986). Thereafter, they were stained for haemoglobin (Hb) as described below. Additional blastodermal fragments composed of all three germinal layers from the

Table 1. *Classes of haemoglobin-staining intensity*

Class	Degree of haemoglobin staining	Assigned value
−	Blue counterstain only (i.e. no Hb-containing cells)	0
−+	Mainly blue counterstain, with traces of pale yellow staining (i.e. staining for Hb uncertain)	1
+	Pale brown Hb staining in few to many cells	2
++	Brown Hb staining in a large number of cells	4
+++	Very intense brown Hb staining in a large number of cells	8

stage-8 to -9 AOV were routinely cultured to ensure that the culturing conditions and Hb staining techniques were always optimal.

Haemoglobin staining

The extent of the erythropoietic differentiation of the explants was determined with the *o*-dianisidine technique, which stains Hb brown (O'Brien, 1961; Wilt, 1965; Miura & Wilt, 1969; Kessel & Fabian, 1986). The counterstain, 1% methyl green, stains the rest of the tissue blue. From microscopical examinations of the explants, each explant was assigned to one of five classes of Hb staining intensity (Table 1), based on the colour produced by the *o*-dianisidine technique. The student *t*-test was employed. For this purpose, numerical values ranging from 0 to 8 were assigned to the five classes of Hb staining (Table 1). Values of P equal to or lower than 0·05 were taken as indicating significant differences in the extent of Hb staining between different types of explants.

Results

Table 2 lists the various types of explants that were cultured and their degree of Hb staining.

Stage-4 AOV explants

The extent of Hb production of stage-4 AOV mesoderm cultured alone (explant type 1) was significantly greater than that of stage-4 AOV ectoderm-plus-mesoderm (explant type 2) ($P < 0{\cdot}001$). This suggests that stage-4 AOV ectoderm has an inhibitory influence on mesodermal erythropoiesis. Coupled with this was the tendency of stage-4 AOV ectoderm-plus-mesoderm explants (explant type 2) to spread out and become flattened over the surface of the coverslips during the culturing period (Fig. 1A).

The situation was different when stage-4 AOV mesoderm was cocultured with stage-4 AOV endoderm (explant type 3). In these explants, there was a significant enhancement in mesodermal erythropoiesis ($P < 0{\cdot}001$). This indicates that stage-4 AOV endoderm stimulates mesodermal erythropoiesis, in agreement with Wilt (1965), Miura & Wilt (1969) and Kessel & Fabian (1986). In explants composed of all three germinal layers (stage-4 AOV ectoderm-plus-mesoderm-plus-endoderm; explant type 4), the degree of Hb production was comparable with that of stage-4 AOV mesoderm-plus-endoderm (explant type 3), being significantly greater than that of stage-4 AOV ectoderm-plus-mesoderm in the absence of endoderm (explant type 2) ($P < 0{\cdot}001$). This suggests that the stimulatory influence of stage-4 AOV endoderm on mesodermal erythropoiesis overshadows the inhibitory influence of stage-4 AOV ectoderm. Moreover, in contrast to the tendency of stage-4 AOV ectoderm-plus-mesoderm explants (explant type 2) to spread out and become flattened in culture, it was found that stage-4 AOV mesoderm cultured alone (explant type 1), cocultured with stage-4 AOV endoderm (explant type 3) or cocultured with stage-4 AOV ectoderm and endoderm (explant type 4) exhibited the tendency to form solid, rounded aggregates of tissue (Fig. 1B).

Table 2. *Degrees of haemoglobin staining by the different types of explants*

Explant type	Total no. of cases	Haemoglobin staining: No. of cases in each staining class*					Haemoglobin staining: Percentage of total no. in each staining class*				
		−	−+	+	++	+++	−	−+	+	++	+++
1. meso (AOV, st. 4)	28	0	1	5	14	8	0·0	3·6	17·9	50·0	28·6
2. ecto + meso (AOV, st 4)	20	7	6	4	3	0	35·0	30·0	20·0	15·0	0·0
3. meso + endo (AOV, st. 4)	22	0	0	1	3	18	0·0	0·0	4·5	13·6	81·8
4. ecto + meso + endo (AOV, st. 4)	19	0	0	0	3	16	0·0	0·0	0·0	15·8	84·2
5. meso (AP, st 4)	22	0	4	7	8	3	0·0	18·2	31·8	36·4	13·6
6. ecto + meso (AP, st. 4)	23	7	4	5	7	0	30·4	17·4	21·8	30·4	0·0
7. ecto + meso + endo (AP, st. 4)	19	3	3	7	6	0	15·8	15·8	36·8	31·6	0·0
8. meso (AP, st. 4) + endo (AOV, st. 4)	20	0	0	1	4	15	0·0	0·0	5·0	20·0	75·0
9. endo (AOV, st. 4)	21	21	0	0	0	0	100·0	0·0	0·0	0·0	0·0
10. ecto + meso + endo (AOV, st. 8–9)	96	0	0	0	0	96	0·0	0·0	0·0	0·0	100·0

* See Table 1 for definitions of staining classes.

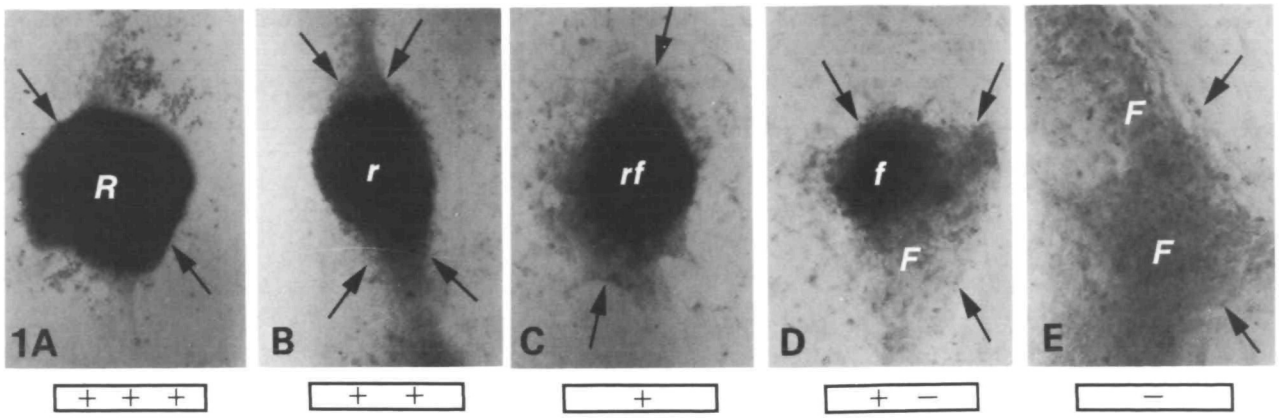

Fig. 1. Sequence of low-power views showing the varying appearances of explants after 72 h of culture on coverslips. The explants ranged in appearance from a solid, rounded aggregate of tissue (*R*) (A), through explants that became progressively less rounded (*r*) (B), to those that tended to become more flattened (*rf*, *f*) (C,D), to the extreme case in which the cells of the explant has spread out to form a flat sheet over the surface of the coverslip (*F*) (D,E). Arrows delimit the boundaries of the explants. The degree of flattening of the explants is correlated with the intensity of Hb staining (see Table 1 for definitions of the five classes of Hb staining). ×117.

Stage-4 AP explants

The extent of Hb production of stage-4 AP mesoderm cultured alone (explant type 5) was significantly greater than that of stage-4 AP ectoderm-plus-mesoderm (explant type 6) ($P < 0{\cdot}01$). This indicates that stage-4 AP ectoderm (like stage-4 AOV ectoderm) has an inhibitory effect on mesodermal erythropoiesis. Again, the inhibitory effect was coupled with the tendency of the ectoderm-plus-mesoderm explants (explant type 6) to spread out and become flattened over the surface of the coverslips (Fig. 1A).

When all three germinal layers of the stage-4 AP were cultured together (explant type 7), the degree of Hb production was not significantly different from that of stage-4 AP ectoderm-plus-mesoderm in the absence of endoderm (explant type 6). Moreover, these explants likewise exhibited the tendency to spread out and become flattened over the surface of the coverslips. It would appear that stage-4 AP endoderm does not counteract either the negative influence of stage-4 AP ectoderm on mesodermal erythropoiesis or the tendency of the explants to spread out and flatten.

On the other hand, when stage-4 AP mesoderm was cocultured with stage-4 AOV endoderm (explant type 8), there was a significant enhancement in Hb production ($P < 0{\cdot}001$): The erythropoietic differentiation of stage-4 AP mesoderm cocultured with stage-4 AOV endoderm was comparable with that of stage-4 AOV mesoderm cocultured with stage-4 AOV endoderm (explant type 3). Coupled with this, these explants did not spread out and become flattened, but formed solid, rounded aggregates of tissue (Fig. 1B).

Control explants

To verify that the isolated stage-4 AOV endoderm was not contaminated with erythropoietic mesodermal cells from the AOV, explants composed of stage-4 AOV endoderm were cultured and monitored for Hb production (explant type 9). None of these endodermal explants exhibited Hb staining (Table 2). Wilt (1965), Miura & Wilt (1969) and Kessel & Fabian (1986) have previously reported that stage-4 AOV endoderm can be separated from the overlying mesoderm and ectoderm without AOV mesodermal cells adhering to it; the mesodermal cells adhere to the ectoderm.

Explants excised from a potentially erythropoietic part of the blastoderm (stage-8 to -9 AOV ectoderm-plus-mesoderm-plus-endoderm) were routinely cultured to ensure that the culturing procedures and *o*-dianisidine staining techniques were always optimal (explant type 10). These explants were always strongly positive for Hb, scoring +++ on our rating system (Table 2).

Discussion

Dorsoventral pattern

The stimulatory influence of stage-4 AOV endoderm and the inhibitory effect of stage-4 AOV ectoderm on mesodermal erythropoiesis that was observed in this *in vitro* study, would seem to be consistent with the dorsoventral distribution of the components of the developing yolk sac vascular system during normal development (see Kessel & Fabian, 1985). The yolk sac blood islands develop in close association with the AOV endoderm, making direct physical contact by means of cellular processes (Mato, Aikawa & Kishi,

1964; Kessel & Fabian, 1985). They show no such association with the ectoderm, becoming physically separated from it by the developing extraembryonic coelom (see Kessel & Fabian, 1985), the parietal coelomic lining underlying the ectoderm, the visceral coelomic lining covering the blood islands and endoderm. This compartmentalization of the developing blood system next to the endoderm and its segregation from the ectoderm, is in developmental harmony with the *in vitro* stimulatory and inhibitory effects of the AOV endoderm and ectoderm, respectively.

Our observations of the tendency of stage-4 AOV ectoderm-plus-mesoderm explants to spread out and flatten, and of their tendency not to spread when they are cocultured with stage-4 AOV endoderm also reflects the dorsoventral patterning. The AOV ectoderm is the cellular layer under which the mesodermal cells spread out and against which they form the flattened, sheet-like coelomic lining; the AOV endoderm is the cellular layer against which the prospective blood island cells aggregate (reviewed in Kessel & Fabian, 1985). Whether aggregation is a consequence of the nature of the endodermal surface, or is due to an aggregation factor emitted by the endodermal layer, has not yet been reported. It may well be that the observed inhibitory influence of the AOV ectoderm on mesodermal erythropoiesis is related to the tendency of the AOV mesoderm, in the absence of endoderm, to spread out below the ectoderm rather than to aggregate into erythropoietic blood islands.

That the local epithelium may exert an effect on the extent of mesodermal cell spreading has been observed in other tissues. For example, in transplantation experiments of Fisher & Solursh (1979), it was found that the normally nonmigratory limb mesenchyme may spread like sclerotome when placed in the somite region. Conversely, somites placed in the limb lose their epithelial organization and become organized into the typical limb-like pattern of dispersed cells surrounding a central condensed core of cells. With reference to the mesodermal cells of the chick yolk sac, transmission and scanning electron microscopic studies indicate that the ectodermal basement membrane may serve as a substratum for the spreading of these cells (Trelstad, Hay & Revel, 1967; Hay, 1968; Wakely & England, 1979). This agrees with our observations and those of Wilt (1965) and Miura & Wilt (1969) that, during the dissection of the stage-4 blastoderm, the mesodermal cells adhere to the ectoderm.

Proximodistal pattern

The inhibitory influence of the ectoderm and endoderm from the AP, and the stimulatory influence of the endoderm from the AOV on mesodermal erythropoiesis *in vitro*, are in developmental harmony with the normal proximodistal pattern in the production of blood cells (see Kessel & Fabian, 1985). The mesoderm migrating through the AP of stage-4 blastoderms is destined for the AOV. This study shows that this AP mesoderm is already potentially erythropoietic. However, during normal development it does not halt its migration in the AP, but continues to migrate into the AOV, where it differentiates erythropoietically. As it passes through the AP, the mesoderm spreads between the AP ectoderm and endoderm (see Kessel & Fabian, 1985). We find that the ectoderm and endoderm of the AP exert an inhibitory influence on the erythropoietic differentiation of stage-4 AP mesoderm, whereas the AOV endoderm stimulates this mesoderm to realize its full erythropoietic differentiation. The inhibitory influence of stage-4 AP ectoderm and endoderm is associated with the tendency of stage-4 AP ectoderm-plus-mesoderm-plus-endoderm explants to spread out and become flattened in culture. Once again, it may well be that the observed inhibitory effects of stage-4 AP ectoderm and endoderm on mesodermal erythropoiesis is related to the tendency of stage-4 AP mesoderm to spread out between these layers rather than to aggregate against them.

The observed inhibitory influence of AP ectoderm and endoderm and stimulatory influence of AOV endoderm on mesodermal erythropoiesis *in vitro* may provide an explanation for the observation from our previous grafting experiment (Kessel & Fabian, 1986) in which we found that the normally nonerythropoietic stage-8 to -9 AP extraembryonic mesoderm can give rise to blood cells when transplanted into the AOV.

Cell shape and cytodifferentiation

One implication of the above findings is the idea that the *in vitro* inhibitory and stimulatory effects on mesodermal erythropoiesis of ectoderm and endoderm from the AP and AOV may be mediated by their different effects on the shape of the mesodermal cells. The literature contains a number of *in vitro* studies that report a relationship between cell shape and gene expression (e.g. Benya & Shaffer, 1982; Solursh, Linsenmayer & Jensen, 1982; Glowacki, Trepman & Folkman, 1983; Greenburg & Hay, 1982, 1986). For example, it has been demonstrated that chondrocytes express their normal biochemical differentiation *in vitro* when in a rounded form (Benya & Shaffer, 1982; Solursh *et al.* 1982; Glowacki *et al.* 1983). 'Dedifferentiated' chondrocytes can be induced to redifferentiate when the cells become rounded by suspension in agarose (Benya & Shaffer, 1982). Likewise, embryonic chick lens epithelial cells

express lens cytodifferentiation *in vitro* when cultured on a medium that enables them to assume a cuboidal morphology (Greenburg & Hay, 1982, 1986). When cultured in a three-dimensional matrix, the explants give rise to mesenchyme-like cells that are migratory and that fail to express lens cytodifferentiation (Greenburg & Hay, 1982, 1986).

Finally, the issues raised above could be extended to suggest that the artificial conditions of tissue culture which we used may have led to artifactual changes of cell behaviour and the misinterpretation of our results. We believe this not to be the case, as our present observations fit in well with the developmental patterns observed *in vivo*, as mentioned above (see Kessel & Fabian, 1985). We are presently undertaking an SEM study to further clarify the relationship between cell shape and phenotypic expression (erythropoiesis) in the extraembryonic mesodermal cells of the early chick blastoderm.

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