Hepatic induction in the avian embryo: Specificity of reactive endoderm and inductive mesoderm

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SUMMARY

Mesoderm of precardiac and cardiac region (‘cardiac’ mesoderm) of chick, quail and mouse embryos could induce hepatic epithelium in the endoderm of the anterior half of young quail or chick embryos (anterior endoderm) in vitro as well as in vivo. No species specificity in the induction of hepatic epithelium by the ‘cardiac’ mesoderm could be observed.

The hepatic induction, was controlled strictly by tissue specificity of both endoderm and mesoderm. Replacement of the ‘cardiac’ mesoderm or the anterior endoderm by non-cardiac mesoderms or endoderms other than the anterior endoderm resulted in failure of hepatic induction. Only the anterior endoderm was found to have competence for hepatic induction, indicating that it was committed, in unknown ways, to react with ‘cardiac’ mesoderm, and can properly be called pre-hepatic endoderm.

Comparison between the development of hepatic endoderm and the hepatic induction potency of ‘cardiac’ mesoderm, which was most intense during 1- to 1.5- incubation days and decreased gradually with the increase of the stage, suggests that in normal development the ‘cardiac’ mesoderm actually induces hepatic epithelium in the competent endoderm. Hepatic-induction potency remained up to 6 days, and was found in truncus arteriosus, ventricle and auricle areas and in endocardial and myocardial layers of the heart.

INTRODUCTION

When fragments of blastoderms cut from definitive-streak- to head-fold-stage embryos are transplanted onto the chorioallantoic membrane (Hunt, 1931, 1932; Willier & Rawles, 1931; Dalton, 1935; Rawles, 1936) or into the hepatic mesenchymal region (Le Douarin, 1964a, b), hepatic epithelium differentiates, accompanied by cardiac tissue. From these studies, it has been suggested that differentiation of the hepatic epithelium may be induced by the heart rudiment. We have previously investigated the hepatic inducing effect of the mesoderm of precardiac and cardiac region (‘cardiac’ mesoderm), and confirmed that ‘cardiac’ mesoderm of head-fold to 11-somite stage can induce hepatic epi-

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thelium in the endoderm, in coelomic implantation or chorioallantoic membrane grafts (Fukuda, 1979).

In the present investigation, we first examined whether cardiac mesoderm can induce hepatic epithelium in vitro. Secondly, we examined the tissue and species specificity of the inductive mesoderm and reactive endoderm. Thirdly, to clarify the role of the hepatic induction potency of 'cardiac' mesoderm in normal development, chronological changes in the induction potency of the 'cardiac' mesoderm were analyzed and compared with the development of hepatogenic potency in the endoderm. Lastly, we attempted to define the population responsible for hepatic induction by analyzing regional and tissue differences in hepatic induction potency of the 'cardiac' mesoderm and heart.

MATERIALS AND METHODS

Embryos

Japanese quail (*Coturnix coturnix japonica*), White Leghorn (*Gallus gallus domesticus*) chicken and ICR strain mouse embryos were used. Eggs were incubated at 38 °C. The conceptus was considered as 0.5 days old at 12.00 h of the day when the vaginal plug was found.

Experimental procedures

Isolation of tissue fragments

Endodermal and mesodermal tissue fragments were isolated with collagenase (Worthington, CLSPA, 0.03 % in Tyrode’s solution for 70 min at 37 °C), if necessary. After separation, endodermal and mesodermal tissue fragments were washed thoroughly in three changes of serum-supplemented Tyrode’s solution and finally in fresh Tyrode’s solution. Then, endodermal tissues of quail embryos were associated with mesodermal tissues of chick or mouse embryos throughout the experiments to exclude cellular contamination of either of the tissue components (Le Douarin, 1969).

Culture and graft

Endodermal and mesodermal tissue fragments were associated on semi-solid agar medium after Wolff & Haffen (1952). The medium consisted of seven parts of 1 % Difco Bacto-Agar in Gey’s solution, three parts of foetal bovine serum (Flow Laboratories Ltd), three parts of medium 199 and one part of Tyrode’s solution containing potassium penicillin G. After cultivation for 1 day, explants were subjected to subsequent cultivation in vivo for 6–8 days in the coelomic cavity (C) of 3-day chick embryos or in vitro for 6–7 days in medium 199 containing 10 % foetal bovine serum, using the Millipore filter roller-tube (MR) method (Sugimoto & Endo, 1969).
The following experiments were performed.

(1) Induction of hepatic epithelium by ‘cardiac’ mesoderm in vitro. Endodermal fragments isolated from anterior half (anterior to the posterior end of the Hensen’s node or of the first somite) of quail embryos of the definitive-streak to 1-somite stage (we call them anterior endoderm for convenience) were used as a responding system throughout the experiments. The areas of secure adhesion between the endodermal layer and overlying tissues in the area of the primitive-streak and the head-process were excluded. Anterior endodermal fragments of these stages were shown to express no hepatogenic potency when explanted alone in the coelomic cavity (Fukuda, 1979). ‘Cardiac’ mesoderm used as an inductive tissue was taken from the precardiac and cardiac region of 1-5-day (4- to 11-somite stage) chick embryos. Anterior endodermal fragments and ‘cardiac’ mesoderm were then associated and cultivated in vivo or in vitro (Fig. 1).

(2) To clarify mesodermal specificity in hepatic epithelial induction, mesoderms such as the cardiac mesoderm of 8.5- to 11.5-day mouse embryo, posterior non-cardiac mesoderm of 1-5-day chick and 8.5- to 9.5-day mouse embryos, 4-day chick forelimb bud and gizzard mesenchymes, and endothelium of 6- to 10-day chick vitelline blood vessels were used instead of the chick ‘cardiac’ mesoderm of experiment (1). Explants were cultured in vivo or in vitro (Fig. 1).
(3) To clarify endodermal specificity in hepatic epithelial induction, the anterior endoderm of experiment (1) was replaced by posterior endoderm of the area pellucida, endoderm of four regions of area opaca (anterior, posterior, right and left as seen in Fig. 2) of definitive-streak to head-fold stage, or allantoic endoderm of the 3-day quail embryo (Fig. 2).

In this series of experiments, the culture period was extended to 14 or 15 days, in case heterotypic differentiation of the epithelium needed a longer culture period.

(4) Chronological changes in hepatic induction potency of the ‘cardiac’ mesoderm and heart isolated from chick embryos. ‘Cardiac’ mesoderm was isolated from the pre-cardiac (Rudnick, 1938; Rawles, 1943; Rosenquist & DeHaan, 1966; Stalsberg & DeHaan, 1969) and cardiac region of 1- (definitive-streak to head-fold stages) and 1-5-day (4- to 11-somite stages) embryos. From 2- (stages 11–14, Hamburger & Hamilton, 1951), 2-5- (stage 15–17) and 3-day (stage 18) embryos, tubular heart was isolated and opened. The anterior endoderm was placed on the endothelial side of the heart.

(5) Hepatic induction potency of various areas of the heart. Anterior and posterior areas of the ‘cardiac’ mesoderm and heart were isolated from 1- to 2-5-day chick embryos. From the heart of chick embryos older than 3 days, three distinct areas, truncus arteriosus, ventricle and auricle were cut out and opened. Anterior endodermal fragments were recombined with ‘cardiac’ mesoderm or the heart fragments.
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(6) Hepatic induction potency of the two layers of the heart. The hepatic induction potency of the endocardial and myocardial layers of the heart was studied. From the truncus arteriosus area of 3- to 5-day embryonic chick heart, inner and outer layers were isolated mechanically. The inner layer of the heart of this area is mainly composed of endocardium and cardiac jelly. The outer layer of the heart of this area is mainly composed of mesenchyme differentiating into myocardium and a small amount of cardiac jelly. The anterior endoderm was associated with the inner or outer layer of the heart. The anterior endoderm was also associated with the outer surface of unopened or the inner surface of opened 3-day chick whole heart.

Histological methods

Grafts and explants were fixed in Bouin’s fluid, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin, or fixed in Gendre’s fluid and sections stained with PAS-haematoxylin to detect glycogen.

Criteria for hepatic epithelial differentiation

The differentiation of hepatic epithelium was identified by the formation of bile canaliculi characteristically bordered by several hepatic cells, and of hepatic cords composed of two rows of hepatocytes, with large eosinophilic cytoplasm containing PAS-positive glycogen granules.

RESULTS

(1) Induction of hepatic epithelium by ‘cardiac’ mesoderm in vitro

When quail anterior endodermal fragments, which are unable to differentiate into hepatic epithelium alone, were cultured in vitro or in vivo in association with chick ‘cardiac’ mesoderm, quail hepatic epithelium with hepatic cords and bile canaliculi differentiated (Figs. 3, 4). Glycogen granules were often present in the cytoplasm of the hepatic cells. However, hepatic sinusoids never differentiated. Besides the quail hepatic epithelium, chick cardiac tissue often developed.

Sequential observations of the induction of hepatic epithelium revealed that the endodermal cells lose their orientation on culture day 1, form tubular structures on day 2, show the first signs of hepatic differentiation on day 3, and hepatic epithelial differentiation on day 4.

(2) Mesodermal specificity in the induction of hepatic epithelium

(a) Regional specificity

In contrast with the effects of association with ‘cardiac’ mesoderm (experiment (1)), hepatic epithelium was minimally induced in anterior endoderm associated with non-cardiac mesoderms such as posterior, limb bud, gizzard or endothelial, regardless of culture methods (Fig. 3). Mostly the endoderm
Origin of the mesoderm (Culture method) | % hepatic induction (no. of observations)
--- | ---
Chick 'cardiac' | (C) | 86 (28)
Mouse 'cardiac' | (MR) | 70 (30)
Chick posterior | (C) | 15 (27)
Mouse posterior | (C) | 7 (14)
Chick limb-bud | (C) | 0 (12)
Chick gizzard | (C) | 0 (16)
Chick endothelium | (C) | 0 (16)

**Fig. 3.** Induction of hepatic epithelium by 'cardiac' and non-cardiac mesoderms. Anterior endoderm of quail embryos of the definitive-streak to the 1-somite stage was used as the responding tissue. After association of the anterior endoderm with 'cardiac' or non-cardiac mesoderm of chick or mouse, the explants were cultivated in the coelomic cavity (C), or by the Millipore filter, roller-tube method (MR).

remained undifferentiated. From these results it can be concluded that only 'cardiac' mesoderm possesses the region-specific ability to induce hepatic epithelium in the anterior endoderm.

(b) **Species specificity**

Mouse 'cardiac' mesoderm could induce hepatic epithelium in the anterior endoderm (Fig. 5), though the incidence of the induction was lower than with chick 'cardiac' mesoderm (Fig. 3). Therefore, the ability of 'cardiac' mesoderm to induce hepatic epithelium was not species specific.

Association of the endoderm with mouse posterior, non-cardiac mesoderm scarcely induced hepatic epithelium (Fig. 3).

(3) **Regional specificity of endoderm to the stimulus of the 'cardiac' mesoderm**

When endoderm other than the anterior endoderm was associated with 1- to 1.5-day 'cardiac' mesoderm possessing intense hepatic inducing potency (Fig. 2), hepatic epithelium was minimally induced regardless of the origin of the endoderm, culture methods, and culture periods (Fig. 6). These results indicate the existence of specific endoderm possessing reactivity to 'cardiac' mesoderm. This endoderm is termed 'pre-hepatic' endoderm.

If non-cardiac or hepatic mesenchyme was associated with non-anterior endoderms, no hepatic tissue was induced (Fig. 6).

Consequently it can be concluded that the induction of hepatic epithelium
requires strict specificity of both endoderm and mesoderm: The capacity to
induce hepatic epithelium which is specific to the 'cardiac' mesoderm, and the
endodermal reactivity which is restricted to the anterior half of the endoderm.

(4) **Chronological changes in the hepatic induction potency**
**of the 'cardiac' mesoderm**

Results are summarized in Fig. 7. The hepatic induction potency of the
chick 'cardiac' mesoderm was most intense at 1 and 1.5 days. The incidence
of hepatic epithelial induction by these stages of mesoderm was 85 and 94 %,
respectively. With increase of stage, the incidence decreased gradually, and it
fell below 10 % at 8 or 10 days. However, it was noted that the heart still
possessed limited hepatic induction potency after its own differentiation.
Mesoderm | Responding endoderm | % hepatic induction (no. of observations)
--- | --- | ---
Anterior | (C) | 0
Allantoic | (C) | 0* (18)
Area opaca | (C) | 0* (24)
Posterior | (C) | 0* (4)
(MR) | 0 (4)
‘Cardiac’ (1-5-day) | Area opaca | 3 (80)
Posterior | (C) | 0 (26)
‘Cardiac’ (1-day) | Area opaca | 9 (11)
Posterior | (C) | 0 (13)
‘Cardiac’ (1-+ 1.5-day) | Area opaca | 0 (12)
Posterior | (C) | 0 (2)
(MR) | 0 (2)
Non-cardiac posterior | Area opaca | 0 (7)
Posterior | (C) | 0 (6)
(MR) | 0 (3)
Hepatic | Allantoic | 0 (29)

Fig. 6. Hepatic induction from the allantoic, area opaca and posterior endoderms by the ‘cardiac’ mesoderm of 1- to 1.5-day embryos. For controls, non-cardiac mesoderm of posterior region or hepatic primordial mesenchyme was used. Culture periods: 6-9 days; *, 14-15 days. Culture methods: C, coelomic implantation; MR, Millipore filter roller-tube.

(5) Hepatic induction potency of various areas of the heart

Results are summarized in Table 1. There were no significant differences between hepatic induction potency of anterior and posterior areas of 1- to 2.5-day ‘cardiac’ mesoderm, when examined by \( \chi^2 \) test. Hepatic induction potency was also found in truncus arteriosus, ventricle and auricle areas of the heart in later stages. With increase of stage, the hepatic induction potency of each area of the heart decreased equally.

(6) Hepatic induction potency of the two layers of the heart

The hepatic induction potency of endocardial and myocardial layers of the chick heart were examined and the results are summarized in Table 2.
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In each type of recombination hepatic epithelium was induced. No significant differences in the incidence of hepatic induction between inner and outer layers of the heart were observed at any stage ($\chi^2$ test). Their hepatic induction potency was as high as that of the whole heart or the whole truncus arteriosus area at the same stage.

DISCUSSION

Endodermal and mesodermal specificity in hepatic induction

In previous (Fukuda, 1979) and present studies, we demonstrated that endoderm younger than the 2-somite stage cannot differentiate into hepatic epithelium by itself, but the ‘cardiac’ mesoderm can induce hepatic epithelium in the anterior endoderm at high frequency in both in vivo and in vitro cultures. The liver-inducing potency was found to be restricted to ‘cardiac’ mesoderm. Mesenchymes other than the ‘cardiac’ mesoderm induce hardly any hepatic epithelium.

Induction of hepatic epithelium takes place even in heteroplastic (quail...
Table 1. Hepatic differentiation in quail endoderm under the influence of various areas and stages of the chick heart
(The ratio indicates number of explants showing hepatic epithelial differentiation relative to the total number of explants.)

<table>
<thead>
<tr>
<th>Areas of the 'cardiac' mesoderm and the heart</th>
<th>Stages of the heart (incubation days)</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>'Cardiac' mesoderm</td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>8/9</td>
</tr>
<tr>
<td>Posterior</td>
<td>9/11</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
</tr>
<tr>
<td>Truncus arteriosus</td>
<td>—</td>
</tr>
<tr>
<td>Ventricle</td>
<td>—</td>
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<tr>
<td>Auricle</td>
<td>—</td>
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</table>
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Table 2. Hepatic induction in the quail anterior endoderm (E) by inner (I) or outer (O) layer of the chick whole heart (3 day) or truncus arteriosus area (3–5 day)

(The ratio indicates the number of explants showing hepatic epithelial differentiation relative to the total number of explants.)

<table>
<thead>
<tr>
<th>Stages of the heart</th>
<th>Modes of association with heart layers</th>
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<tbody>
<tr>
<td></td>
<td>E</td>
</tr>
<tr>
<td>3 day</td>
<td>9/14</td>
</tr>
<tr>
<td>4 day</td>
<td>—</td>
</tr>
<tr>
<td>5 day</td>
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—, Not examined.

endoderm–chick mesoderm) or interclass (quail endoderm–mouse mesoderm) combinations. Our experimental data also show that in the reverse heteroplastic combination (chick endoderm–quail mesoderm), hepatic epithelium was induced in seven cases out of eight. These results indicate that a common mechanism exists in hepatic epithelial induction among these three kinds of animals.

In the present investigation, hepatic epithelium did not differentiate from allantoic, area opaca, or embryonic posterior endoderm when cultured in combination with the inductively active ‘cardiac’ mesoderm, even if the culture period was prolonged up to 2 weeks. Since the allantoic endoderm is known to be able to differentiate heterotypically (Mizuno & Yasugi, 1973; Yasugi & Mizuno, 1974), these results indicate that the inducing potency of the ‘cardiac’ mesoderm is limited and only the anterior endoderm is competent for the induction of hepatic epithelium.

In contrast, the endoderm and mesenchyme of the embryonic digestive tract possess extensive reactivity and induction potency, respectively. It has been known that, when they are recombined heterotypically, the endoderm can be affected by heterotypic mesenchymes (Sigot & Marin, 1970; Yasugi & Mizuno, 1978), and the digestive-tract mesenchyme can induce heterotypic epithelia from the ureter (Bishop-Calame, 1966), from the bronchial epithelium (Dameron, 1968), and from the allantoic endoderm (Mizuno & Yasugi, 1973; Yasugi & Mizuno, 1974).

During organogenesis of the digestive tract, it has been suggested (Mizuno, 1975) that in early embryonic stages, undifferentiated endoderm is roughly determined and, later, the mesoderm of the embryonic digestive tract exerts its regionally specific action on cytodifferentiation and morphogenesis. In the present investigation, it was found that during development of hepatic epithelium, the anterior endoderm is committed, and that later the ‘cardiac’
mesoderm acts with the competent endoderm and induces hepatic differentiation.

The development of self-differentiation potency in the hepatic endoderm has been investigated extensively in the chick embryo, in an in vitro system with isolated endoderm enveloped in vitelline membrane (Sumiya, 1976; Mizuno & Sumiya, 1977). Hepatic epithelium developed from ventral foregut endoderm after the 7-somite stage when this was cultured alone. However, by intracoelomic transplantation of quail embryo endodermal fragments we could demonstrate that they can self-differentiate from the 2-somite stage (Fukuda, 1979). In the present investigation, it was shown that anterior endoderm younger than the 2-somite stage has no hepatic self-differentiation potency but has been committed to be able to react to the inductive stimulus of the 'cardiac' mesoderm.

Le Douarin (1964a, b) called the endoderm at early stages 'pre-hepatic endoderm' in the sense that it is not yet determined to be hepatic. We restrict this terminology to the endoderm which can react to cardiac mesoderm and differentiate into the hepatic epithelium.

Comparison between the development of hepatogenic potency in the endoderm and chronological changes in hepatic induction potency of the cardiac mesoderm

In the present investigation, stage-dependent changes in the hepatic induction potency of the 'cardiac' mesoderm were demonstrated. It was the highest at 1 to 1.5 days (from the definitive-streak to 11-somite stages) and then fell gradually with advancing stage. It must be noted that the ability to induce hepatic epithelium has already appeared in precardiac mesoderm at the definitive-streak stage. Since the self-differentiation potency of the precardiac mesoderm is known to appear at the late medium- (Chacko & Rosenquist, 1974) or definitive-streak (Fukuda, unpublished data) or head-process stage (Le Douarin, Le Douarin & Cuminge, 1965; Renaud & Le Douarin, 1968), hepatic induction potency seems to appear in the precardiac mesoderm as soon as it acquires self-differentiation potential.

In a previous paper we showed that hepatogenic potency first appears in the endoderm during the 2- to 5-somite stage, indicated by a low incidence of hepatic differentiation when the endoderm is cultured alone or with heterologous mesenchymes (Fukuda, 1979). The hepatogenic potency is then gradually stabilized during hepatic morphogenesis (about 2.5 days) and is quite stable by 4 days, since, at this stage, the hepatic epithelium does not show any sign of differentiation into any other type of epithelium even under the influence of inductively active digestive-tract mesenchymes (5- or 6-days oesophageal, proventricular, gizzard and small intestinal mesenchymes) (Fukuda, unpublished data). Thus, as shown in Fig. 7, increasing hepatogenic potency in the anterior endoderm is inversely related to changes in hepatic induction potency of the
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precordial and cardiac mesoderm. Since precardiac and cardiac mesoderm is in contact with the anterior endoderm throughout early developmental stages and the anterior endoderm is the only competent endoderm for hepatic induction, it is strongly suggested that the precardiac and cardiac mesoderm induce hepatic epithelium in the anterior endoderm \textit{in vivo} during normal development.

Though the ‘cardiac’ mesoderm gradually loses its hepatic induction potency during its differentiation into cardiac tissue, the heart maintains hepatic induction potency up to at least 6 days. However, at these later developmental stages, the heart is so separated from the hepatic endoderm that it is virtually impossible for it to exert an inductive action directly on the endoderm.

\textit{Hepatic induction potency of various areas and tissues of the heart}

Hepatic induction potency was found in all areas examined (anterior, posterior, truncus arteriosus, ventricle and auricle) and layers (endocardium and myocardium) of the chick ‘cardiac’ mesoderm and heart. If the ‘cardiac’ mesoderm contains diverse cell populations which differentiate into truncus arteriosus, ventricle, auricle, endocardium or myocardium, all these cell populations must have hepatic induction potency. It is also possible that undifferentiated cells possessing hepatic induction potency are still present in the differentiated heart tissues, and that these cells keep their hepatic induction potency to fairly late embryonic stages. Le Douarin (1964a, b) has suggested that the hepatic mesenchyme originates from the precardiac mesodermal region. However, the hepatic mesenchyme isolated experimentally by insertion of an obstacle in the hepatic mesenchymal region could not induce hepatic epithelium (Le Douarin, 1964a, b). In the present study, we showed that the ‘cardiac’ mesoderm can induce hepatic epithelium and that this induction potency is quite tissue specific but not species specific. Properties specific to cardiac tissue, but common to cell populations of various areas and layers of the ‘cardiac’ mesoderm and heart must be responsible for the induction of hepatic epithelium.

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