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Intestinalization of the area-vitellina endoderm cultured in association with digestive-tract mesenchymes

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SUMMARY

Small-intestine-type differentiation of the area-vitellina endoderm of quail embryos, cultured in association with digestive-tract mesenchymes of chick embryos, was analysed chronologically with special attention to the appearance of a striated border and its enzymes (alkaline phosphatase and α-glucosidase). Undifferentiated endoderm of the area vitellina differentiated first into yolk-sac parenchyma expressing cysteine lyase, then gradually lost the cysteine lyase activity, and eventually manifested small intestine-type differentiation with the expression of the striated border and its enzymes.

INTRODUCTION

A previous study (Masui, 1981*a*) demonstrated that endoderm of the area vitellina can self-differentiate *in vitro* into yolk-sac parenchyma according to its own presumptive fate, in the absence of mesenchyme. In contrast, when the endoderm was cultured *in vitro* in association with digestive-tract mesenchymes, all the endodermal cells developed transiently into yolk-sac parenchyma. Then, basophilic cells appeared among them, and these cells differentiated morphologically into mesenchyme-specific epithelia as well as small intestine-type epithelium (Masui, 1981*a*, *b*; Mizuno & Masui, 1982). However, in these studies the intestinalization was judged mainly by histological criteria. It has been reported that striated border of the small intestine possesses enzymes, such as alkaline phosphatase (ALP) and neutral α-glucosidase (α-GLD) (Pearse, 1968; Fishman, 1974; Gossrau, 1976), and that cysteine lyase (CL) is expressed in the differentiated yolk-sac endoderm of the chick embryo (Chapeville & Fromageot, 1967; Bennett, Dubois & Chapeville, 1972; Bennett, 1973).

In the present investigation, the intestinalization of the area-vitellina endoderm under the influence of digestive-tract mesenchymes was investigated with special attention to the appearance of the activities of ALP, α-GLD, and CL. Striated border was also examined by the electron microscopy.

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MATERIALS AND METHODS

Embryos, the isolation of tissue fragments, and organ culture methods for the tissue fragments were the same as those described in the previous paper (Masui, 1981*a*). The 3- and 5-day area-vitellina endoderm of quail embryo was cultured for 2–35 days in combination with digestive-tract mesenchymes of 5-day chick embryos. Quail and chick tissues can each be identified after they have developed (Le Douarin, 1969).

Histochemical methods

Samples were fixed in a cold 90% ethanol for 30 min to 1 h, and embedded in paraffin after rapid dehydration. Benzene was used as a paraffin solvent. Serial sections of explants were cut at 8 μm and mounted on several slides in serial order. Histochemical detection of enzyme activities was applied in parallel to these slides.

CL activity was visualized by the method of Bennett *et al.* (1972) on slides incubated for 1·5–3 h at 37 °C in a reaction mixture containing L-cysteine, 1 g; sodium sulphite, 750 mg; lead acetate, 250 mg; pyridoxal phosphate, 2 mg in Tris-HCl buffer (pH 8·5, 0·15 M) 500 ml. After CL activity was visualized, some sections were stained with haematoxylin-mucicarmine to examine the origin of tissues and the differentiation of goblet cells.

For detection of non-specific ALP, the method of Burstone (1962) was used. Sections were incubated for 30 min at 37 °C in a mixture of 4 mg naphthol AS-BI phosphate (Sigma, dissolved in 0·25 ml dimethyl sulphoxide) and 30 mg fast red violet LB salt (Sigma) in 50 ml 0·1 M Tris-HCl buffer, pH 9·0.

The detection of neutral (microvillous) α-GLD was performed by the method of Gossrau (1976). Sections were incubated for 1 h at 37 °C in a mixture of 12 mg β-naphthyl-α-D-glucoside (Sigma, dissolved in 0·5 ml *N*,*N*-dimethyl-formamide) and 0·6 ml hexazonium-*p*-rosanilline in 10 ml maleic buffer, pH 6·5.

For these histochemical procedures, control experiments were performed with substrate-free reaction mixtures.

Electron microscopy

For scanning electron microscopy (SEM), specimens were washed gently in Tyrode's solution to remove mucus on the epithelial surface. When the specimens were heavily covered with mucus, they were rinsed in 1 M-HCl with mild agitation (Lim & Low, 1977) for one min. They were fixed with 2·5% glutaraldehyde in 0·1 M-cacodylate buffer (pH 7·4) for 2 h at 4 °C and rinsed in 0·1 M-cacodylate buffer. They were then post-fixed with 1% OsO_4 in 0·1 M-cacodylate buffer (pH 7·4) for 2 h at 4 °C. The specimens were dehydrated through a graded series of alcohols and amylacetate, dried in a critical-point dryer with

CO_2, coated with gold in an ion coater, and examined with a Hitachi S-430 SEM.

For transmission electron microscopy (TEM) specimens were fixed in a modified Karnovsky's fixative (1·5% paraformaldehyde and 2% glutaraldehyde in 0·1 M-phosphate buffer, pH 7·4) for 1 h at room temperature. They were then rinsed in 0·1 M-phosphate buffer, pH 7·4 containing 0·22 M-sucrose at room temperature. They were post-fixed with 1% OsO_4 in 0·1 M-phosphate buffer, pH 7·4 for 1 h at 4 °C. Thereafter they were carried through a graded series of alcohols and embedded in Spurr's low-viscosity embedding medium (Spurr, 1969). Sections were cut on a Porter-Blum MT-2 microtome, stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12.

RESULTS

(1) *Appearance of CL activity during self differentiation and normal development of area-vitellina endoderm*

The endoderm of the area vitellina *in situ* consists of large cells swollen with yolk droplets and irregularly arranged in several layers, and exhibits no CL activity. When cultured directly on the medium, the isolated endoderm self-differentiated into typical yolk-sac parenchyma and expressed intense CL activity. However, the endoderm demonstrated neither ALP nor α-GLD during self differentiation or throughout normal development.

(2) *Appearance and disappearance of enzyme activities during small-intestine-type differentiation of area-vitellina endoderm*

Isolated quail endoderm of the area vitellina was cultured in association with 5-day chick digestive-tract mesenchymes, and enzyme activities in the endoderm were investigated chronologically. The total of 339 explants were examined. The results are summarized in Fig. 1, in which three types of endodermal differentiation were recognized by expression of enzyme activities: explants expressing CL activity alone, both CL and ALP activities, and ALP activity alone. The epithelial cells examined were derived from the quail.

Most of the endodermal cells of all explants became CL positive at the beginning of culture, and they differentiated into yolk-sac parenchyma. Thereafter, the percentage of explants exhibiting CL activity alone decreased rapidly.

After 10 days of cultivation, explants expressing CL and ALP activities in neighbouring sections appeared (Figs. 2A, B). The percentage of such explants increased during 2 and 3 weeks of cultivation and dropped considerably after 4 weeks. After 3 weeks of cultivation, explants expressing ALP activity alone appeared. The ALP activity was observed in the apical surface of the simple columnar epithelium.

We tested whether the simple columnar epithelium could also express

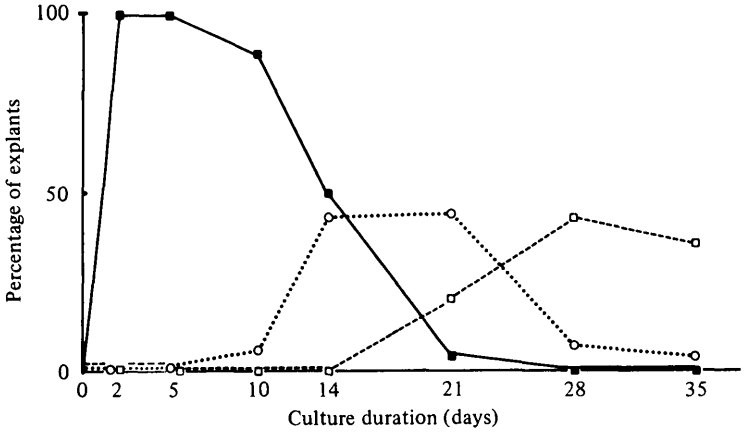

Fig. 1. Chronological changes in percentages of explants expressing CL alone (■——■), ALP alone (□-----□), and both CL and ALP (○···○) in area-vitellina endoderm cultured *in vitro* in association with digestive-tract mesenchymes.

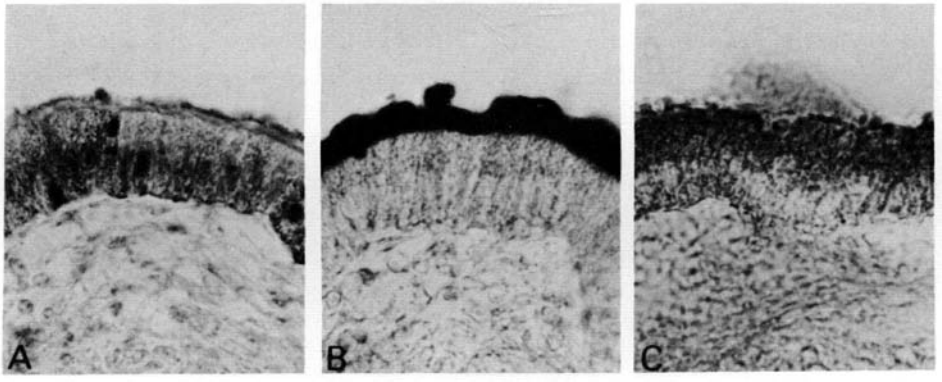

Fig. 2. Sections of an association of area-vitellina endoderm with proventricular mesenchyme, cultured *in vitro* for three weeks. A, B, and C are the neighbouring sections of the same explant. ×720. (A) CL activity in the epithelium. (B) ALP activity of apical surface of the epithelium. (C) α-GLD activity of apical area of the epithelium.

activity of α-GLD, and found that epithelium demonstrating α-GLD activity also expressed ALP activity (Figs. 2B, C).

(3) *Demonstration of striated border during intestinalization of area-vitellina endoderm*

TEM and SEM observations demonstrated that striated border developed in the free surface of the columnar cells which originated from area-vitellina endoderm cultured in association with digestive-tract mesenchymes for 3 weeks (Figs. 3A, B). Goblet cells also developed in epithelium in all types of recombinations.

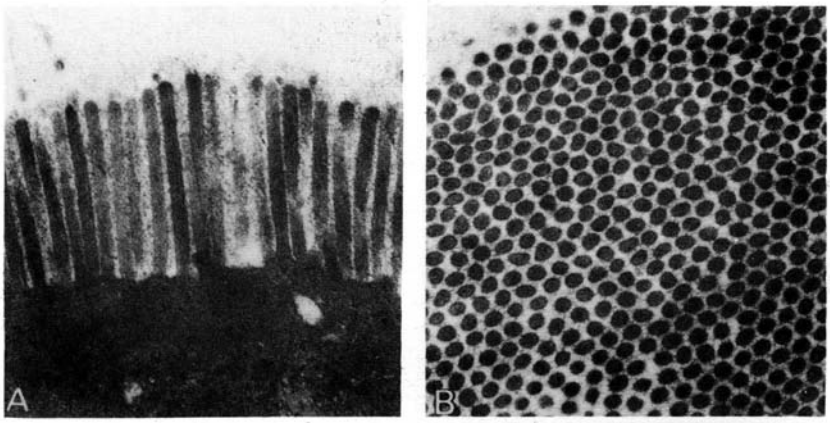

Fig. 3. Sections of the epithelium formed by combining area-vitellina endoderm and small intestinal mesenchyme, after culture *in vitro* for three weeks. TEM. ×20000. (A) Typical striated border developed on the surface of the endoderm. (B) A transverse section of the striated border.

DISCUSSION

The present study demonstrates that, when endoderm of the area vitellina is cultured in association with various mesenchymes of embryonic digestive tract, the endoderm differentiates into yolk-sac parenchyma which expresses CL activity at the beginning of the cultivation. This CL activity then decreases as cultivation proceeds. During cultivation, some areas of epithelium undergo morphological differentiation into mesenchyme-specific epithelia of digestive-tract type (Masui, 1981*a*, *b*; Mizuno & Masui, 1982). This intestinalization occurs in multiloci in the epithelium in some explants. The question arises, then, as to how intestinalization occurs in the area-vitellina endoderm under the influence of digestive-tract mesenchymes.

Intestinalization potency of area-vitellina endoderm

In normal development and self differentiation of yolk-sac endoderm, striated border and goblet cells are not observed (Bellairs, 1963; Bennett, 1973; Masui, 1978, 1981*a*, *b*; Mobbs & McMillan, 1979). The present study demonstrates that the activities of small intestinal enzymes (ALP and α-GLD) cannot be detected histochemically in yolk-sac endoderm during normal development or in area-vitellina endoderm cultured alone *in vitro*. However, when area-vitellina endoderm was cultured *in vitro* in association with various mesenchymes of digestive tract, striated border and goblet cells differentiated, and ALP and α-GLD activity appeared in the apical surface of the epithelium. Intestinalization of the area-vitellina endoderm, did not vary when recombined with different mesenchymes. These results suggest that area-vitellina endoderm has a tendency to differentiate into small intestine-type epithelium, and this is

realized when it is cultured for more than 3 weeks under non-organ-specific stimuli of various mesenchymes of embryonic digestive tract, though intestinalization is not expressed during normal development. Since the quail embryo normally hatches at 17 days and the yolk sac is absorbed, the culture duration of 35 days may seem to be highly aberrant. However, under the experimental conditions of the present study, the intestinalization occurred in the latter half of the culture period.

A similar situation has been found in the endoderm of the allantois, one of the embryonic membranes, which can differentiate towards intestinal epithelium in culture (Fell, 1954). Goblet cells, which do not differentiate in the endoderm during normal development of the allantoic epithelium, often appear under non-organ-specific stimuli of various mesenchymes of the digestive tract (Mizuno & Yasugi, 1973; Yasugi & Mizuno, 1974; Yasugi, 1976*a*, *b*, 1979; Gumpel-Pinot, Yasugi & Mizuno, 1978).

Expression of activities of specific enzymes in yolk-sac parenchyma and small intestine during the course of intestinalization

The sequence of intestinalization *in vitro* of the area-vitellina endoderm might include three phases of enzyme expression: yolk-sac parenchymal phase (CL activity alone), transitional phase (both CL and ALP activities), and small intestinal phase (ALP activity alone).

In the first phase the associated mesenchymes permit normal development of area-vitellina endoderm, since all cells of the endoderm can express CL activity. In normal development and self-differentiation experiments, only the yolk-sac parenchymal phase was observed.

In the second phase, digestive-tract mesenchymes do not support expression of CL activity, since the CL activity expressed in the endoderm decreases with time of cultivation. However, it is not known whether the mesenchymes inhibited the expression of CL activity. The present study also showed that CL activity is preferentially preserved in areas which express ALP and α-GLD, suggesting that favourable conditions for intestinalization of the endoderm may also be suitable for preservation of CL activity. In the second phase, some areas of the endoderm begin to differentiate morphologically into the mesenchyme-specific epithelium of digestive tract under the 'directive' influence of digestive-tract mesenchymes (Masui, 1981*a*, *b*; Mizuno & Masui, 1982), and some of the other areas come to express intestinal enzyme activities under 'non-organ-specific' stimuli of the digestive-tract mesenchymes.

In the third phase, expression of CL activity ceases and the endoderm differentiates into small intestine-type epithelium expressing ALP and α-GLD. Preliminary experiments (Masui & Matsushita, unpublished data) showed that an anti-serum against striated border of chick small intestine reacted with the apical surface of the area-vitellina endoderm which manifested intestinalization *in vitro* in association with digestive-tract mesenchymes.

From normal development and the self-differentiation experiments, it might be conceivable that the yolk-sac parenchymal phase of the area-vitellina endoderm should be regarded as 'terminal differentiation'. But our results revealed that the fully differentiated yolk-sac parenchyma type can differentiate into the intestine-type epithelium under certain experimental conditions. It is still uncertain whether a given cell can express both CL activity and striated border when its enzyme characteristics are in the second phase. Our results suggest that cells might be able to exhibit both CL and small intestinal enzymes, since a section expressing CL activity in the epithelium may be found between sections expressing ALP and α-GLD activities or vice versa, and the striated border develops on the epithelial surface in patches, with diameters of few dekamicrometres. Although a cell can exhibit activities of CL, ALP, and α-GLD at the same time, it does not necessarily imply that these enzymes are synthesized at the same time. Mechanism switching enzyme expression might explain the intestinalization of the area-vitellina endoderm, and this problem is worth further study.

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