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Cytokeratin Expression in the Stomach Epithelia of the Chicken Embryo is Regulated by Epithelial-Mesenchymal Interactions

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ABSTRACT—Regulatory mechanisms of cytokeratin expression in the stomach (the proventriculus and the gizzard) epithelia of chicken embryos were examined by epithelial-mesenchymal recombination experiments. In the proventriculus, antigens detected by monoclonal antibodies (PKK1 and AE3) against cytokeratins invariably decreased in gland cells which synthesize pepsinogen, while luminal and duct epithelial cells were positive to these antibodies. When recombined and cultured with the proventricular mesenchyme, the esophageal, proventricular and gizzard epithelia formed proventriculus-type, pepsinogen-expressing glands and the expression patterns of PKK1 and AE3 antigens were identical to that in the normal proventriculus. Under the same experimental conditions, the small-intestinal and allantoic epithelia also formed complex glands but did not express pepsinogen, and PKK1 and AE3 antigens were actively expressed throughout the epithelia. These results indicate that the proventricular mesenchyme can regulate cytokeratin expression and that the expression of cytokeratins in epithelial cells is closely related to the physiological characteristics of cells such as pepsinogen production.

INTRODUCTION

Cytokeratins are components of epithelial intermediate filaments. In the human tissues, there exist at least 19 kinds of cytokeratins (Moll *et al.*, 1982). Patterns of cytokeratin expression in various mammalian tissues have been widely investigated biochemically and immunohistochemically, and the different types of epithelial cells have been found to express different cytokeratin molecules.

As to the mammalian digestive organ epithelia, cytokeratins 8, 18 and 19 in the catalogue of human cytokeratins are expressed in human small intestine and colon (Moll *et al.*, 1982), and cytokeratins 5 or 8, and 18 in the stomach (Ramaekers *et al.*, 1987). Cytokeratins in the esophageal and intestinal epithelia are examined also in other animals (Calnek and Quaroni, 1993; Franke *et al.*, 1981, 1987), but reports about cytokeratins in the stomach epithelium are relatively few in number. Moreover, the roles played by cytokeratins and the regulatory mechanism of their expression in the digestive tract are little analyzed.

In the chicken, cytokeratins have been well analyzed in relation to the morphogenesis and cytodifferentiation of skin derivatives, but there is no report on the expression of

cytokeratins in the development of digestive tract.

It is well-known that epithelial-mesenchymal interactions are important in organogenesis of vertebrate digestive organs (for reviews see Mizuno and Yasugi, 1990; Yasugi, 1993, 1994; Yasugi and Mizuno, 1990). As to the morphogenesis and cytodifferentiation of the digestive tract epithelia of the chicken embryo, it has been demonstrated that the small-intestinal mesenchyme can induce in the stomach epithelium intestinal morphology and expression of intestinal enzymes (Haffen *et al.*, 1982; Ishizuya-Oka and Mizuno, 1984). It has been also shown that the mesenchymes of the two stomachs (the proventriculus and gizzard) and the esophagus can induce various epithelia to form mesenchyme-dependent morphology (Masui, 1981; Matsushita, 1993; Sigot, 1971; Sigot and Marin, 1970; Urase and Yasugi, 1993; Yasugi and Mizuno, 1974).

We have shown that the proventricular mesenchyme induces characteristic proventriculus-type complex gland and pepsinogen, a marker of the differentiated proventricular epithelial cells (Yasugi and Mizuno, 1981a, b), when recombined and cultured with the esophageal, proventricular and gizzard epithelia (Takiguchi *et al.*, 1986, 1988; Takiguchi-Hayashi *et al.*, 1988), while the small-intestinal and allantoic epithelia form similar glands but never express pepsinogen under the same experimental conditions (Hayashi *et al.*,

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1988; Yasugi *et al.*, 1985).

These lines of evidence lead us to investigate the distribution of cytokeratins and regulation of their expression in the chicken digestive tract. In the present study, we examined, firstly, the expression of cytokeratins in epithelial cells of the chicken digestive tract in the normal development by using monoclonal antibodies to cytokeratins and, secondly, the regulatory mechanism of cytokeratin expression in the stomach epithelial cells by tissue recombination experiments, paying special attention to the relationships with pepsinogen expression.

MATERIALS AND METHODS

Animals

Embryos of White Leghorn chicken (*Gallus gallus domesticus*) at various developmental stages were used during the experiments.

Epithelial-mesenchymal recombination

Epithelial-mesenchymal recombination techniques are the same as those described in a previous paper (Takiguchi *et al.*, 1986). In brief, epithelia of 6-day esophagus, proventriculus, gizzard, small intestine and 3.5-day allantois and 6-day proventricular mesenchyme were isolated with the aid of collagenase (Cooper Biomedical, Malvern, USA; Code CLS, 0.03 % in Tyrode's solution, 40 min at 38°C). Epithelial and mesenchymal fragments were recombined after inactivation of collagenase by washing in serum-containing Tyrode's solution and the recombinants were cultured according to the method of Wolff and Haffen (1952) for half a day to ensure the adhesion of two tissue fragments. They were then grafted onto the chorio-allantoic membrane (CAM) of 9-day chicken embryo and cultured for 9 days. Reaggregated proventricular or gizzard mesenchymal cells were prepared as described (Takiguchi-Hayashi *et al.*, 1988; Urase and Yasugi, 1993) and recombined with 6-day proventricular or gizzard epithelium and cultured *in vitro* for 6 days.

Antibodies

The monoclonal antibodies PKK1 (Labsystems Oy, Helsinki, Finland) and AE3 (ICN Immunobiologicals, Lisle, Illinois, USA) were used

to examine cytokeratin expression. PKK1 was raised against cytoskeletal protein isolated from a pig kidney epithelial cell line (Holthofer *et al.*, 1983) and recognizes cytokeratin 8, 18 and 19 in the catalogue of human cytokeratins (Moll *et al.*, 1982). AE3 was prepared against human epidermal cytokeratin (Woodcock-Mitchell *et al.*, 1982) and recognizes most of the basic (type II) cytokeratins. The monoclonal antibody Y37 was raised against embryonic chicken pepsinogen, a secretory protein of the proventricular epithelial cells (Yasugi *et al.*, 1987). As a secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cooper Biomedical) was used.

Immunohistochemistry

Tissues were removed just after the killing of animals or after culturing and fixed in ice-cold 95% ethanol for 4 hr, embedded in paraffin, and 5 µm sections were subjected to the indirect immunofluorescence according to the method of Sainte-Marie (1962). In brief, deparaffinized sections were first treated with primary antibodies diluted with phosphate-buffered saline for 1 hr at 38°C, and then treated with secondary antibody for 1 hr. Antibody-stained sections were mounted in a glycerin buffer and examined with an Olympus photomicroscope equipped with an epi-illumination fluorescence system.

RESULTS

Distribution of cytokeratins during proventricular epithelial differentiation of chicken embryo

When the proventriculus of 16-day embryo was stained with AE3, both pepsinogen-positive and -negative epithelial cells showed positive reactivity, but the staining pattern of pepsinogen-positive cells was distinctly different from that of pepsinogen-negative cells; in pepsinogen-positive gland cells AE3-positive substances localized only in the apical portion of the cells, while in luminal epithelial cells these substances distributed in entire cytoplasm (Fig. 1a, b). PKK1 stained pepsinogen-negative luminal cells but not pepsinogen-positive gland cells (Fig. 1a, c).

Distribution of PKK1 antigen was examined from just after the beginning of the proventricular gland formation. At

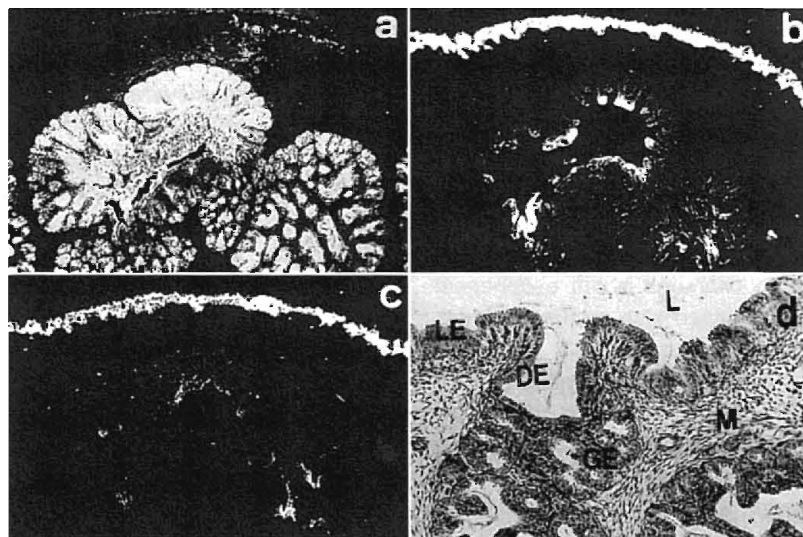


Fig. 1. Distribution of pepsinogen and cytokeratin antigens in the proventriculus of 16-day chicken embryo. Immunofluorescence of the pepsinogen antigen (Y37, a), AE3 antigen (b) and PKK1 antigen (c). (d) Histology (hematoxylin-eosin staining) of 16-day proventriculus showing lumen (L), luminal surface epithelium (LE), duct epithelium (DE), gland epithelium (GE) and mesenchyme (M). $\times 48$.

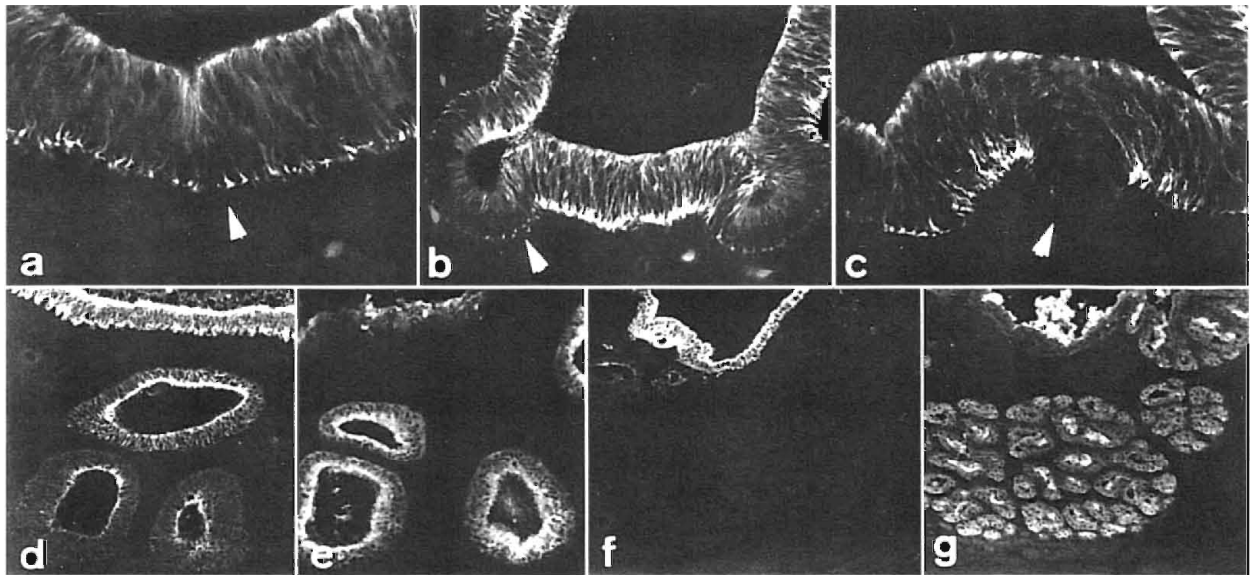


Fig. 2. Distribution of PKK1 antigen during the proventricular gland formation in normal chicken embryos. (a) Day 6. Arrowhead indicates an intra-epithelial invagination. $\times 413$. (b) Day 7. Arrowhead indicates the tip of an elongated gland. $\times 206$. (c) Day 8. Arrowhead indicates a small gland in the luminal epithelium. $\times 413$. (d,e) Day 12. $\times 103$. (f,g) Day 16. $\times 103$. (a-d,f) Immunofluorescence of the PKK1 antigen. (e,g) Immunofluorescence of the pepsinogen antigen in the neighboring sections.

about 6 days of incubation, several cells in the pseudostratified epithelium lessened in height and form an intra-epithelial invaginations, but these cells in gland rudiments were PKK1-positive and cytokeratins were observed as fibrils running from apical to basal surface (Fig. 2a). During gland elongation, PKK1 antigen decreased at the basal portion and became faint in epithelial cells at the tip of elongated glands (Fig. 2b). At 8 days of incubation, epithelial cells of small glands in luminal epithelium, which began to

express pepsinogen detected with Y37 antibody, became PKK1-negative (Fig. 2c). From 9 days of incubation onward, PKK1 antigen was detected only at the apical portion of epithelial cells of glands, while in luminal surface cells and duct cells of glands, PKK1 antigen persisted from apical to basal surface of the epithelial cells (Fig. 2d, e). From 14 days of incubation onward, PKK1 antigen was not detected in glandular epithelial cells even at the apical portion (Fig. 2f, g). On the other hand, PKK1 antigen was detected in the gizzard

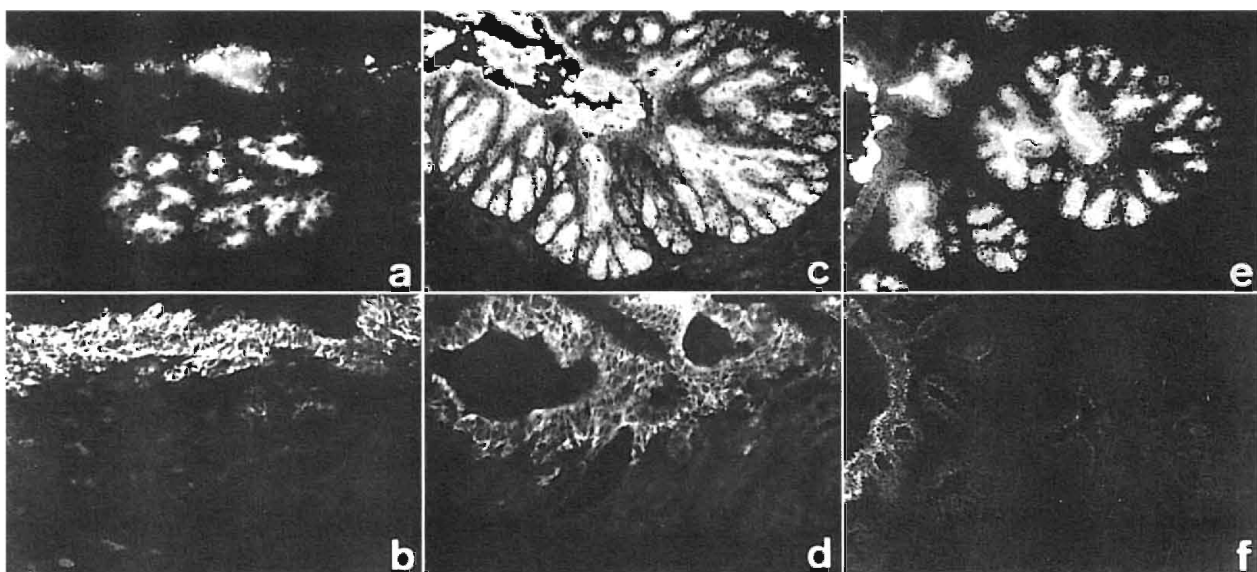
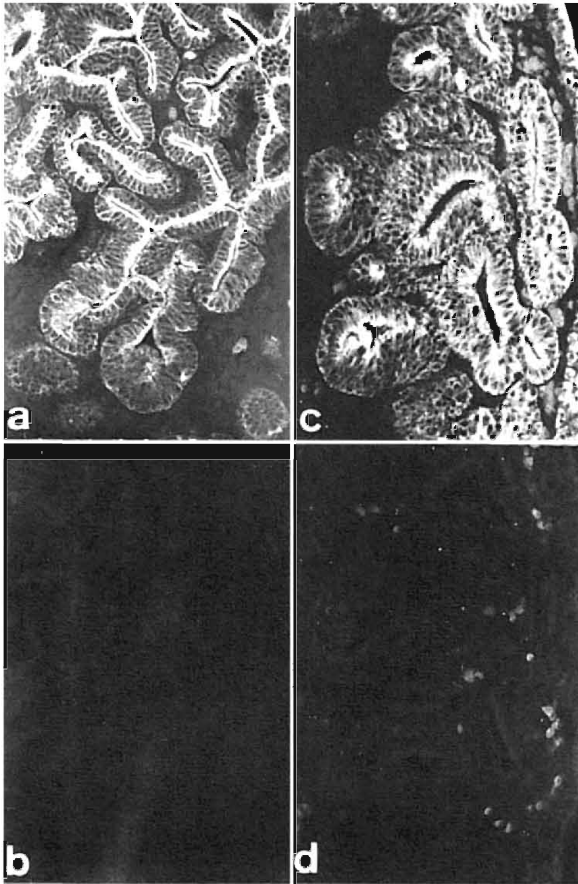


Fig. 3. Distribution of pepsinogen (a,c,e) and PKK1 (b,d,f) antigens in the recombinants of 6-day esophageal (a,b), proventricular (c,d) or gizzard (e,f) epithelium and 6-day proventricular mesenchyme cultured for 9 days on the CAM. Pepsinogen-producing cells do not express PKK1 antigen. (a,b,d) $\times 206$. (c,e,f) $\times 103$.



epithelium throughout the development (data not shown). All cells other than epithelial cells were PKK1-negative.

Regulatory influence of the proventricular mesenchyme on the expression of cytokeratins in various digestive tract epithelia

Six-day esophageal, proventricular, gizzard or small-intestinal epithelium or 3.5-day allantoic epithelium was recombined with 6-day proventricular mesenchyme and cultured on the CAM. The esophageal, proventricular and gizzard epithelia showed proventriculus-type complex gland morphology and gland epithelial cells expressed pepsinogen. PKK1 antigen was not detected in gland epithelial cells expressing pepsinogen (Fig. 3). The distribution of AE3 antigen was similar to that in the normal proventriculus (data not shown).

Six-day small-intestinal or 3.5-day allantoic epithelium recombined and cultured with the proventricular mesenchyme also formed glands but did not express pepsinogen and PKK1 antigen was detected throughout these epithelia, even in the epithelial cells forming complex glands (Fig. 4).

When 6-day proventricular or gizzard epithelium was cultured in recombination with 6-day gizzard mesenchyme,

Fig. 4. Distribution of pepsinogen (b,d) and PKK1 (a,c) antigens in the recombinants of 6-day small-intestinal (a,b) or 3.5-day allantoic (c,d) epithelium and 6-day proventricular mesenchyme cultured for 9 days on the CAM. Gland epithelial cells are PKK1-positive and do not express pepsinogen. $\times 103$.

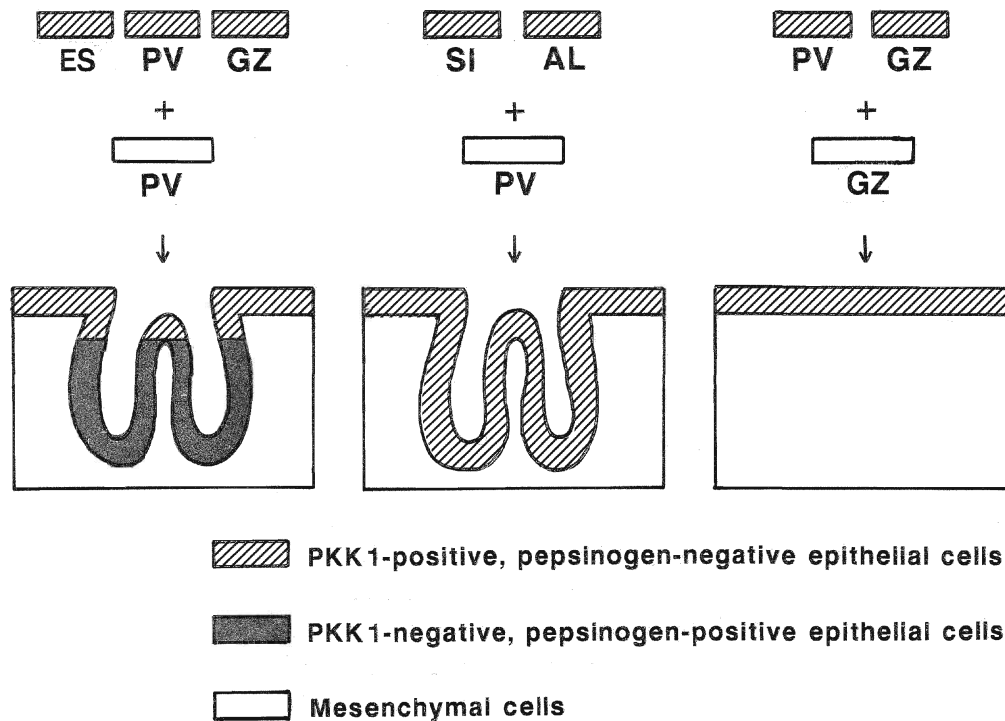


Fig. 5. Summary of the epithelial-mesenchymal recombination experiments. Four parts of digestive tract epithelium and the allantoic epithelium were isolated and associated with the proventricular mesenchyme. The recombinants were cultured for 9 days on the CAM. Pepsinogen and cytokeratin expression was observed by immunohistochemistry. ES, esophagus; PV, proventriculus; GZ, gizzard; SI, small intestine; AL, allantois.

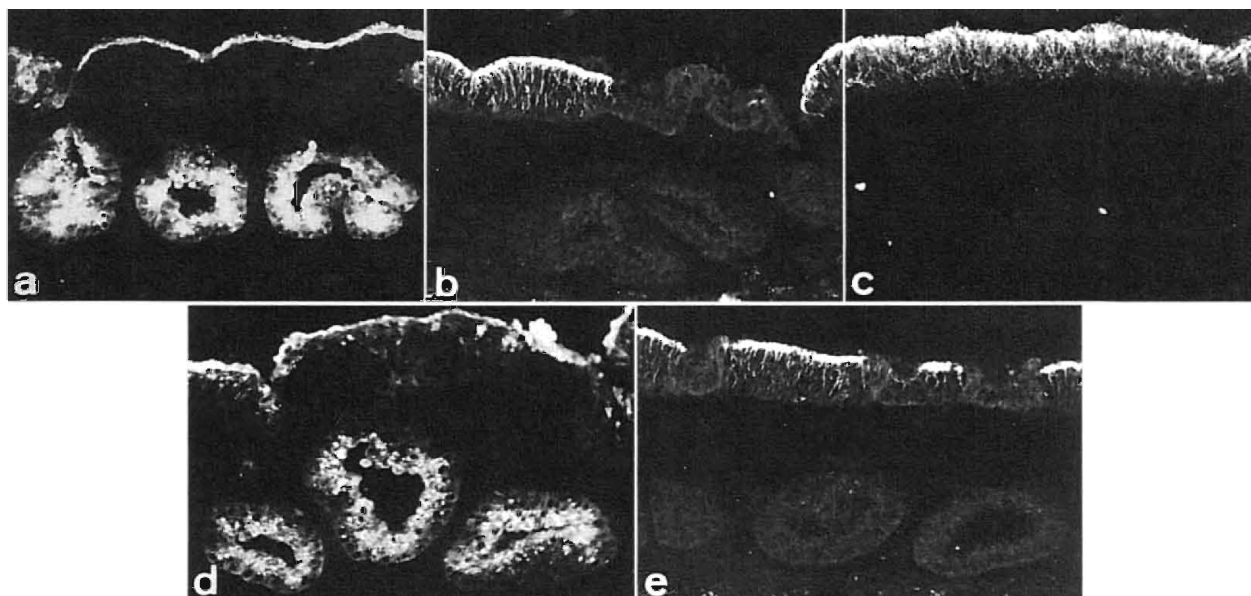


Fig. 6. Distribution of the pepsinogen (a,d) and PKK1 (b,c,e) antigens in the recombinants of 6-day proventricular (a,b) or gizzard (d,e) epithelium and reaggreated 6-day proventricular mesenchymal cells, or in the recombinants of 6-day gizzard epithelium and reaggreated 6-day gizzard mesenchymal cells (c). Immunofluorescence at the apical surface of luminal epithelial cells (a,d) is due to pepsinogen secreted by the gland epithelial cells; the luminal epithelial cells themselves are pepsinogen-negative. $\times 206$.

these epithelia did not form complex glands nor express pepsinogen, and PKK1 antigen was detected throughout these epithelia (data not shown). These results as to the expression of PKK1 antigen and pepsinogen in various recombinations are summarized in Fig. 5.

Regulatory influence of reaggreated proventricular mesenchymal cells on the expression of cytokeratins in digestive tract epithelia

In *in vitro* organ culture, 6-day proventricular and gizzard rudiments showed their typical morphology, and the distribution of PKK1 antigen was consistent with that during early development of the digestive tract; PKK1 antigen disappeared only in the gland cells of the proventriculus (data not shown). The reaggreated 6-day proventricular mesenchymal cells induced glands and pepsinogen in the homologous proventricular epithelium and PKK1 antigen disappeared in the pepsinogen-expressing epithelial cells (Fig. 6a, b), while reaggreated 6-day gizzard mesenchymal cells suppressed the gland formation and pepsinogen expression in the homologous gizzard epithelium and PKK1 antigen was detected throughout the epithelium (Fig. 6c). When 6-day gizzard epithelium was recombined and cultured with the reaggreated proventricular mesenchymal cells, the gizzard epithelium formed glands and expressed pepsinogen, and PKK1 antigen disappeared in the pepsinogen-expressing epithelial cells (Fig. 6d, e).

DISCUSSION

Nature of cytokeratins present in the chicken digestive tract epithelial cells

Our present results showed that the majority of epithelial cells of the embryonic chicken digestive tract possess substances recognized by PKK1 and AE3 monoclonal antibodies, which were raised against mammalian cytokeratins. Whether these substances are in fact cytokeratins is crucial for evaluating the results of the present study. We tried several times to detect the antigen recognized by PKK1 on immunoblots after SDS-PAGE of the crude extracts or of the keratin fractions (Mathinsen and Miller, 1987) of the digestive organs but could not obtain positive signals. AE3 antibody gave clear band of 54kDa on the immunoblot (data not shown). For this reason we could not identify the molecular weight of cytokeratins recognized by PKK1. We can conceive, however, that the substances recognized by PKK1 is cytokeratin because its localization is restricted to endodermal epithelial cells and it appeared as fine fibrils on the sections and because this antibody recognizes also fish cytokeratins (manufacturer's information). Moreover the reported amino acid sequences of mammalian and chicken cytokeratins show high similarity (Charlebois *et al.*, 1990).

We have recently cloned a cDNA of chicken cytokeratin gene from cDNA library of embryonic digestive organs. It has a sequence similarity with human cytokeratin 19 and its expression pattern was almost identical to that of PKK1-reactive substances (Sato *et al.*, unpublished results). These results suggest that the reactivity to PKK1 antibody found in the chicken digestive tract epithelium is in fact due to the presence of cytokeratin in these cells.

Correlation of pepsinogen expression and cytokeratin disappearance in the proventricular epithelial cells

Proventricular epithelial cells can be subdivided into gland cells, duct cells and luminal epithelial cells (Fig. 1d). Among these cell types, only gland cells are PKK1-negative, while duct and luminal epithelial cells are PKK1-positive (Figs. 1 and 2). In all vertebrates studied except mammals, pepsinogen-expressing cells in glandular stomach are PKK1-negative, while in mammals pepsinogen-expressing cells are PKK1-positive (Takiguchi-Hayashi and Yasugi, 1988).

It is still unclear why pepsinogen-expressing proventricular epithelial cells are negative to PKK1. We cannot exclude the possibility that the PKK1-negative epithelial cells may contain very small amount of PKK1-reactive cytokeratins. However it is known that the structure of gland epithelial cells, which actively secrete pepsinogen zymogen, is different from that of other epithelial cells (Toner, 1963). Also the distribution of AE3-reactive cytokeratin is different between pepsinogen-expressing and -non-expressing cells (Fig. 1a, b). These facts suggest that disappearance of PKK1 antigen and altered localization of AE3 antigen in proventricular gland epithelial cells may be related to some cellular physiological functions, such as production and transport of zymogen granules.

Recently we have found that expression of some genes coding substances important for development such as *cSox 2* (Ishii *et al.*, unpublished result) and *sonic hedgehog* (Narita *et al.*, unpublished result) decreases dramatically in proventricular gland epithelial cells from just after the onset of gland formation. Also the expression of a mucus-associated antigen decreases abruptly in proventricular gland epithelial cells (Yoshida *et al.*, 1996). These results indicate that proventricular gland epithelial cells constitute special subtypes among digestive tract epithelium.

Regulation of cytokeratin expression by the proventricular mesenchyme

We demonstrated that the proventricular mesenchyme can regulate cytokeratin expression in the digestive tract epithelia of chicken embryos. Disappearance of PKK1 antigen and altered distribution of AE3 antigen in the esophageal, proventricular and gizzard epithelia cultured in recombination with the proventricular mesenchyme were shown by grafting of recombinants on the CAM (Fig. 4). It was also shown that reaggregated proventricular mesenchymal cells can regulate cytokeratin expression in the stomach epithelia just as intact proventricular mesenchyme (Fig. 6). These results indicate that the regulatory influences on cytokeratin expression in the digestive tract epithelia comes from the mesenchymal cells, not from hormone-like factors.

We also revealed that the competence of the epithelium to respond to the cytokeratin-regulating action of the proventricular mesenchyme is closely related to whether pepsinogen can be induced in the epithelium or not. It was

also demonstrated that the change of cytokeratin expression in the epithelium is not necessary for gland formation since small-intestinal and allantoic epithelia formed complex glands while they maintain the reactivity to PKK1. Various epithelial-mesenchymal recombination experiments have shown that, in some cases, epithelial morphogenesis can be induced by the heterologous mesenchymes but the epithelial cytodifferentiation cannot be altered by the same mesenchyme (Lawson, 1972; Mizuno and Yasugi, 1990; Sakakura *et al.*, 1976). As for the digestive tract of chicken embryo, the small-intestinal and allantoic epithelia formed proventriculus-like complex glands but did not express pepsinogen when recombined and cultured with the proventricular mesenchyme (Haffen *et al.*, 1982; Yasugi, 1984; Yasugi *et al.*, 1985). These results suggest that the process of epithelial cytodifferentiation requires some mechanisms different from those of epithelial morphogenesis.

It was claimed that appropriate cell shape or polarity is prerequisite for the expression of some genes. For example, mammary gland epithelial cells express casein actively when cultured on floating collagen gels, but not on attached collagen gels, and cell shape and polarity are quite different in these two cases (Emerman and Pitelka, 1977; Haeuptle *et al.*, 1983; Lee *et al.*, 1985). The importance of laminin was also stressed for the synthesis of casein in mammary epithelial cells (Streuli *et al.*, 1995). It was also reported that the cytoskeleton is important for the polarity of intestinal epithelial cells (Fath *et al.*, 1993). The present study may also suggest that the disappearance of PKK1 antigen and altered distribution of AE3 antigen are necessary to maintain the specific physiological characteristics of gland cells which secrete pepsinogen.

An experiment showing the importance of tissue interactions in the regulation of cytokeratin gene expression is reported (Kurpakus *et al.*, 1992), in which bovine conjunctival epithelium was cultivated on the corneal substrate with or without basement membrane. The conjunctival epithelium does not express K12 cytokeratin normally whereas the corneal epithelium does. Under the presence of the corneal basement membrane, the conjunctival epithelium came to express this cytokeratin, but without basement membrane it remained silent as to the expression of K12 cytokeratin, indicating the involvement of basement membrane in the cytokeratin gene expression.

In conclusion, the present study reveals that the mesenchymal influences regulate at the same time the expressions of pepsinogen and cytokeratin in epithelial cells. Our recent study showed that the mesenchymal influence on the expression of pepsinogen gene is mediated by the promoter region of this gene (Fukuda *et al.*, 1994). Whether the regulatory pathways are common for the expressions of these two different proteins is very important in considering the nature of the mesenchymal influences on the epithelial differentiation, and the problem deserves further analysis. It has been demonstrated, using cDNA for embryonic chicken

pepsinogen as a probe, that the proventricular mesenchyme regulates the expression of pepsinogen gene at transcriptional level (Hayashi *et al.*, 1988). The same kind of study is necessary for cytokeratin genes. In this context, it is noteworthy to mention that the tissue-specific expression of K18 cytokeratin is regulated by DNAase-hypersensitive sites located in promoter regions and exon 6 of this gene (Nenzaniv and Oshima, 1993).

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