The maturation of cortisone-treated embryonic duodenum in vitro. I. The villus

by RAYMOND L. HAYES Jr. 1

From the Department of Anatomy, Harvard Medical School, Boston

WITH SIX PLATES

Mitotic expansion of the mucosa coupled with contraction of intrinsic longitudinal and circular muscle fibers produces previlous ridges on the luminal surface of the embryonic chick duodenum by the thirteenth day of incubation. Subsequently, remodelling by periodic indentation of the mucosal surface yields primitive villi which expand and elongate to form the tall, finger-like projections characterizing the absorptive surface of the mature duodenum (Hilton, 1902; Pap, 1933; Coulombre & Coulombre, 1958).

Coincident with the morphogenesis of the villus is the acquisition of enzymatic activity in its epithelium. As early as the 14th day of incubation of the chick embryo, alkaline phosphatase is detectable in the epithelial free border of the duodenum (Hancox & Hyslop, 1954), although other investigators report later appearances for this enzyme (Moog, 1950; Hebert, 1950).

The onset of this physiological activity in the duodenal epithelium may be accelerated by exposure to adrenocorticoids as originally postulated by Hébert (1950). Administration of these hormones in vivo or in vitro induces precocious enzyme activity (Moog et al. 1954, 1955, 1957, 1962).

In this investigation, the maturation of the intestinal villus has been studied in vitro to ascertain the effects of cortisone acetate upon the morphological aspects of differentiation. Alkaline phosphatase activity has been used as a marker for functional maturation since this is known to be influenced by hormone treatment. It follows that if the morphological and physiological phases of the differentiative process were linked in any way, stimulation of the one with cortisone should produce coincident stimulation of the other.

METHODS AND MATERIALS

Duodenal loops from White Leghorn chicken embryos of Stages 37-45 (Hamburger & Hamilton, 1951) were removed aseptically and minced into

1 Author's address: Department of Anatomy and Cell Biology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213, U.S.A.
fragments 1–2 mm³ in size. These explants were maintained as organ cultures aboard a Millipore filter disc supported by a stainless steel grid (modified from Trowell, 1959). The nutritive medium was #199 (Morgan, Morton & Parker, 1950) supplemented with 10 per cent. calf serum. Cortisone acetate was added to the medium in concentrations ranging from 0.01 to 1.0 μg./ml.

All explants were fixed after 48 hr. of incubation at 37° C. They, as well as the unincubated control tissues, were fixed in 2 per cent. glutaraldehyde buffered with sodium cacodylate for one hour at 4° C. and subsequently washed overnight in an isotonic solution of sucrose in cacodylate buffer (Gordon et al. 1963).

Tissues were prepared for histological observation by post-fixation in 1 per cent osmium tetroxide in cacodylate buffer for 1 hr. at 4° C. Following alcoholic dehydration, they were embedded in Epon 812 (Luft, 1961), sectioned on a Porter-Blum microtome at 1.5 μ and stained with toluidine blue.

RESULTS

11 Days (stage 37)

Control

The luminal surface of the 11-day chick duodenum is covered by a simple columnar epithelium supported by a submucosa of compacted mesenchymal cells and an ill-defined muscularis externus. In transverse section, the mucosa is flat but occasional high, thin previllous ridges project into the lumen (Plate 1, Fig. A).

Cultures

After maintenance as an organ culture for 48 hr., the epithelium remains simple columnar. Relative to the controls, cells of the submucosa, however, are dispersed due to an increase in intercellular fluid-filled space. The mucosal surface is thrown into a number of low, irregular serpentine folds. The muscularis externus thickens and both longitudinal and transverse orientations of the primitive muscle cell bundles are distinguishable (Plate 1, Fig. B).

The morphology of the cortisone-treated cultures is significantly different from the untreated 48-hr. cultures. The hormone-treated epithelium is still simple columnar, but the mucosal surface is folded into previllous ridges separated by shallow sulci. These ridges are of uniform height but of irregular width. Although vascular spaces which parallel the epithelial surface are apparent in the submucosa, no vascular channels were found to penetrate the lamina propria of the folds (Plate 1, Fig. C). Application of cortisone in vitro promotes mucosal folding and submucosal vascularization, but no significant change occurs in the muscularis externus.

13 Days (stage 39)

Control

The high simple columnar epithelium of the 13-day duodenum is folded into narrow, tapering projections and lower secondary foldings appear between the
Key to abbreviations used in Plates
Epithelium (e); submucosa (sm); muscularis externus (me); lamina propria (lp); vascular space (vs).

PLATE 1
Duodenum of 11-day chick embryo. Fig. A. Unincubated control. Fig. B. 48-hr. untreated culture. Fig. C. Explant cultivated for 48 hr. in medium containing 1·0 μg./ml. of cortisone. Cortisone enhances mucosal folding which has begun in untreated culture. × 200.

RAYMOND L. HAYES Jr.
Duodenum of 13-day chick embryo. Fig. D. Unincubated control. Fig. E. 48-hr. untreated culture. Fig. F. Explant cultivated for 48 hr. in medium containing 1·0 μg./ml. of cortisone. Note phosphatase reaction along luminal surface of epithelium in hormone-treated culture. × 200.

RAYMOND L. HAYES Jr.
Duodenum of 15-day chick embryo. **Fig. G.** Unincubated control. **Fig. H.** 48-hr. untreated culture. × 200.

RAYMOND L. HAYES Jr.
48-hr. culture of 15-day chick duodenum. Explants maintained in medium containing the following concentrations. Fig. I. 0·025 µg./ml. Fig. J. 0·1 µg./ml. Fig. K. 0·25 µg./ml. As the concentration of cortisone is increased, the mesenchymal core of the villus is constricted and the villi increase in length. × 750.
primary ones. Cells of the muscularis externus are aggregated to a greater extent than in the 11-day control but two fiber directions cannot yet be distinguished. The submucosa consists of a loose association of disorganized mesenchymal cells (Plate 2, Fig. D).

**Cultures**

During cultivation, previllous ridges or folds expand in width. The epithelial cells remain high columnar; the submucosa condenses and develops vascular spaces paralleling the epithelial surface. The cells of the muscularis externus aggregate and may be divided into layers according to the orientation of their longitudinal axes (Plate 2, Fig. E).

In response to hormone exposure in vitro, the previllous ridges increase in height. These folds are not tapered nor apically expanded, but are of constant thickness. The epithelium remains high columnar but the cells of the submucosa are more diffuse than in the untreated explant. Vascular channels do not extend into the folds. The earliest detectable alkaline phosphatase staining by the Gomori method (1941) occurs on the luminal surface of the epithelial cells following treatment of explants of this age with cortisone (Plate 2, Fig. F).

Hence, in response to hormone application, previllous ridges of the 13-day explant increase in height and adopt a more uniform shape. Accompanying these changes in form, physiological maturation may be deduced from the appearance of enzymatic activity on the apical membrane of the epithelial cell. Both normal morphological and functional maturation appears to be promoted by hormone treatment of the 13-day duodenum in vitro.

**15 Days (stage 41)**

**Control**

By 15 days, the previllous ridges have divided, and definitive finger-like villi cover the luminal surface of the duodenum. A condensed lamina propria fills the center of the villus and a simple columnar epithelium envelopes this core. Cells of the submucosa are loosely organized and a bilaminar muscularis externus is well-defined. In addition to the single villus, bifurcated villi are prevalent (Plate 3, Fig. G).

**Cultures**

With cultivation, the distal ends of the villi expand so that they appear to shorten and their apices are blunted. The lamina propria remains condensed and the submucosal region is congested with cells in contrast to that of the uncultivated control. Distinction between the muscular layers is lost although the cells of the muscularis externus remain aggregated (Plate 3, Fig. H).

In the cultures exposed to hormone, the cells of the simple columnar epithelium increase in height. The villi of these treated explants are transformed from a low, broad and plateaued form to an elongated and finger-like morphology as
in the 15-day control tissues. This change becomes more evident as hormone concentration increases (Plate 4, Fig. I, J & K). Villi of the explant treated with \( \cdot 25 \text{ gamma of cortisone per milliliter of nutritive medium} \) (Plate 4, Fig. K) are taller than those of the unincubated control, the apices of the villi are rounded and the cells are higher and more uniformly columnar than in the controls. No significant difference in cell number within the lamina propria or submucosa is found after hormone exposure. As the concentration of hormone increases, the core of lamina propria is constricted radially and elongated, thereby effecting a change in the shape of the villus. Consequently, cortisone applied to the 15-day mucosa \textit{in vitro} not only maintains but accelerates morpho-differentiation through the elongation of villi and the augmentation of epithelial cell height.

17 Days (stage 43)

Control

The 17-day duodenal epithelium is a simple columnar sheet. The villi are tall and roughly cylindrical with expanded apices. The tips of these villi are packed with blood cells within vascular spaces in the lamina propria. The basal core of the villus consists of a dense accumulation of cells. Fasciculi of the two layers of muscularis externus are evident (Plate 5, Fig. L).

Cultures

After 48 hr. in cultivation with no hormone, villous structure is disrupted through extensive dispersion of cells of both the lamina propria and the submucosa. This cellular dispersion presumably is due to an edematous fluid uptake by the tissue. Epithelial cell height appears to be increased (Plate 5, Fig. M).

If cortisone is added to the nutritive medium, villi elongate and their apical ends expand as in the untreated cultures. This expansion, however, is due to the enlargement of endothelially-lined vascular channels. The lamina propria is compressed into a thin peripheral region immediately subjacent to the epithelium (Plate 5, Fig. N). Hence, cortisone stimulates the vascularization of villi of the 17-day duodenal explant. Moreover, the hormone maintains the morphological integrity of the villus by counteracting cellular dispersal within the lamina propria.

19 Days (stage 45)

Control

The 19-day duodenal villus is definitively finger-shaped. These villi contain vascular channels and a condensed lamina propria. The epithelium is simple columnar; the submucosa is condensed and the muscularis externus is bilaminar (Plate 6, Fig. O).
Duodenum of 17-day chick embryo. Fig. L. Unincubated control. Fig. M. 48-hr. untreated culture. Fig. N. Explant cultivated for 48 hr. in medium containing 0.25 μg./ml. of cortisone per milliliter. Addition of hormone to the medium prevents dispersion of cells of the lamina propria and submucosa as in Fig. M but promotes expansion of vascular spaces. × 200.
Duodenum of 19-day chick embryo. Fig. O. Unincubated control. Fig. P. 48-hr. untreated culture. Fig. Q. Explant cultivated for 48 hr. in medium containing 0.025 gamma of cortisone per milliliter. × 200.

RAYMOND L. HAYES Jr.  

(Facing page 165)
Cortisone-treated embryonic duodenum

Cultures
During 48 hr. of *in vitro* maintenance, the villi of the 19-day duodenum expand radially and elongate. No significant alterations occur in the submucosa or muscular laminae (Plate 6, Fig. P).

The major effect of cortisone on the 19-day tissue is upon the epithelial cells, which are transformed from a low to a high columnar morphology. The hormone also appears to promote the expansion of villi both in breadth and length. This expansion is not consequent to dispersal of mesenchymal cells but to the enlargement of vascular channels within villi. Alteration of the submucosal and muscular layers is not evident following hormonal exposure (Plate 6, Fig. Q).

**DISCUSSION**

Morpho-differentiation of the embryonic duodenal villus consists of several developmental processes which may occur concurrently, but which appear in the following sequence: (1) mucosal folding; (2) interruption of previllous ridges to form primitive villi; (3) villous elongation accompanied by constriction of the radial dimension of the villus; and (4) vascularization of the villus. In addition to these parameters, there are maturational changes in each of the layers of the duodenal wall: The cells of the epithelium increase in height and the epithelium itself is transformed into a high simple columnar sheet. The lamina propria is altered from an accumulation of loose mesenchymal cells into a condensed and central vascular core for the villus. Also, the submucosa condenses and develops extensive vascular channels. The musculature of the duodenal wall fasciculates and two fiber directions are distinguishable.

When added to the culture medium in sufficient concentration, cortisone acetate has several morphological effects. First, it prevents dispersion of cells of the lamina propria and thereby maintains villous structure. Secondly, it promotes epithelial modulation to a high columnar type. Cortisone also stimulates the maturation of the tissue by accelerating mucosal folding as well as villous elongation and vascularization in comparison with untreated cultures. Consequently, the rate of maturation of villi on duodenal explants treated with cortisone *in vitro* approximates the *in vivo* rate. Moreover, the sequence of emergence of morpho-differentiative phenomena in steroid-treated villi is not altered when induced *in vitro*.

Moog & Kirsch (1955) have reported that hydrocortisone not only induces the elongation of villi beyond the length at explantation, but also maintains villi when cultivation is extended beyond 48 hr. In the absence of hormone, these villi from 16-day duodenal explants shorten and disappear if cultivated longer than 2 days, leaving a simple cuboidal epithelium over the original luminal surface of the tissue.

An edematous reaction is evident in the lamina propria and submucosa of
duodenal cultures. This swelling has been regarded previously as active absorption and fluid storage by the tissue (Hancox & Hyslop, 1954) and is undoubtedly attributable to the abnormal conditions of the in vitro environment. The administration of cortisone does not counteract the swelling, but the site of fluid retention is altered. The fluid accumulates within the endothelially-lined vascular spaces and not among the mesenchymal cells of the lamina propria and submucosa. Further work is being done to ascertain the mode of fluid absorption and the specific influence of cortisone on this phenomenon.

Those explants cultivated in the absence of cortisone do mature beyond the stage when removed from the embryo, but the in vivo maturative rate is retarded markedly. Similar results have been demonstrated in chick embryos which were hypophysectomized by partial decapitation and allowed to mature in situ (Hinni & Watterson, 1963). These workers report that the formation of previllous ridges, the breakdown of these into villi and the elongation of villi are retarded in their experimental series. In addition, the maturation of mucosal cells and the onset of enzymatic activity are retarded when the hypophyseal-adrenal axis is interrupted.

Postnatal administration of adrenocorticoids also alters intestinal structure. Doell & Kretchmer (1964) report the elongation of villi plus the precocious induction of intestinal invertase activity with hydrocortisone in vivo. Clark (1959) has observed a similar change in villous morphology after cortisone treatment of suckling rats and mice.

An understanding of agents promoting tissue maturation in the embryo not only contributes to our knowledge of embryogenesis, but also might elucidate those factors maintaining the differentiated state in adult tissue. The gross alterations of villous morphology manifested after cortisone application may be explained on the basis of chemical or physical effects of the steroid molecule on the cell surface. The question remains as to how the hormone might effect such changes in the character of the cell surface.

**SUMMARY**

1. Morphogenesis of the intestinal villus of the chick embryo involves the following processes which overlap in duration but which emerge in the following sequence: (i) mucosal folding; (ii) formation of primitive villi; (iii) elongation of villi accompanied by radial constriction; and (iv) vascularization of villi.

2. Alterations of villous structure accompanying in vitro maintenance of duodenal tissue are described.

3. Cortisone acetate applied to duodenal explants in vitro promotes morphodifferentiation of the villus in comparison with untreated cultures and uncultivated controls. The specific morphological effect of the hormone varies with the degree of maturation of the tissue at explantation. The principal effects of cortisone on the tissue, according to the age at explantation, are as follows:
Cortisone-treated embryonic duodenum

formation of previllous ridges (11 and 13 days); elongation of villi (15 days); vascularization of villi (17 and 19 days); and augmentation of height of epithelial cells (19 days). In addition to these morphological effects, alkaline phosphatase activity is induced precociously in 13-day explants treated with hormone.

RÉSUMÉ

La maturation in vitro du duodenum embryonnaire traité par la cortisone.

1. Les villosités

1. La morphogenèse des villosités intestinales de l'embryon de Poulet s'accomplit selon les processus suivants, qui se chevauchent dans le temps mais apparaissent dans l'ordre indiqué ci-après: (i) formation de replis de la muqueuse; (ii) formation des villosités primaires; (iii) allongement des villosités accompagné d'une constriction radiaire; (iv) vascularisation des villosités.

2. Des altérations de la structure villeuse allant de pair avec le maintien en culture in vitro du tissu duodénal sont décrites.

3. L'acetate de cortisone appliqué aux explants de duodenum active la différenciation morphologique des villosités par rapport à celle des explants non traités et des témoins non cultivés. L'effet morphologique spécifique de l'hormone varie avec le degré de maturation des tissus au moment de l'explantation. Les principaux effets de la cortisone sur le tissu, en fonction de l'âge de l'organe au moment de l'explantation, sont les suivants: formation de crêtes previlleuses (11 et 13 jours); allongement des villosités (15 jours); vascularisation des villosités (17 et 19 jours); et augmentation de la hauteur des cellules épithéliales (19 jours). Outre ces effets morphologiques, l'activité de la phosphatase alcaline est induite précocement dans les explants de 13 jours traités par l'hormone.

ACKNOWLEDGEMENTS

The author expresses his sincere appreciation to Dr Don W. Fawcett and Dr Peter Gould for critically reviewing these manuscripts. The helpful suggestions of Dr Susumu Ito are also gratefully acknowledged.

This work was supported by U.S.P.H.S. Grant RG-6729.

REFERENCES


168 R. L. HAYES, JR.


(Manuscript received 28th April, 1965)