Biochimica et Biophysica Acta, 601 (1980) 343—348

BBA 78918

MICROVILLOUS MEMBRANE VESICLE ACCUMULATION IN MEDIA DURING CULTURE OF INTESTINE OF CHICK EMBRYO

BETTY L. BLACK *, YOSHITAKA YONEYAMA and FLORENCE MOOG **

Department of Biology, Washington University, St. Louis, MO 63130 (U.S.A.)

(Received February 12th, 1980)

Key words: Alkaline phosphatase; Maltase; Thyroxine; Microvesicle; (Embryo intestine)

Summary

Explanting chick embryo duodenal tissue elicits an increase in the activities of alkaline phosphatase and maltase, an effect which is greatly enhanced by the addition of thyroxine. A large part of the elevated enzyme activity is released into the culture medium, from which it can be sedimented by centrifugation at 200 000 × *g*. The resulting pellet contains 87% or more of the alkaline phosphatase and maltase activity present in the medium at the end of 72 h of culture, but only about 25% of the protein. Negative staining of the pellet reveals the presence of microvesicles, the surfaces of which bear tiny protrusions resembling the knobs that have been seen on isolated microvilli and in preparations of purified microvillous membrane. The microvesicles appear to be derived from fragmentation of microvilli. Microvesicles with similar properties can be washed out of the duodenal lumen of embryos near hatching, suggesting that vesiculation may be a normal process that plays a useful role in intestinal function.

Introduction

When the duodenum of the 14-day-old chick embryo is cut into fragments and cultured in a chemically defined medium, both structural and enzymic differentiation proceed faster than in ovo [1—3]. Addition of 0.1 nm or more L-thyroxine further enhances the acceleration [3] but appears to overstimulate the production of microvilli and of alkaline phosphatase and maltase, which are bound to the microvillus membrane [4,5]. In particular, the microvilli become exceptionally long and appear to undergo fragmentation [2], while

* Present address: Department of Zoology, North Carolina State University, Raleigh, NC 27650, U.S.A.
** To whom reprint requests should be addressed.

much of the enzyme activity is discharged into the medium [3]. Preliminary studies have indicated that most of the released activity is not in soluble form [6]. In this paper, we present evidence that the released activity is largely bound to vesicles that have the characteristics of microvillus membrane, and are probably the fragments revealed by transmission electron microscopy [2].

Materials and Methods

White Leghorn eggs obtained from Spafas, Inc., Norwich, CT, were used for culture at 14 or 18 days of incubation at 38°C (i.e., 6 or 2 days before hatching). The duodena were removed under sterile conditions and cut into rings, 2—3 mm in length, which were then split open. Strips totalling approx. 12 mg at 14 days or 40 mg at 18 days were placed in 3 ml of culture fluid in 25-ml Erlenmeyer flasks that were gassed with 95% O_2/5% CO_2, tightly stoppered, and maintained at 38°C, as previously described [2,3]. The culture fluid was Medium 199 (Grand Island Biological Co.), either unsupplemented or containing L-thyroxine (Sigma) at a final concentration of 1 or 10 nM. The latter concentration was chosen for quantitative determinations because it elicits maximal enzyme activities with maximal release into the medium [3]. After 72 h, during which the medium was not changed, the tissue was removed, rinsed, weighed, and stored at −20°C in 0.9% NaCl. Media were also stored at −20°C.

Preparation and assay of vesicles

After thawing, the media from four cultures were pooled, centrifuged at 500 × *g* for 10 min to remove dissociated cells [3], and dialyzed against 0.9% NaCl for 48 h in the cold. 2-ml of the dialyzed medium were removed for assay, and the remainder was centrifuged at 200 000 × *g* for 1 h (Spinco, Model L, rotor 50). The pellet was resuspended in 3 ml of cold 0.9% NaCl.

Pellet suspensions, supernatants, whole media, and tissue strips homogenized to a concentration of 10 mg/ml in 0.9% NaCl in a TenBroeck grinder were assayed for alkaline phosphatase and maltase activities. Alkaline phosphatase determinations were made with disodium phenylphosphatase (Sigma) as substrate, according to the method of King and Armstrong [7], as previously employed in this laboratory [3]. Maltase activity was measured with maltose (Calbiochem-Behring) according to the method of Dahlqvist [8], as previously reported [3]. Protein content of homogenates, media, supernatants and pellet suspensions was determined by using the method of Lowry et al. [9], with bovine serum albumin as the standard.

Negative staining of vesicles

To examine the structure of material precipitated from the ultracentrifuged medium, pellets were fixed in 3% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, for 1 h. Fixed material was rinsed and suspended in 0.5 ml of buffer. Aliquots were transferred to Formvar-coated grids, negatively stained with 1% ammonium molybdate, pH 7.1, air-dried, and immediately viewed in a Hitachi HU-11C electron microscope.

To determine whether sedimentable enzyme activity is similarly released

from intestinal epithelium in vivo, duodena of 20-day-old embryos were perfused with saline, with the wash being centrifuged at 500 × *g* for 10 min to remove intact cells, and then at 200 000 × *g* for 1 h. The pellet was prepared for electron microscopy as described.

Results

Determinations of enzyme activity in tissues and media verified earlier findings that the addition of 10 nM thyroxine to the medium in which the chick embryo duodenum is cultured has a strong stimulatory effect on alkaline phosphatase, and a milder effect on maltase, with much of the activity being released into the medium [3]. Partitioning the medium into sedimentable and soluble components revealed that with 14-day cultures, more than 80% of the total alkaline phosphatase activity originally in the medium was found in the pellet after centrifugation at 200 000 × *g* for 1 h, and 89% with 18-day cultures; approx. 77% of the maltase activity was in the pellet in both cases. Since only about 25% of the released protein was precipitated, the highest specific activities were found in the pelleted vesicles. These results are summarized in Table I, which represents one experiment begun at 14 days, and one begun at 18 days; each of these experiments was repeated once, with concordant results. It is noteworthy that the vesicles were enriched roughly 3-fold in specific activities of both enzymes, relative to the medium, whether or not total activity was elevated by the presence of thyroxine (the fact that maltase fell short of 3-fold enrichment in the unstimulated 14-day cultures is probably

TABLE I

PROTEIN CONCENTRATION AND ENZYME ACTIVITIES OF EXPLANTS, MEDIA, SUPERNATANTS AND PELLETED VESICLES

Duodenal segments were explanted at 14 or 18 days and maintained in vitro for 72 h. Enzyme activities are given as μmol phenylphosphate or maltose/mg protein per h. Protein concentrations are given as μg/mg tissue. Each value is the mean ± S.E. of determinations on samples from three pools of four cultures each. Samples were assayed as described under Materials and Methods. n.m., activity too low for accurate measurement.

Age (days)	Thyroxine (10 nM)	Tissue	Medium	Supernatant	Pellet
			Protein		
14	—	79.3 ± 1.20	20.2 ± 0.47	12.4 ± 0.65	5.5 ± 0.60
14	+	71.5 ± 1.60	33.2 ± 0.43	22.8 ± 0.52	8.6 ± 0.47
18	—	76.7 ± 0.94	21.8 ± 0.10	18.0 ± 0.10	5.0 ± 0.10
18	+	82.8 ± 2.47	29.9 ± 0.74	23.3 ± 1.05	7.2 ± 0.30
			Alkaline phosphatase		
14	—	4.79 ± 0.13	7.28 ± 0.52	n.m.	20.7 ± 2.43
14	+	18.02 ± 1.34	104.0 ± 3.79	5.82 ± 0.41	331.0 ± 12.36
18	—	26.70 ± 2.02	89.4 ± 6.87	5.14 ± 0.18	349.0 ± 14.02
18	+	42.13 ± 2.86	248.0 ± 5.90	15.20 ± 0.10	919.0 ± 41.35
			Maltase		
14	—	0.65 ± 0.022	1.78 ± 0.16	n.m.	3.93 ± 0.35
14	+	0.81 ± 0.044	6.30 ± 0.12	0.96 ± 0.077	18.87 ± 0.88
18	—	0.86 ± 0.022	4.58 ± 0.02	0.91 ± 0.022	15.36 ± 0.64
18	+	0.68 ± 0.110	6.30 ± 0.21	0.88 ± 0.008	20.23 ± 0.40

TABLE II

DISTRIBUTION OF ALKALINE PHOSPHATASE (ALP), MALTASE, AND PROTEIN IN MATERIAL WASHED FROM THE DUODENA OF 20-DAY-OLD, CHICK EMBRYOS

Duodenal loops excised from 15 embryos were each flushed with 0.5 ml of cold 0.9% saline. The wash was diluted to 10.5 ml, of which 9.0 ml was centrifuged at 500 × *g* for 5 min to remove cells, and then at 200 000 × *g* for 30 min. All embryos used were in the process of hatching.

	Wash	Supernatant	Pellet
Protein (mg in 9 ml)	3.72	3.28	0.134
ALP (μmol phenylphosphate/mg protein per h)	15.7	3.2	238
Maltase (μmol maltose/mg protein per h)	1.01	0.22	17.6

incidental to the marginally small amount of enzyme activity found in these cultures).

To determine whether enzymes are also released in vesicular form in vivo, the duodenal lumina of 20-day-old embryos were gently flushed with cold saline, the wash being subjected to the same procedure as the media in other experiments. In the experiment reported in Table II, the wash proved to be rich in protein (approx. 330 μg per g duodenum), of which only 3.6% was sedimented. The specific activity of alkaline phosphatase in the pellet was, however, increased 15-fold compared to that of the wash fluid, and the specific

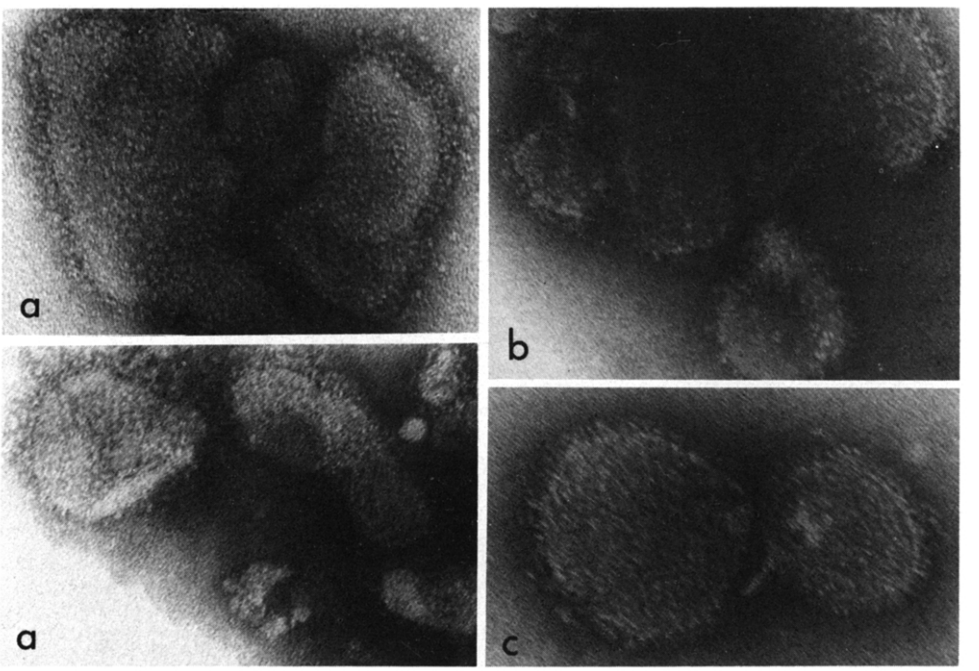

Fig. 1. Negatively stained preparations of pelleted vesicles obtained by ultracentrifugation of culture media and duodenal contents. (a) Vesicles from cultures maintained in the presence of 1 nM thyroxine. (b) Vesicles from control cultures without thyroxine. (c) Vesicles from fluid washed out of duodena of 20-day-old embryos. Magnification, × 160 000.

activity of maltase 17-fold. This experiment was repeated once, with similar results.

Inspection in the electron microscope of sedimented material that had been negatively stained revealed masses of vesicles, the surfaces of which were studded with knobs (Fig. 1a,b). Vesicles were more abundant in cultures with thyroxine, but did not otherwise differ consistently from those released in the absence of the hormone. The wash fluid also yielded knobby vesicles similar to those collected from culture media (Fig. 1c).

Discussion

Explants of embryonic chick duodenum maintained in organ culture release part of their alkaline phosphatase and maltase activity into the medium in a form sedimentable by the high gravitational force that is commonly used in the isolation of microvillous membrane [10]. The pelleted material, although containing some amorphous substance, is composed principally of vesicles having the knob-studded surfaces characteristic of isolated microvilli [11] or of membrane precipitated from brush border preparations [12,13]. Such knobs appear to be the loci of enzyme activity in microvilli and membrane derived from adult intestine [11—13], and may reasonably be assumed to have the same significance in embryonic vesicles.

The output of vesicles apparently results from the fragmentation of microvilli [3]. Vesiculation occurs in the absence of hormones, but the quantity of vesicles (membrane) released per mg of culture tissue is enhanced by thyroxine, a fact which is in agreement with the influence of this hormone in increasing both the length and density of microvilli [2] and the incorporation of [^{3}H]glucosamine into vesicles (Yoneyama, Y., unpublished results). Although these findings do not yet shed any light on the question as to whether the observed increases in enzyme activity elicited by thyroxine reflect de novo synthesis or activation of precursor molecules, they do demonstrate that thyroxine, even at the concentration (10 nM) that causes maximal displacement of enzymes into the medium [3], does not appear to alter the percentage of enzyme activity that is membrane-bound (i.e., insoluble). This conclusion is not contradicted by the fact that in both 14- and 18-day cultures, a larger percentage of maltase is soluble than phosphatase. The latter enzyme is deeply seated in the lipid bilayer [14], whereas disaccharidases are more superficially situated [12], and thus might be more readily dissipated, even in the absence of pancreatic proteases [15].

Fragmentation of microvilli is not peculiar to our system, having been observed also in cultured fetal rat jejunum [16] and 18-day-old chick duodenum overstimulated by cortisone [17]. In normal adult intestine in situ, vesicles have been seen among the microvilli [18], and in the hamster their number has been reported to increase in proportion to the intensity of functional activity [19]. Vesiculation thus appears to be a normal property of intestine, whether partly or fully differentiated. In the duodenal fluid of human patients, vesicles have been found to contain leucine aminopeptidase as well as maltase and alkaline phosphatase [20]. Only 33% of the alkaline phosphatase was bound to vesicles, in contrast to 80% or more in our cultures,

but the difference is probably explained by the presence of proteases in adult duodenal fluid [21]. It is interesting that in HeLa cells, in which the possibility that cells may release fragments of plasma membrane was first suggested [22], 86% of the alkaline phosphatase was bound to membrane [20].

All these results indicate that the high turnover rates of brush border enzymes [23,24] may be due primarily to the pinching off of microvillous vesicles rather than to molecular degradation. Whether or not the vesicles serve a digestive function is problematical. A protective function may be more likely, the release of membrane-bound immunoglobulins providing a possible model for such action [25]. In the intestine, immunoglobulins protect against cholera toxin by preventing toxin molecules from binding to the receptor sites at which they exert their action [26]. As probable carriers of both immunoglobulins and receptors, vesicles might serve as advance agents of the brush border, serving to intercept and inactivate noxious molecules before they reach the cell surface.

Acknowledgements

We are indebted to Joseph Goellner for performing some of the assays, and to G. Michael Veith for assistance with the electron micrographs. This work was supported by research grant HD-03490 from the National Institutes of Health.

References

1 Black, B.L. and Moog, F. (1977) Science 197, 368—370
2 Black, B.L. (1978) Am. J. Anat. 153, 573—599
3 Black, B.L. and Moog, F. (1978) Dev. Biol. 66, 232—249
4 Forstner, G.G., Sabesin, S.M. and Isselbacher, K.J. (1968) Biochem. J. 106, 381—390
5 Eichholz, A. (1969) Fed. Proc. 28, 30—34
6 Black, B.L. (1976) Ph.D. Thesis, Washington University, St. Louis (Dissertation Abstracts 37 : 12B, 6008—6009; No. 77-12,450)
7 King, E.J. and Armstrong, A.R. (1934) Can. Med. Assoc. J. 31, 376—381
8 Dahlqvist, A. (1968) Anal. Biochem. 22, 99—107
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
10 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Gerda, J.J. and Crane, R.K. (1973) Biochem. Biophys. Acta 323, 98—112
11 Johnson, C.F. (1967) Science 155, 1670—1672
12 Louvard, D., Maroux, B., Vannier, C. and Desnuelle, P. (1975) Biochim. Biophys. Acta 375, 236—248
13 Maestracci, D. (1976) Biochim. Biophys. Acta 433, 469—481
14 Colbeau, A. and Maroux, S. (1978) Biochim. Biophys. Acta 511, 39—51
15 Kwong, W.K.L., Seetharam, B. and Alpers, D.H. (1978) Gastroenterology 74, 1277—1282
16 DeRitis, G., Falchuk, Z.M. and Trier, J.S. (1975) Dev. Biol. 45, 304—317
17 Hayes, R.L. (1965) J. Embryol. Exp. Morphol. 14, 169—179
18 Dougherty, W.J. (1976) Anat. Rec. 185, 77—83
19 Misch, D.W., Giebel, F.E. and Faust, R.G. (1977) J. Cell. Biol. 75, 363a
20 DeBroe, M., Wiema, R.J., Logghe, G.N. and Roels, F. (1977) Clin. Chim. Acta 81, 237—245
21 Alpers, D.H. and Tedesco, F.J. (1975) Biochim. Biophys. Acta 401, 28—40
22 Marcus, P. (1962) Cold Spring Harbor Symposium 27, 351—365
23 James, W.P.T., Alpers, D.H., Gerber, J.E. and Isselbacher, K.J. (1971) Biochim. Biophys. Acta 230, 194—203
24 Alpers, D.H. (1972) J. Clin. Invest. 51, 2621—2630
25 Vitetta, E.S. and Uhr, J.W. (1972) J. Exp. Med. 136, 676—696
26 Walker, W.A., Field, M. and Isselbacher, K.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 320—324