THE EFFECT OF VITAMIN D₃ AND DIETARY CALCIUM LEVEL ON THE CADMIUM- INDUCED MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN RAT INTESTINAL MUCOSA

Setsuko NODA,* Kura KUBOTA,* Kazuhiko YAMADA, Setsuko YOSHIKAWA,
Sachiko MORIUCHI, and Norimasa HOSOYA¹

**Department of Anatomy, Tokyo Women's Medical College, Shinjuku-ku, Tokyo 160, Japan
Department of Nutrition, School of Health Sciences Faculty of Medicine,
University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*

(Received March 3, 1978)

Summary The effect of vitamin D₃ and dietary calcium level on the cadmium-induced changes was observed in the duodena of rats raised on various diets differing in vitamin D and calcium levels.

Observation with scanning electron microscopy revealed that vitamin D and dietary calcium were required for normal intestinal villi and microvilli formation. The damaged cells were observed in the intestinal villi of cadmium-exposed rats. The length of microvilli was shortened in the cadmium-exposed rats. Furthermore, dietary cadmium reduced the enzyme activities in microvilli. Especially, alkaline phosphatase activity was reduced in the cadmium-exposed groups, even though it was still responsive to vitamin D₃. These effects with cadmium were modulated by vitamin D₃ and dietary calcium level. That is, in the presence of vitamin D₃ and calcium, the effect of cadmium on intestinal villi and microvilli was reduced.

Cadmium toxicity is modulated by dietary factors, such as dietary calcium, phosphorus, protein and vitamin D (1-6). Recent evidence clearly demonstrated that cadmium interfered with vitamin D-stimulated calcium transport (6, 7), especially in rats raised on low calcium diets (7).

In order to determine that the modulation of cadmium toxicity by dietary factors is ascribed to the effect on the sites of intestinal calcium transport, the effect of vitamin D₃ and dietary calcium on the cadmium-induced morphological changes of the intestinal epithelial surface was examined by scanning electron microscopy.

¹ 野田節子, 久保田くら, 山田和彦, 吉沢節子, 森内幸子, 細谷憲政
All correspondence should be addressed to Sachiko Moriuchi.

This approach provides a clear three-dimensional view of the villi and microvilli. Furthermore, the biochemical changes of intestinal microvilli were also investigated.

METHODS

1. *Animals.* Female weanling albino rats of the Wistar strain, weighing 40–50 g, were raised on either a vitamin D deficient diet (8) or a vitamin D deficient diet supplemented with 200 ppm Cd in a dark room. CdO was used for the Cd source. After five weeks, each group was divided into 4 groups based on dietary calcium (low Ca: 0.002% or normal Ca: 0.47%) and vitamin D level (vitamin D deficient or vitamin D₃ dosed) for additional 2 weeks. Doses of either 100 IU of vitamin D₃ dissolved in 0.1 ml of propylene glycol and ethanol solution (9:1) or propylene glycol and ethanol solution, were given orally 5 times during 2 weeks. Twenty-four hours after the last dose, the animals were sacrificed by decapitation and used for the following experiments. Food and distilled water were given *ad libitum*. Animals were fasted for 18 hours before sacrifice.

2. *Scanning electron microscopy.* Eight groups of rats were sacrificed by decapitation and the duodenum was quickly removed and placed in cold 0.2 M sodium phosphate buffer (pH 7.4). A micropipet was used to direct a stream of buffer into the gut in order to remove mucus. The gut was slit open lengthwise on the filter paper and fixed in 2% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.4). After fixation, the tissues were rinsed in the phosphate buffer and followed by the conductive staining (9). After the conductive staining, the tissues were rinsed for one hour in distilled water and dehydrated in a graded series of ethanol and amyl acetate, and dried in a critical pointdryer (HCP-1, Hitachi) using CO₂ as the transitional fluid. For the examination of microvilli, the upper villi were cut carefully with a stereoscopic microscope. All samples were coated in a gold vapor coater (Eiko 1B-3). The tissues were examined in a scanning electron microscope (MSM4C-201, Akashi).

3. *Preparation of intestinal microvilli.* Microvilli were prepared from duodena of rats raised on various diets according to the method of KESSLER *et al.* (10). Mucosal scrapings from rat duodena were suspended in 30 vol. of ice-cold 50 mM mannitol in 2 mM Tris-HCl buffer (pH 7.1), and homogenized in a Waring blender at the maximum speed for 2 min. Solid CaCl₂ was added to the homogenate so as to give a final concentration 10 mM. After standing for 15 min in the cold, the suspension was centrifuged at $3,000 \times g$ for 15 min. The supernatant was centrifuged at $27,000 \times g$ for 30 min. The pellet was then resuspended in 10 ml of 50 mM mannitol in 10 mM Tris-HCl buffer (pH 7.1) and used for enzyme assay and electrophoresis.

4. *Polyacrylamide disc gel electrophoresis.* The microvilli were solubilized in 1% sodium dodecyl sulphate solution containing 10% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM ethylene diamine tetra acetic acid (pH 8.0), 40 mM dithiothreitol, and 10 µg/ml of pyronin Y (tracking dye) for 30 min at 37°C. The solubilized proteins (ca. 50 µg) were separated on polyacrylamide gel (5.6% in acrylamide) in the

presence of 1% sodium dodecyl sulphate according to the method of FAIRBANKS *et al.* (11). Electrophoresis was performed for 2 hours at 6 mA/one gel column. Two different gels of the same sample were run simultaneously. One gel was stained for protein with Coomassie brilliant blue (11). The other gel was immediately frozen and then cut into 1 mm slices with a Yeda macrotome (Yeda Scientific Instruments, Rehovot-Israel). The sliced gels were soaked in 0.3 ml of redistilled water for 1 hour at room temperature, then, maltase, sucrase and alkaline phosphatase activities were assayed as described under "Assays."

5. *Assays.* Maltase and sucrase activities were assayed by the method of DAHLQVIST (12). Substrate concentration was 28 mM in 50 mM sodium maleate buffer (pH 6.0). Activity was expressed as μ moles disaccharide hydrolyzed/hr/mg protein. Alkaline phosphatase activity was assayed using *p*-nitrophenylphosphate as a substrate at pH 10.0. Substrate concentration was 8 mM in 0.5 M 2-amino-2-methyl-1-propanol buffer (pH 10.0) (13). Enzyme activity was expressed as μ moles *p*-nitrophenol produced/min/mg protein.

Protein concentration was determined by the method of LOWRY *et al.* using bovine serum albumin as a standard (14).

6. *Chemicals.* Glutaraldehyde was obtained from TAAB Laboratories (Reading). OsO₄ was purchased from E. Merck (Darmstadt, Germany). SDS, 2-amino-2-methyl-1-propanol, and Coomassie brilliant blue were from Tokyo Kasei Ind. Co., Ltd.. Tris, dithiothreitol were from Sigma Chem. Co.. N,N,N',N'-Tetramethylethylenediamine was from Daiichi Pure Chem. Co. Ltd., acrylamide from Eastman Kodak Co., N,N'-methylenebisacrylamide from Nakarai Chem. Ltd., EDTA and sucrose from Kokusan Chem. Works, Ltd., ammonium persulfate from Kishida Chem. Ltd., glucose oxidase from Worthington Biochem. Co., pyronin Y, maltose, *p*-nitrophenylphosphate and other chemicals were from Wako Pure Chem. Ind. Ltd.. All reagents were analytical grade. Vitamin D₃ was kindly supplied by Dr. G. Katsui (Eisai Co. Ltd.).

RESULTS

1. *The effect of vitamin D₃ and dietary calcium level on the cadmium-induced morphological changes in rat intestinal mucosa*

To investigate the effect of vitamin D₃ and calcium on the cadmium-induced changes in rat intestinal mucosa, rats were raised on various diets differing in vitamin D and calcium level. Group 1-4 are the cadmium non-exposed groups and group 5-8 are the cadmium exposed groups. Among these eight groups, group 4 was raised on sufficient vitamin D₃ and a normal calcium diet and is supposed to be normal.

Duodenal villi of rats raised on sufficient vitamin D₃ and normal calcium diet (group 4) showed a characteristic shape, namely, large, tongue-like shape of a uniform height. However, duodenal villi of the rats raised on vitamin D deficient diet (groups 1 and 3) did not show a typical villous shape irrespective of calcium

content in diets. The tips of villi were flatten and atrophy of villi was prominent. Furthermore, the villi were connected to each other in a zigzag way or as linear bands along a long axis of the intestine. Duodenal villi of the rats raised on low calcium diet did not show a typical shape even in the presence of vitamin D (group 2). Although the tips of villi are not flat, the villi were connected to each other along the long axis of the intestine as those in the vitamin D deficient rat. (Fig. 1. 1–4).

In cadmium exposed groups, the atrophy and erosion of the villi was significant and the villous tips showed bulbous shapes. This effect of cadmium on intestinal villi were prominent especially in the vitamin D deficient rats. The bifurcation of villi was observed and the villi were connected to each other more complicately in the groups 5 and 7. Vitamin D₃ reduced the effect of cadmium especially in the presence of calcium (Fig. 1. 5–8).

Examination of the surface of intestinal epithelial cells with higher magnification revealed intestinal epithelial cell microvilli appearing as knob-like structures arranged predominantly in parallel rows when viewed directly from above. The intestinal epithelial cells are covered by such knob-like microvilli arranged in orders in the absence of cadmium (Fig. 2. 4a and 4b). However, in the cadmium exposed groups, intestinal epithelial cells which have lost most of their microvilli were observed mainly in the region of middle to upper intestinal villar surface in groups 5, 6 and 7, especially in group 5. Namely, such damaged cells exhibit rather pitted surfaces, protrude somewhat into the lumen, and may be in the process of sloughing off (Fig. 2. 5a and 5b).

2. *The effect of vitamin D₃, dietary calcium level and cadmium on the length of rat intestinal microvilli*

Examination of intestinal microvilli perpendicular to their long axis gives their view as parallel cylindrical entities. From this, the length of microvilli was estimated.

Vitamin D deficiency and cadmium led to a significant decrease in the length of the microvilli. The length of microvilli in the cadmium-exposed groups was shorter as compared with that in the cadmium non-exposed groups. With vitamin D₃ repletion, the microvilli increased in length significantly. However, in the presence of

Fig. 1. Scanning electron micrographs of duodenal villi of rats raised on various diets differing in vitamin D and calcium level in the absence or presence of cadmium. Numbers indicate the name of groups. 1–4 are the cadmium non-exposed groups and 5–8 are the cadmium exposed groups. Groups 1, 3, 5 and 7 are vitamin D deficient, and groups 2, 4, 6 and 8 are vitamin D₃ dosed groups. With respect to dietary calcium level, groups 1, 2, 5 and 6 are low calcium and groups 3, 4, 7 and 8 are normal calcium groups. Horizontal bars indicate 100 μ m.

Fig. 2. The surface of duodenal epithelial cells. The duodenal epithelial cells are covered by knob-like microvilli arranged in orders in the absence of cadmium (4a and 4b, group 4). The border of cell is observed as the fissure of the microvilli (4a and 4b). In the cadmium exposed groups, duodenal epithelial cells which have lost of their microvilli are observed mainly in the region of middle to upper intestinal villous surface in groups 5, 6 and 7, especially in group 5 (5a and 5b group 5). Horizontal bars indicate 1 μ m.

Fig. 1.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4. The effect of vitamin D₃, dietary calcium and cadmium on the length of rat duodenal microvilli. The length of microvilli was measured from scanning electron micrographs as shown in Fig. 3. Values are the means $\pm$ S.E. of 10 microvilli. Difference between the group 6 and 8 is significant at $p < 0.05$. Differences between groups 1 and 2, 3 and 4, 5 and 6, 7 and 8, 1 and 5, 2 and 6, 3 and 7, and 4 and 8 are significant at $p < 0.01$. Cd -, Cd no added; Cd +, Cd 200 ppm added; Ca -, 0.002%; Ca +, 0.47%; D -, vitamin D deficient; D +, 100 IU of vitamin D₃ was dosed 5 times in 2 weeks orally.

cadmium, the effect of vitamin D₃ on the microvilli was significantly repressed in the rats raised on low calcium diet compared with those raised on normal calcium diet (Fig. 3, 4).

3. The effect of vitamin D₃ and dietary calcium level on the cadmium-induced changes in the hydrolytic enzymes activities and the membrane proteins of rat intestinal microvilli

Microvilli are the sites of the terminal digestion and absorption of nutrients. Many hydrolytic enzymes, which are involved in this process, are located in the microvilli (15, 16). Therefore, after the microvilli were prepared from duodenal mucosa of the rats raised on various diets, the effect of vitamin D₃ and dietary

Fig. 3. Scanning electron micrographs of duodenal microvilli of rats raised on low calcium diets in the absence or presence of vitamin D and cadmium. Numbers indicate the name of groups. The length of microvilli in the cadmium exposed groups (5 and 6) was shorter as compared with that in the cadmium non-exposed groups (1 and 2). With vitamin D₃ repletion, the microvilli increased in the length significantly (2 and 6). Horizontal bar indicate 0.5 μ m.

Table 1. The effect of vitamin D₃, dietary calcium level and cadmium on the hydrolytic enzymes in rat duodenal microvilli.

Group	Diet			Enzyme activities		
	Cd	Ca	D	Sucrase*	Maltase*	Alkaline phosphatase**
1	—	—	—	56.8 ± 6.5	213.5 ± 18.0	96.8 ± 4.2
2	—	—	+	45.6 ± 3.8	219.0 ± 20.1	206.0 ± 15.2 ^b
3	—	+	—	50.3 ± 4.7	229.5 ± 18.5	114.7 ± 21.7
4	—	+	+	49.6 ± 3.4	192.0 ± 22.0	197.3 ± 27.0 ^b
5	+	—	—	32.3 ± 6.1 ^a	144.5 ± 14.5 ^a	25.1 ± 2.9 ^a
6	+	—	+	28.5 ± 3.2 ^a	112.2 ± 18.9 ^a	64.8 ± 7.3 ^{a,b}
7	+	+	—	29.0 ± 3.2 ^a	141.0 ± 14.0 ^a	35.6 ± 6.2 ^a
8	+	+	+	27.0 ± 2.2 ^a	110.9 ± 14.1 ^a	62.1 ± 5.8 ^{a,b}

Values are the means ± S.E. of 3 rats. Enzyme activities were expressed as either μ moles substrate hydrolyzed/mg protein/hr (*) or μ moles *p*-nitrophenol produced/mg protein/min (**).

^a Significantly different from respective Cd non-exposed group at $p < 0.05$.

^b Significantly different from respective vitamin D deficient group at $p < 0.05$.

Cd —, no addition; Cd +, 200 ppm added; Ca —, 0.002%; Ca +, 0.47%; D —, vitamin D deficient; D +, 100 IU of vitamin D₃ was dosed 5 times in 2 weeks orally.

Fig. 5. The effect of vitamin D₃, dietary calcium and cadmium on the membrane proteins of rat duodenal microvilli. The membrane proteins were separated on polyacrylamide disc gel electrophoresis in the presence of 1% sodium dodecyl sulfate. Migration is from top to bottom. The diagrams are schematically described from the gels stained by Coomassie brilliant blue. Numbers of both sides of gels indicate protein bands, and numbers in the bottom of gels indicate the name of groups.

Fig. 6. Patterns of enzyme activities of proteins from duodenal microvilli (group 4) after gel electrophoresis. For the comparison of protein pattern of gel and enzyme activities, the protein pattern of gel was illustrated under the figure. Enzyme activities were expressed in absorbance of hydrolyzed products.

calcium on the cadmium-induced changes in the hydrolytic enzymes activities was observed.

Alkaline phosphatase activity of duodenal microvilli was not influenced by dietary calcium, but it was significantly increased by vitamin D₃ and repressed by dietary cadmium. Despite the decreases of alkaline phosphatase activity in cadmium-exposed rats, the enzyme activity was partly recovered by vitamin D₃ (Table 1).

On the other hand, maltase and sucrase activities of the duodenal microvilli were not influenced by vitamin D₃ and dietary calcium. They were significantly repressed by dietary cadmium (Table 1).

Furthermore, membrane proteins of microvilli were analyzed on SDS-electrophoresis. The profiles are essentially similar in all groups, however, those of the cadmium-exposed and vitamin D deficient groups (groups 5 and 7) showed the splitting of protein band 4. In the cadmium-exposed and calcium-deficient groups (groups 5 and 6), protein band 6 was also split (Fig. 5).

In order to identify the membrane proteins influenced by cadmium, vitamin D and dietary calcium level, unstained gels were sliced and soaked in water, and their enzyme activities were assayed. It revealed that band 1 corresponded to maltase, band 2 to sucrase and band 4 to alkaline phosphatase, however, band 6 was not identified (Fig. 6).

DISCUSSION

The effect of vitamin D and dietary calcium level on the cadmium-induced changes in rat intestine was observed morphologically and biochemically in comparison with the cadmium non-exposed group.

Scanning electron microscopic observation provides a clear three dimensional view of the changes of villi and microvilli. It revealed that vitamin D₃ and calcium were essential factors to maintaining a normal villous structure and function and to prevent the effect of cadmium.

Duodenal villi of rats raised on vitamin D deficient or low calcium diets did not show a typical villous shape. Villi were connected to each other in a zigzag way or in linear bands along long axis of intestine which could probably restrict the movement of villi. This change in villi appears as a kind of retrograde change of villous development. In the course of development, longitudinal folds (previllous ridges) appear along long axis of intestine initially. The previllous ridges differentiate to show a zigzag pattern. Then finally, they developed into tongue-like shaped distinctive villi (17). In addition to a kind of retrograde change of villous development, the intestinal absorptive cells, which have lost most of their microvilli, were observed in the villous surface of cadmium-exposed group except for group 8. In the low calcium dietary group exposed to cadmium, damaged cells were frequently seen even in the presence of vitamin D₃. This suggests that prolonged feeding of a low calcium diet with cadmium, even in the presence of vitamin D₃, might lead to easier development of cadmium toxicity than that of normal calcium diet with cadmium in the presence of vitamin D as reported by many investigators (1-5). Namely, even in the presence of vitamin D₃, the prolonged feeding of low calcium diet containing cadmium would develop cadmium poisoning easily compared with the normal calcium diet. It is not known how cadmium deteriorates the membranes, however, its effect is enhanced in the fragile membranes such as those in vitamin D deficient and/or calcium deficient rats.

Furthermore, the effect of vitamin D₃ and dietary calcium level was observed in cadmium-induced changes in the microvilli. The length of microvilli in the cadmium-exposed rats was shorter than that in the cadmium non-exposed rats. In good agreement with the finding of Jande that the length of microvilli in chick intestine was lengthened by vitamin D₃ (18), the length of microvilli in rat intestine was also lengthened by vitamin D₃. However, the effect of vitamin D₃ was modulated by dietary calcium level in the presence of cadmium. Namely, the effect of vitamin D₃ on the length of microvilli was repressed by cadmium especially in the absence of calcium in the diet.

The morphological changes of microvilli was reflected in the biochemical properties of microvillous membranes. Maltase, sucrase and alkaline phosphatase activities were decreased in the cadmium-exposed rats compared with those in the cadmium non-exposed rats. Among these enzymes, alkaline phosphatase is thought to be one of the essential factors in calcium transport. The activity of alkaline

phosphatase was significantly inhibited in the cadmium-exposed group although it was still responsive to vitamin D₃. Membrane protein which has alkaline phosphatase activity was split on the gel of polyacrylamide disc gel electrophoresis in vitamin D deficient and cadmium-exposed group. This suggests that the inhibition of alkaline phosphatase activity by cadmium would be ascribed to the changes in the enzyme protein induced by cadmium.

The results appear to be reflected on the fact that intestinal vitamin D stimulated calcium transport is inhibited by cadmium, especially in rats raised on low calcium diet (7). Namely, the modulation of cadmium toxicity by dietary factors could be ascribed to the effect on the sites of intestinal calcium transport. Among the dietary factors, vitamin D is one of essential factors to preventing cadmium poisoning especially in the beginning of cadmium exposure although the dietary calcium level also modulates the effect of cadmium.

This work was supported by a grant (144027) from the Scientific Research Fund of the Ministry of Education, Science and Culture of Japan. We would like to thank Dr. G. Katsui (Eisai Research Laboratory, Japan) for providing vitamin D₃.

REFERENCES

- 1) LARSSON, S. E., and PISCATOR, M. (1971): Effect of cadmium on skeletal tissue in normal and calcium deficient rats. *Metab. Bone Dis.*, **7**, 495–498.
- 2) ITOKAWA, Y., ABE, T., and TANAKA, S. (1973): Bone changes in experimental chronic cadmium poisoning. Radiological and biological approaches. *Arch. Environ. Health*, **26**, 241–244.
- 3) ITOKAWA, Y., ABE, T., TABEL, R., and TANAKA, S. (1974): Renal and skeletal lesions in experimental cadmium poisoning. Histological and biochemical approach. *Arch. Environ. Health*, **28**, 149–154.
- 4) WASHKO, P. W., and COUSINS, R. J. (1975): Effect of low dietary calcium on chronic cadmium toxicity in rats. *Nutr. Rep. Int.*, **11**, 113–127.
- 5) OMORI, M., and MUTO, Y. (1977): Effect of dietary protein, calcium, phosphorus, and fiber on renal accumulation of exogenous cadmium in young rats. *J. Nutr. Sci. Vitaminol.*, **23**, 361–373.
- 6) SUDA, T., SUZUKI, M., FUJII, K., SASAKI, A., and YOSHIKI, S. (1975): Abnormal calcium metabolism induced by oral administration of cadmium. *Bone Metab. (Kotsutaisha, in Japanese)*, **7**, 65–71.
- 7) TSURUKI, F., WUNG, H. L., TAMURA, M., SHIMURA, F., MORIUCHI, S., and HOSOYA, N. (1978): The effect of dietary calcium and vitamin D₃ on the cadmium accumulation in the tissues. *Vitamins (in Japanese)*, **52**, 161–166.
- 8) SUDA, T., DELUCA, H. F., and TANAKA, Y. (1970): Biological activity of 25-hydroxyergocalciferol in rats. *J. Nutr.*, **100**, 1049–1052.
- 9) WATANABE, T., NAGATANI, T., and MURAKAMI, T. (1975): New non-coating observation techniques in scanning electron microscopy. *The Cell*, **7**, 118–125.
- 10) KESSLER, M., ACUTO, O., STORELLI, C., MURER, H., MÜLLER, M., and SEMENZA, G. (1978): A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport system. *Biochim. Biophys. Acta*, **506**, 136–154.
- 11) FAIRBANKS, G., STECK, T. L., and WALLACH, D. F. H. (1971): Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*, **10**, 2606–2624.
- 12) DAHLQVIST, A. (1964): Method for assay of intestinal disaccharidases. *Anal. Biochem.*, **7**, 18–25.

- 13) LOWRY, O. H. (1957): Micromethods for the assay of enzymes. II. Specific procedure, alkaline phosphatase. *in* Methods in Enzymology, Vol. 4, ed. by Colowick, S. P., and Kaplan, N. P., Academic Press, New York, pp. 371–372.
- 14) LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- 15) UGOLEV, A. M. (1965): Membrane (contact) digestion. *Physiol. Rev.*, **45**, 555–595.
- 16) CRANE, R. K. (1969): A concept of digestive-absorptive surface of the small intestine. *in* Handbook of Physiology, Vol. V, Section 6, ed. by Code, C. F., American Physiological Society, Washington, pp. 2535–2542.
- 17) GREY, R. D. (1972): Morphogenesis of intestinal villi. I. Scanning electron microscopy of the duodenal epithelium of the developing chick embryo. *J. Morphol.*, **137**, 193–214.
- 18) JANDE, S. S., and BREWER, L. M. (1974): Effect of vitamin D₃ on duodenal absorptive cells of chicks. An electron microscopic study. *Z. Anat. Entwicklungsgesch.*, **144**, 249–265.