Localisation of vitamin D receptor in normal human duodenum and in patients with coeliac disease

K W Colston, A G Mackay, C Finlayson, J C Y Wu, J D Maxwell

Abstract

Immunocytochemistry using a specific monoclonal antibody 9A7γ was used to identify receptors for calcitriol (1,25 $(OH)_2$ D_3), the active metabolite of vitamin D, in sections of duodenal mucosa. Specific staining for vitamin D receptors was largely restricted to nuclei of enterocytes lining crypts in duodenal biopsy specimens from normal mucosa. Vitamin D receptors were also abundant in crypts from duodenal mucosa in coeliac disease patients with mucosal damage and villous atrophy. In contrast, alkaline phosphatase, a vitamin D regulated protein, was absent from crypts but present on brush borders of normal villi, and on surface enterocytes in coeliac disease. Oestrogen receptor could not be identified in duodenal mucosa. These findings suggest that calcium malabsorption in coeliac disease does not result from the absence of vitamin D receptors, but rather from reduction in vitamin D regulated proteins and functions essential for active calcium absorption that are located in the enterocytes of the villi.

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Animal studies have shown that the highest capacity for active calcium absorption is in the duodenum. Calcium absorption entails uptake by the enterocyte, transport across the intestinal cell mediated by calcium binding protein, and an energy dependent calcium pump in the enterocyte basolateral membrane responsible for calcium efflux into the circulation. These processes are regulated by calcitriol (1,25 $(OH)_2$ D_3), the active hormonal form of vitamin D.

Studies in experimental animals have shown that intestinal vitamin D receptor concentrations are highest in the duodenum,[1] which also has the highest capacity for active calcium transport. In animal studies active calcium absorption has been shown to correlate well with circulating concentrations of calcitriol and with the cellular content of vitamin D dependent calcium binding protein calbindin D9K, whose concentration is greatest in the duodenum in comparison with other segments of the intestine.[2 3] The distribution of rat intestinal calbindin 9K mRNA has been shown by the use of a complementary DNA probe[4] and these studies have shown the greatest abundance of message in the duodenum. Furthermore, in situ hybridisation histochemistry showed the mRNA to be localised only to the absorptive cells.[5]

As with the other steroid hormones, calcitriol interacts with a specific receptor protein, which results in transcriptional activation of specific target genes, leading to synthesis of vitamin D regulated proteins (including brush border alkaline phosphatase, and calbindin 9K in the cytosol). Vitamin D receptors have been shown in the small intestinal mucosa of experimental animals (where they are most abundant in the duodenum), but a detailed understanding of the mechanisms by which vitamin D influences intestinal calcium absorption at the cellular and molecular level is awaited.

Little is known of the cellular localisation of vitamin D receptors or vitamin D regulated proteins in the human intestine, or of the effects of disease on vitamin D regulated functions. Such studies might provide insight into the mechanisms by which vitamin D influences calcium absorption. In gluten induced enteropathy (coeliac disease) mucosal damage resulting in villous atrophy is most noticeable in the proximal small bowel, as the duodenum and jejunum bear the brunt of the enteropathy induced by ingested gluten. Calcium malabsorption with hypocalcaemia, osteopenia, and fractures are well recognised features.[6 7]

We have used immunocytochemical methods to examine whether changes in the distribution of vitamin D receptors and a calcium regulated protein (alkaline phosphatase) occurred in villous atrophy seen in patients with coeliac disease. As calcium absorption declines after the menopause, we were also interested to look for the presence of oestrogen receptors in duodenal mucosa.

Methods

BIOPSY SPECIMENS

Distal duodenal biopsy specimens were obtained at endoscopy from eight patients with gluten induced enteropathy. Four untreated patients showed severe (3) and moderate (1) mucosal damage. Only one of four patients who had received a gluten free diet had a normal mucosa. The others showed persisting severe (2) or mild (1) mucosal damage. Biopsy specimens showing normal distal duodenal architecture were obtained from 12 patients being investigated for anaemia in whom a final diagnosis of a non-gastrointestinal disorder was made. Biopsy specimens were orientated

Departments of Clinical Biochemistry
K W Colston
A G Mackay

Histopathology and
C Finlayson

Medicine, St George's Hospital Medical School, London
J D Maxwell

Institute of Animal Physiology, Babraham, Cambridge
J C Y Wu

Correspondence to:
Dr J D Maxwell, Department of Biochemical Medicine, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE.

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under a dissecting microscope, and snap frozen in liquid nitrogen for subsequent study. Patient consent was obtained and approval for the study was granted by the local research ethics committee.

IMMUNOCYTOCHEMICAL STAINING PROCEDURES

Six to eight μM sections of duodenal biopsy specimens were cut on a cryostat at −20 to −30°C, and thaw mounted onto poly-l-lysine coated slides. The slides were fixed immediately in 4% formaldehyde in phosphate buffered saline (PBS; pH 7·2) at room temperature for 10 minutes, followed by two washes with PBS. The sections were then immersed in methanol (−20°C) for four minutes, followed by one minute in acetone (−20°C), and washed twice in PBS. The sections could be stored at −20°C), after fixation for up to four weeks in glycerol-sucrose storage medium before staining.

CELL CULTURE

Three human osteosarcoma cell lines, Te85c, Hs811, and HTB96 (generously provided by Dr Tim Arnett, University College, London) and MCF7 breast cancer cells were studied. For biochemical analysis of receptor, cells were grown to 70% confluence in 75cm^2 plastic flasks and harvested by scraping into phosphate buffered saline. For immunocytochemical studies cells were plated at a density of $1-4\times10^4$ cells/ml in Lab-Tek tissue culture chamber slides (Miles Scientific, Napaville IL) in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. When cultures approached confluence (5–7 days) cell sheets were washed three times with PBS. Fixation was carried out as described above.

SUCROSE DENSITY GRADIENT ANALYSIS OF VITAMIN D RECEPTORS

Extracts of cells were prepared by sonication in KTEDM (TRIS - potassium chloride EDTA - sodium molybdate) buffer and high speed supernatants obtained. Frozen human duodenal biopsy tissue (10–20 mg wet weight) was pulverised in a polytron and KTEDM extracts of cytosols similarly prepared, followed by immediate binding of radioactive ligand. Mucosal cytosol extracts were incubated with (^{3}H)1,25(OH)$_2$D$_3$ (1·0 nM) at 0°C for 3·5 hours. Non-specific binding was determined in duplicate samples by addition of a 500-fold excess of radioinert hormone. Cold 25-(OH)D$_3$ was added to both total and non-specific binding tubes to eliminate any interfering cross reaction from a possible contaminating 6S binding protein to 25-(OH)D$_3$ from serum. Bound and free steroid was separated by charcoal adsorption and extracts were layered onto chilled 5–20% sucrose gradients, centrifuged, and fractions collected as previously described.[8]

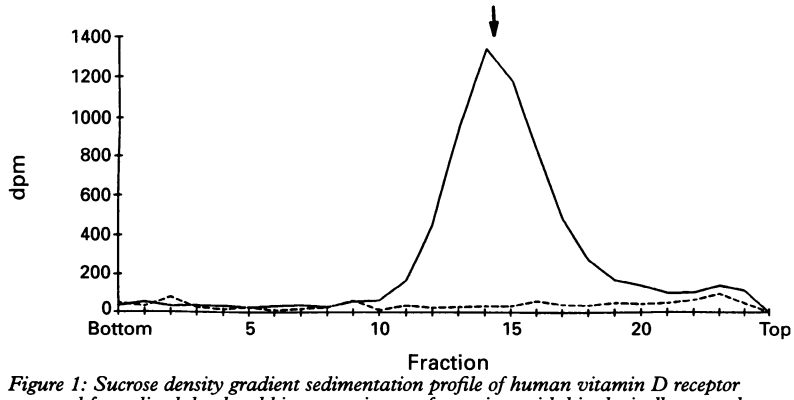

Figure 1: Sucrose density gradient sedimentation profile of human vitamin D receptor prepared from distal duodenal biopsy specimens of a patient with histologically normal duodenal mucosa.

MONOCLONAL ANTIBODY TO VITAMIN D RECEPTOR

The monoclonal antibody 9A7γ to chick intestinal 1,25(OH)$_2$ D receptor (vitamin D receptor) was obtained from confluent cultures of rat spleen/mouse myeloma hybrids. It is a rat immunoglobulin G2b (IgG2b). 9A7γ migrates in the 7–8S region on 10–30% sucrose density gradient centrifugation. The antibody reacts with vitamin D receptors from a variety of mammalian tissues including rat, pig, and human. The antibody does not react with oestrogen or glucocorticoid receptors.[9]

Vitamin D receptor – staining was carried out using a modification of the method for visualising oestrogen receptor.[10] Briefly, after washing and reaction with normal goat serum in PBS, slides were incubated with 9A7γ antibody (diluted 1:750 in PBS-BSA) at room temperature for 60 minutes. Each sample had in addition, a negative control slide in which the primary antibody was replaced by a normal (rat) IgG, and the slides were incubated for 60 minutes. They were then washed with PBS, and the antibody was visualised using a modification of the peroxidase – antiperoxidase technique with visualisation of the antigen by diamino-benzidine tetrahydrochloride-hydrogen peroxide (DAB) as the chromogen substrate, and counterstaining with 1·5% Harris haematoxylin.

Alkaline phosphatase – adjacent cryostat tissue sections were stained for alkaline phosphatase activity using naphthol-ASBI phosphate as substrate, revealed with fast red violet LB base (Sigma) counterstained with haematoxylin.

Oestrogen receptor – in experiments to examine for the presence of oestrogen receptor in the duodenum, the 9A7γ primary antibody was replaced by H222 anti-oestrogen receptor mAB. Specimens of MCF7 breast cancer cells (which are both vitamin D receptors+ve and oestrogen receptor+ve) were included in each staining as a positive control.[11]

The specificity of the staining reaction for 1,25 (OH)$_2$ D$_3$ receptors was confirmed by immunostaining studies using the 9A7γ monoclonal antibody preabsorbed with a purified vitamin D receptor protein preparation

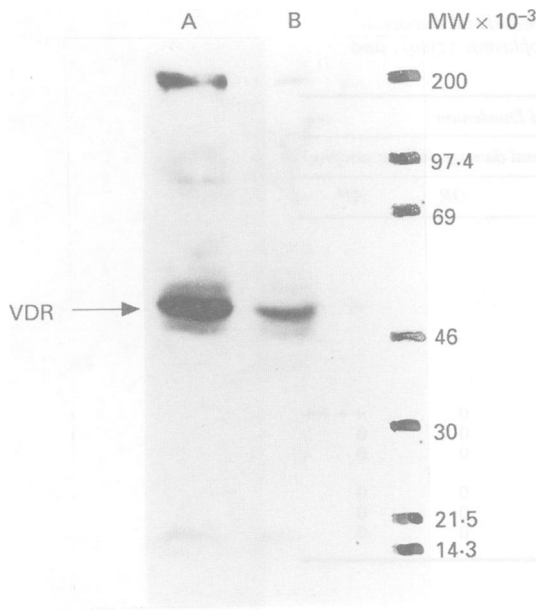

Figure 2: Western analysis of human duodenal vitamin D receptor (VDR) from two different patients A and B. A single immunoreactive band of about 55 000 MW was seen in both samples.

obtained by expression of the protein in *Saccharomyces cerevisiae*.[12] The preabsorption of the monoclonal antibody with this receptor enriched protein was carried out with 9A7γ antibody at a concentration of 2 μg/ml, incubated with excess antigen at 4°C for 18 hours before the staining procedure.

CHARACTERISATION OF VITAMIN D RECEPTOR IN HUMAN DUODENAL BIOPSY SPECIMENS BY WESTERN ANALYSIS

The binding of the anti-vitamin D receptor monoclonal antibody 9A7γ to the receptor in human duodenal biopsy specimens was assessed by SDS polyacrylamide gel electrophoresis and immunoblotting.

Briefly, human duodenal mucosa was pulverised under liquid nitrogen and sonicated in PBS containing aprotinin, diluted in SDS buffer, and denatured by boiling. Fifty–100 μg of total cell protein was run on 7·5% polyacrylamide minigels and transferred to nitrocellulose in a wet blotting apparatus using standard methodology (Hybond C-extra, Amersham in a Bio-rad trans blot cell). Transfer was monitored with Rainbow markers (Amersham). Filters were blocked and probed with the vitamin D receptor monoclonal antibody 9A7γ (1/750). Antibody binding was revealed with a sheep anti-rat IgG secondary antibody labelled with horse radish peroxidase and detected by autoradiography of enhanced chemiluminescence (Amersham).

QUANTITATIVE ASSESSMENT OF IMMUNOREACTIVE STAINING

A semi-quantitative assessment of vitamin D receptor, alkaline phosphatase, and oestrogen receptor staining of duodenal mucosa was carried out, ascribing scores of 0 to ++++ to the degree of staining present in the nuclei, cytoplasm, and lumenal border of the duodenal epithelium, and comparing the staining within crypts with that of the bases, midportion, and tips of the villi.

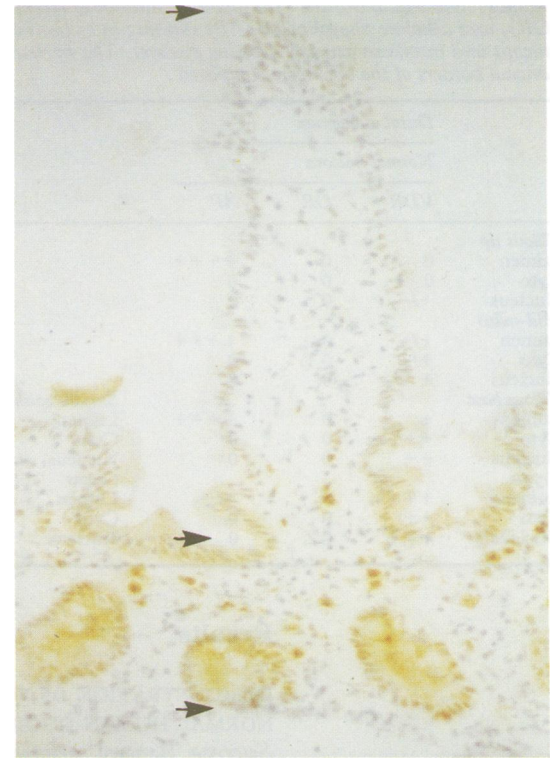

Figure 3: Vitamin D receptor staining in normal distal duodenal mucosa ×100 (frozen section). Staining is maximal in the crypts, diminishing along the villus. Arrows mark the crypt neck from which nuclear absorbance readings are taken upwards to the villous tip (arrow), and downwards to the villous base (arrow). See Figure 2.

A quantitative image analysis of enterocyte staining for vitamin D receptors was also performed using microdensitometry (Vickers Instruments, York, England). Absorptive cell (enterocyte) nuclei were sequentially measured from the crypt base to the villous tip (or surface mucosa in the case of coeliac patients) using a 5 μm scanning disc at a final magnification of ×1000.[13]

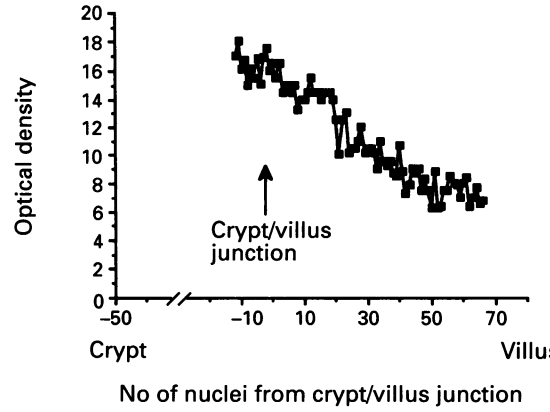

Figure 4: Quantitative densitometry of vitamin D receptor staining in enterocyte nuclei from crypt base to villous tip in 18 quantifiable crypt/villous regions from six subjects with normal duodenal mucosa. Numbers on vertical axis represent mean nuclear absorbance readings for the nuclei at that position. Position of nuclei relative to crypt/villus junction (arrow) shown on horizontal axis.

TABLE I *Semi-quantitative assessment of vitamin D receptor (VDR), oestrogen receptor (OR), and alkaline phosphatase (AP) staining in sections of normal distal duodenal mucosa and in villous atrophy (coeliac disease). The nuclear, cytoplasmic (cyto), and lumenal borders of the cells were compared*

	Distal duodenum				*Distal Duodenum*		
	Normal mucosa				*Mucosal damage (villous atrophy)*		
	VDR	*OR*	*AP*		*VDR*	*OR*	*AP*
Villous tip							
Lumen	0	0	++++				
Cyto	0	0	0				
Nucleus	+/−	0	0				
Mid-villus							
Lumen	+/−	0	++++				
Cyto	+/−	0	0				
Nucleus	+	0	0				
Villous base				*Surface epithelium*			
Lumen	+	0	++++	Lumen	+	0	++++
Cyto	++	0	0	Cyto	+	0	0
Nucleus	++	0	0	Nucleus	+	0	0
Crypts				*Crypts*			
Lumen	++	0	+/−	Lumen	++	0	0
Cyto	++	0	0	Cyto	++	0	0
Nucleus	+++	0	0	Nucleus	++	0	0

Results

DEMONSTRATION OF VITAMIN D RECEPTORS IN NORMAL HUMAN DUODENAL BIOPSY SPECIMENS

Sucrose density gradient analysis – Figure 1 shows the sucrose density gradient sedimentation profile of the 1,25-$(OH)_2$ D_3 receptor prepared by cytosol extraction from histologically normal duodenal biopsy specimens of a single patient. The arrow points to the ^{14}C ovalbumin marker protein (3·6S). Radioactivity was associated with a sedimentation coefficient of about 3·6S, totally quenched by the addition of an excess of inert ligand. This is in good agreement with results reported for human vitamin D receptor in surgical specimens of the gut.[14]

Western analysis – Vitamin D receptor in duodenal biopsy specimens was characterised by western analysis using the anti-vitamin D receptor monoclonal antibody 9A7γ with a peroxidase labelled secondary antibody and enhanced chemiluminescence. This technique showed a predominant band MW 55000 in samples of human duodenal mucosa (Fig 2). A single band of identical molecular weight was also obtained with western analysis of the vitamin D receptor positive breast cancer cell line MCF7.

Localisation of vitamin D receptor like immunoreactivity – incubation of sections of histologically normal human duodenum with 9A7γ showed the presence of immunoreactive vitamin D receptors in duodenal enterocytes.

TABLE II *Concordance between vitamin D receptor like immunoreactivity in duodenal sections and cultured human osteosarcoma cell lines of known vitamin D receptor (VDR) phenotype*

	VDR immunostaining	*VDR ligand binding**
Normal distal duodenum:		
Villous tip	−	
Mid-villus	+/−	+ In duodenal mucosal homogenate
Villous base	+	
Crypt	++	
Human osteosarcoma cell cultures:		
Te85C	++	+
HTB96	++	+
Hs811	−	−

*Denotes typical specific vitamin D receptor binding in 3·2S region as analysed on sucrose density gradient.

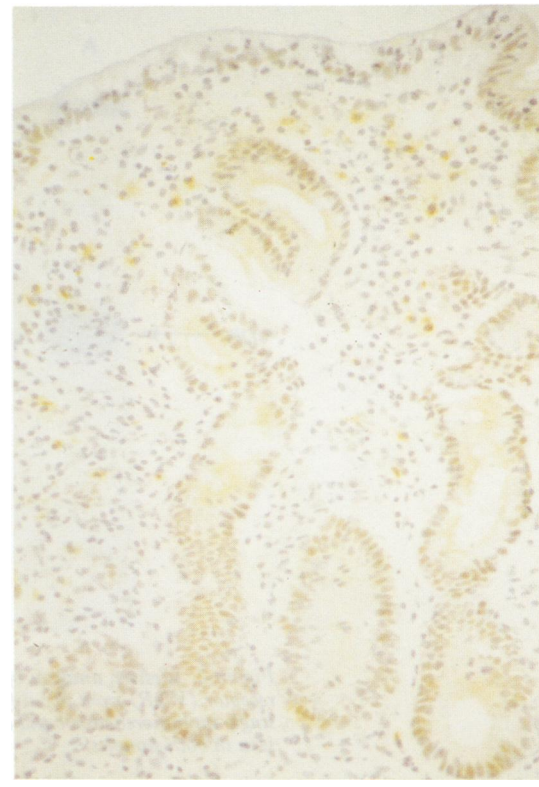

Figure 5: Vitamin D receptor staining in subtotal villous atrophy (coeliac disease) ×160 (frozen section). Staining is maximal in the crypts and diminishes towards the surface.

The staining was located predominantly in the nuclei of positively reacting cells, with very little cytoplasmic staining. A striking gradation of immunoreactivity was seen (Figs 3 and 4 and Table 1) with staining predominantly located in crypt cells.

The intensity of staining decreased abruptly along the villi, and little or no vitamin D receptor immunoreactivity was seen above mid-villi or at the villous tips. Some weak cytoplasmic staining was seen in crypt cells, which seemed significant and was reproducible.

SPECIFICITY OF STAINING REACTION

We carried out experiments to assess the specificity of vitamin D receptor staining in

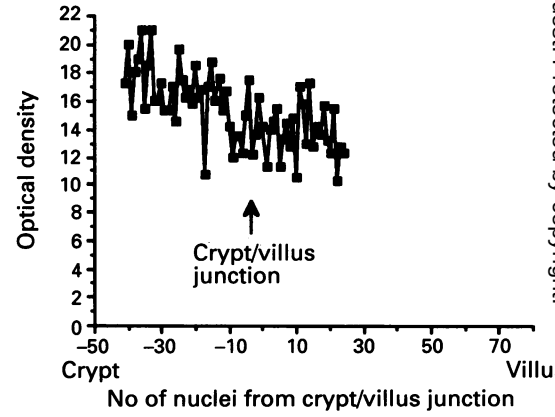

Figure 6: Quantitative densitometry of vitamin D receptor staining in enterocyte nuclei from crypt base to surface mucosa in six quantifiable regions from three patients with untreated coeliac disease. Mean nuclear absorbance on vertical axis, and position relative to crypt/villous junction on horizontal axis.

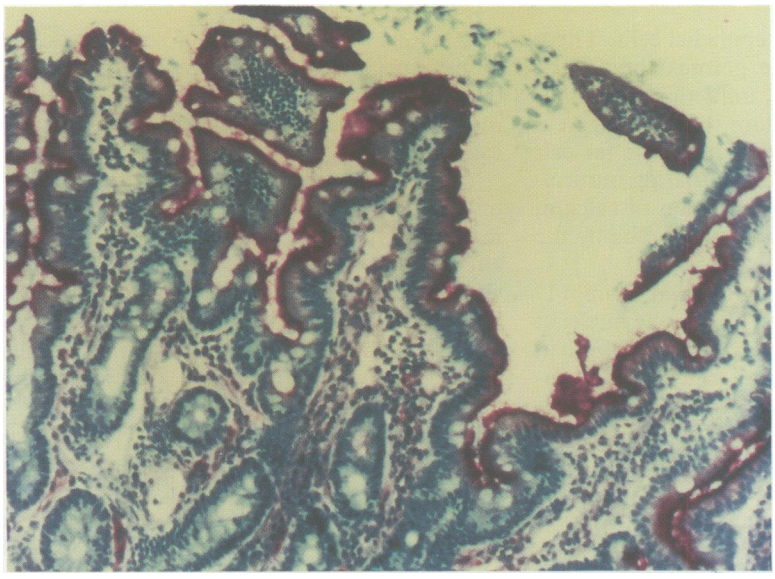

Figure 7: Alkaline phosphatase staining in normal duodenal mucosa ×100 (frozen section). Staining is only seen in villi (brush border) and not in crypts.

three ways. Firstly no nuclear staining was seen when normal rat IgG was substituted for the monoclonal antibody 9A7γ on control slides. Secondly, the nuclear staining associated with 9A7γ was effectively inhibited by preabsorption of the antibody with pure antigen. Preabsorption of the monoclonal antibody before the staining procedure eliminated the specific staining reaction in MCF7 cells (not shown). Thirdly, no staining was seen in a receptor negative cell line (Hs811), but present in receptor positive culture lines (Te85C and HTB96).

Table II shows that there is concordance between the presence of hormone binding and immunocytochemical staining in the three human osteosarcoma cell lines examined.

DUODENAL VITAMIN D RECEPTOR STAINING IN COELIAC DISEASE

Biopsy specimens from four patients with untreated coeliac disease, and a further four coeliac patients after treatment with gluten free diet were sectioned and incubated with monoclonal antibody 9A7γ or normal rat IgG.

In specimens obtained from coeliac patients before treatment with a gluten free diet, vitamin D receptors like immunoreactivity was mostly localised to nuclei of cells lining the crypts. Some weak focal staining was also detectable on surface enterocytes (Figs 5, 6, and Table I). Specimens with villous atrophy, regardless of treatment status, showed similar features.

DISTRIBUTION OF ALKALINE PHOSPHATASE STAINING

In contrast with the predominantly nuclear localisation of vitamin D receptors, alkaline phosphatase staining was almost entirely confined to the enterocyte brush border in histologically normal duodenal mucosa. In further contrast with the location of vitamin D receptor immunoreactivity, very little staining reaction was seen in the crypts (Fig 7 and Table I) and this was extremely superficial.

Biopsy specimens from patients with coeliac disease showed strong lumenal (brush border) staining for alkaline phosphatase on surface enterocytes. In some of the villous atrophy sections, however, crypts close to the surface showed focal lumenal staining. The intensity of staining was uniformly strong throughout (Fig 8 and Table I).

SEARCH FOR IMMUNOREACTIVE OESTROGEN RECEPTOR PROTEIN IN DUODENAL BIOPSY SPECIMENS

Fourteen specimens from histologically normal duodenal mucosa and eight specimens from patients with coeliac disease were incubated with H222, a monoclonal antibody to the oestrogen receptor protein. No immunoreactivity to oestrogen receptor was seen in any specimen.

Discussion

In animals and humans active calcium absorption occurs primarily in the duodenum and is regulated by the secosteroid hormone 1,25 $(OH)_2$ D_3. This active metabolite of vitamin D functions by a classic steroid hormone mechanism through receptors localised to the nuclei of intestinal cells. Nuclear localisation is associated with increased RNA synthesis, the induction of vitamin D dependent proteins, and an increase in calcium absorption.[2 13 15]

1,25 $(OH)_2$ D_3 regulates intestinal brush border alkaline phosphatase activity,[16] and participates in various steps in the transport of

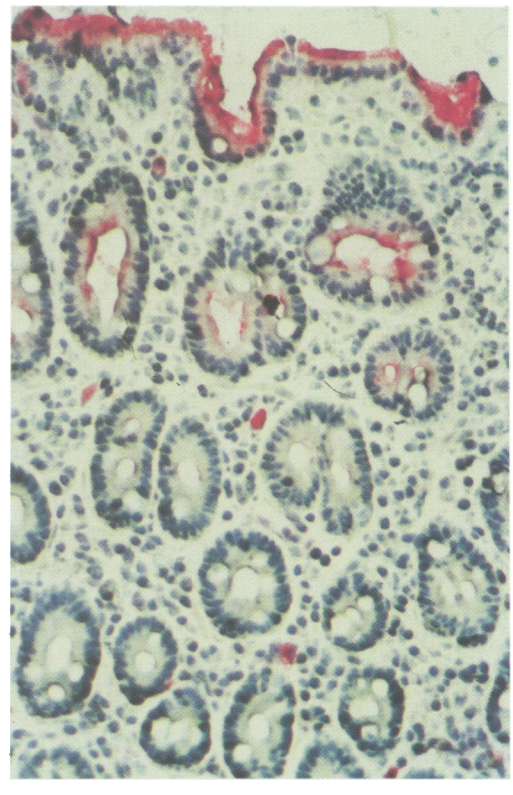

Figure 8: Alkaline phosphatase staining in subtotal villous atrophy (coeliac disease) ×160 (frozen section). Staining is only seen on surface mucosa and gland necks, but not in crypts.

calcium across intestinal epithelial cells. These include regulation of the permeability of the brush border membrane, induction of synthesis of a cytosolic calcium binding protein (which acts as an intracellular shuttle for calcium and may play a part in buffering intracellular calcium), and also regulation of calcium efflux across the basolateral membrane by the calcium pump.[2 3 17 18]

In earlier studies we reported that vitamin D receptors are widely distributed in the nuclei of epithelial cells from a variety of normal human tissues.[10] The predominant nuclear localisation of vitamin D receptors seen in immunocytochemical studies of tissue sections, typical of steroid hormones, is consistent with the results of previous biochemical studies.

Our studies show that in both normal and abnormal duodenal mucosa there is a preferential distribution of vitamin D receptors to the nuclei of cells lining the crypts, while staining for alkaline phosphatase, a vitamin D regulated protein, was seen almost exclusively lining the brush border of villi and surface enterocytes. Animal studies have shown that other vitamin D regulated proteins such as calbindin are present in enterocytes lining the villi, and that there is a gradient of vitamin D regulated enzyme activity, and active calcium transport in the duodenal mucosa, with the proliferative crypt and villous base regions having lower calcium transport activity than the functional mid and villous tip regions.[2 16]

Interestingly, vitamin D receptor is not detected in those enterocytes most active in calcium transport. Taken together these findings suggest that the major function of calcitriol on small intestine is to promote differentiation of immature enterocytes in the crypts and at the base of villi. During migration up the villi these cells become mature enterocytes capable of expressing vitamin D regulated functions required for calcium absorption.

Our immunocytochemical findings may also shed some light on the pathogenesis of the calcium malabsorption commonly seen in coeliac disease. The conventional explanation for impaired calcium absorption in gluten induced enteropathy is that it is a consequence of steatorrhoea and malabsorption of fat soluble vitamins resulting in reduced bioavailability of vitamin D and calcium.[19 20] The recognition, however, that dietary vitamin D makes a negligible contribution to body stores in comparison with the contribution from endogenous photosynthesis[21 22] makes this explanation unconvincing.

Our immunocytochemical studies suggest that calcium malabsorption in patients with coeliac disease cannot be explained by an absence of 1,25 D responsive cells from the small intestinal mucosa. The malabsorption seems to result from the loss of vitamin D regulated proteins and enzymes involved in active calcium absorption, which are preferentially located in the functional zone of the small intestinal mucosa (especially the most mature enterocytes of the mid and tip villous regions), as a result of gluten induced mucosal damage and villous atrophy. Assay of small intestinal biopsy specimens from patients with coeliac disease has shown significant reductions in mucosal alkaline phosphatase[18 23] and mucosal calbindin.[18]

Although vitamin D supplements are recommended for the treatment of hypocalcaemia in coeliac disease (and might have an effect by upregulating available intestinal vitamin D receptors and vitamin D regulated proteins,[24 25] gluten withdrawal is the appropriate treatment, as recovery of normal villous architecture will restore the cellular machinery for active calcium absorption.

As the postmenopausal period is also associated with impaired calcium absorption, which can be corrected by oestrogen administration,[26] we searched for oestrogen receptors in duodenal mucosa from healthy premenopausal women and in patients with coeliac disease. The negative findings in our study using conventional immunocytochemistry are in keeping with autoradiography for oestrogen receptor in baboon intestine.[27] In view of the known clinical responsiveness of the intestine to oestrogen and its permissive action on vitamin D induced calcium absorption, more sensitive molecular techniques should be applied to human intestine to search for low abundance oestrogen receptors. It is of interest that recent studies on cultured rat small intestinal crypt cells using a reverse transcriptase polymerase chain reaction technique showed that rat intestinal epithelial cell lines contained mRNA for oestrogen receptor.[28] These findings from animal studies raise the possibility that impaired calcium absorption and osteoporosis after the menopause might, in part, be mediated through an oestrogen effect on the gut.

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