Virchows Arch (2000) 436:466–472

ORIGINAL ARTICLE

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The pattern of prion-related protein expression in the gastrointestinal tract

Received: 15 September 1999 / Accepted: 23 December 1999

Abstract Prion diseases or transmissible spongiform encephalopathies have been shown to be communicated by oral ingestion of the infectious agent. However, the exact route of transmission is still unknown. In order to better understand the pathophysiology of these diseases, it is crucial to identify cell types of peripheral tissues in which the infectious agent may propagate. Since expression of cellular prion protein (PrP^c) is a prerequisite for prion replication, we determined the expression of PrP^c in the mucosa of the gastrointestinal tract using immunohistochemistry. Expression of PrP^c was negative or weak in the neck region of the gastric mucosa and moderate to strong in crypts of both the small and the large bowel. PrP^c was found to be upregulated in the mucosa of patients with *Helicobacter pylori* gastritis. In contrast, PrP^c staining appeared to be downregulated in patients with inflammatory disorders of the large bowel and it remained moderate to strong in inflammatory disorders of the small bowel. Our results support the notion that epithelial cells of the gastrointestinal tract may represent a possible target for prion entry and replication.

Key words Prion protein · Gastrointestinal mucosa · Epithelia · Inflammation

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Introduction

Transmissible neurodegenerative encephalopathies, also named prion diseases, comprise Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease, and Kuru in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep [27, 29]. Prion diseases in humans are rare; the incidence is one per million people. Recently, new variant Creutzfeld-Jakob disease (nvCJD), possibly transmitted from cattle infected with BSE to humans [9, 33], raised the interest in these infrequent disorders. Since more than a million cattle with BSE may have entered the human food chain [1] and the incubation time in humans is up to 40 years in these diseases [29], further cases of nvCJD might occur in the future.

According to the prion hypothesis developed by Prusiner [27], the agent of transmissible neurodegenerative encephalopathies is composed of protein. This protein named PrP^{Sc} is a converted form of a normally occurring glycoprotein (PrP^c) expressed in the central nervous system and many other parts of the human body. Through a profound conformational change, PrP^c is thought to be converted into the infectious agent. This conformational change is induced by the infectious protein PrP^{Sc} [27]. Accordingly, PrP^c expression by target cells is a prerequisite for the occurrence and spread of these diseases [8, 28].

PrP^c was found to be expressed in various extraneural tissues [4, 19]. However, until now, it is still unknown where the initial steps of prion infection occur. Possible sites are cells lining the luminal surface of the gastrointestinal tract, such as squamous epithelial cells of the oral mucosa and the esophagus that have been shown to express PrP^c [26]. That also epithelial cells of the remainder of the gastrointestinal tract might be involved in transmission of the disease is supported by the immunoelectron microscopic finding that normal gastric mucous and parietal cells [12] are positive for PrP^c. Further evidence comes from the recent demonstration that gastrointestinal epithelia of primates fed with BSE-infected cattle brain stained for PrP^{Sc} [5, 6]. Since the distribution

of PrP^c in the mucosa of the entire normal and diseased gastrointestinal tract has not yet been studied in detail, we investigated the expression of this protein by the gastrointestinal mucosa.

Methods

Immunohistochemistry

In this retrospective study, formalin-fixed, paraffin-embedded tissues were obtained from the files of the Institute of Clinical Pathology. Biopsy specimens taken from the esophagus, stomach, small bowel and colon, and resection specimens of the small bowel and colon (Table 1) were studied.

Paraffin sections were immunostained for PrP^c using monoclonal antibodies (mAb) 3F4 (1:400, Chemicon, Temecula, Calif.), 6H4 (1:300, Prionics AG, University of Zurich, Switzerland [21]) and MA1-750 (1:300, Affinity Bioreagents, Colden, Co.). Antigen retrieval was performed by boiling sections in 0.01 mol/l citrate buffer (pH 6) at 450 W for 20 min in a microwave oven (mAb 3F4 and 6H4) or in an autoclave (MA1-750). Primary mAbs were applied overnight. The immunohistochemical staining was performed using a biotinylated anti-mouse immunoglobulin and, subsequently, a streptavidin-biotin complex alkaline phosphatase system (Super Sensitive Detection System, Biogenex, San Ramon, Calif.).

Staining was performed on sections of all cases with mAb 3F4, on 20 sections with mAb 6H4 and on 26 sections with mAb MA1-750. Negative controls were carried out on consecutive tissue sections of all specimens using isotype antibodies (IgG1 or IgG2a, Coulter, Hialeah, Fla.). Sections were counterstained with hematoxylin.

Staining intensity of glandular tissue was semi-quantitatively determined and graded 0 to 3 (0, negative; 1, weak; 2, moderate; 3, strong expression). Staining intensities were assessed independently by two pathologists (J.P. and G.O.). In cases with differing results, a final conclusion was found after discussion of the findings. G-cells of the antro-pyloric mucosa were detected with a polyclonal anti-gastrin/cholecystokinin (CCK) ab (B 36-1, Accurate Chemical and Scientific Corp., Westbury, N.Y.) using the horseradish peroxidase (HRP)-labeled streptavidin-biotin staining technique (Vector, Burlingame, Calif.).

Statistical analysis was performed using the Mann-Whitney Wilcoxon test; the level of significance was set at $P<0.05$.

Table 1 Specimens investigated for cellular prion protein (PrP^c) expression

	Number of cases
Esophagus	
Regular	5
Epithelial hyperplasia	2
Ulceration	5
Corpus	
Regular	3
Mild chronic inactive gastritis	4
Chronic active H.pylori gastritis	7
Antrum	
Regular	3
Chemical gastritis	2
Mild chronic inactive gastritis	8
Chronic active *Helicobacter pylori* gastritis	12
Small bowel	
Regular	7
Celiac disease	6
Unspecific duodenal ulceration	1
Crohn's disease	3
Large bowel	
Regular	10
Infectious colitis	3
Crohn's disease	7
Ulcerative colitis	2

Cell culture and Western blotting

Human colon tumor cell lines Caco-2/15 [2], Caco-2/AQ, HT-29 (ATCC HT-38), PC52, and PC53 [30] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (all GIBCO BRL, Gaithersburg, Md.), containing 10 mmol/l Hepes, 4.0 mmol/l glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The neuroblastoma cell line HTB-10 (ATCC SK-N-MC) and the medulloblastoma cell line HTB-186 (ATCC Daoy) were grown in minimal essential medium including Earle's BSS supplemented with 0.1 mM non-essential amino acids (K0293, Schöller Pharma, Vienna, Austria). Primary human and bovine keratinocytes were cultured as described previously [26]. For stimulation experiments, cells were grown in medium supplemented with interferon gamma (IFN-γ; 1000 U/ml, Imukin, BenderMed, Vienna, Austria), transforming growth factor alpha (TGF-α; 100 ng/ml, Eubio, Vienna, Austria), or tumor necrosis factor alpha (TNF-α; 200 U/ml, Biomol, Hamburg, FRG) for 24 h.

Cell lines were lysed in phosphate-buffered saline (PBS) containing 1% NP40, 1% sodium dodecyl sulfate (SDS) and 1 mM phenylmethylsulfonyl fluoride (PMSF). In one set of experiments, maximal amounts of protein were loaded in each lane (Fig. 3A) to detect PrP^c also at low concentrations. In another set of experiments, equal amounts of protein were loaded after measurement of protein concentrations by a Micro BCA Protein Assay Reagent (Pierce, Rockford, Ill.) (Fig. 3B) to allow comparison of the relative amounts of protein. SDS/polyacrylamide gel electrophoresis and Western transfer to nitrocellulose membranes were performed under standard conditions [25]. Membranes were reacted to mAbs 3F4, 6H4, MA1-750, and SAF70 (provided by CEA, Service de Pharmacologie et d´Immunologie, Saclay, France), followed by HRP-labeled sheep anti mouse-IgG (Amersham Life Science, UK). After rinsing in enhanced chemiluminescence reagent (Amersham Life Science), membranes were exposed to a X-OMAT-AR film (Eastman Kodak, N.Y.).

Results

PrP^c expression in esophageal squamous epithelium

Cytoplasmic PrP^c expression in regular squamous epithelia of the esophagus was moderate and mainly confined to basal cells with only a few suprabasal cells expressing PrP^c weakly (Fig. 1A). In specimens with epithelial hyperplasia taken from patients with gastrointestinal reflux

Fig. 1 Expression of cellular prion protein (PrP^c) by epithelial cells of the gastrointestinal tract. Whereas PrP^c expression is confined to basal cells of regular esophageal epithelium (A), it is distinctly upregulated in hyper-regeneratory epithelium (B). This epithelium shows both cytoplasmic and membranous staining for PrP^c. Neuroendocrine cells of the antrum label strongly for PrP^c (C, *arrows*). PrP^c expression is upregulated in the neck region in chronic active *Helicobacter pylori* gastritis of the corpus (D, *arrows*) and the antrum (E). Crypts of the small bowel (F) and the colon (G) stain distinctly for PrP^c. A focal downregulation of PrP^c is found in this case of infectious colitis (H). Original magnifications: **A**, **B**, **D–G** 200×, **C** 160×, **H** 125× ▶

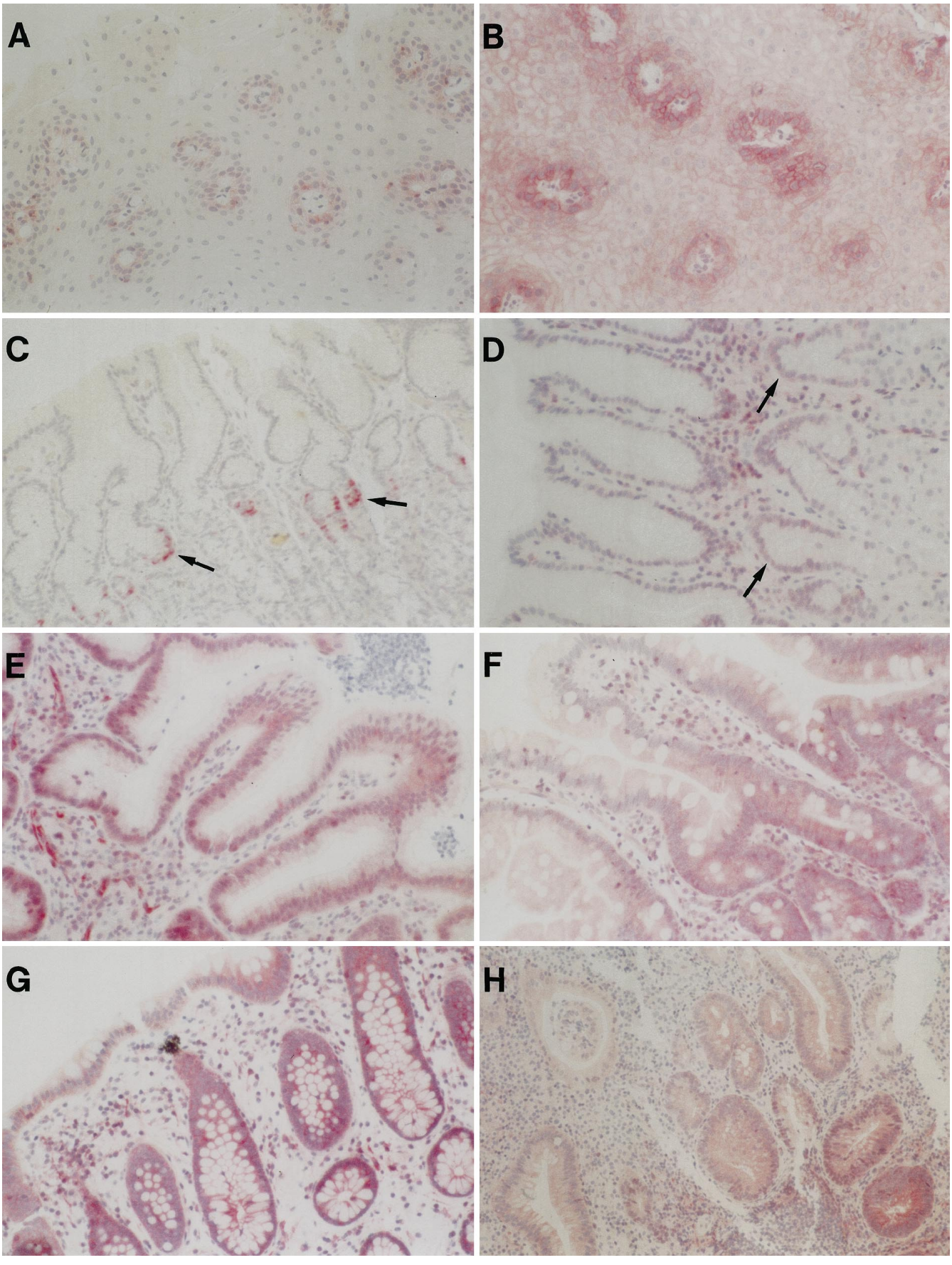
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Table 2 Expression of cellular prion protein (PrP^c) in glandular epithelia of the gastrointestinal tract. Results were obtained with mAb 3F4. Cases with inflammation in the large and small bowel include celiac disease, Crohn's disease, ulcerative colitis, and infection. Significance of differences in staining intensities of different histologic types are as assessed using the Mann-Whitney Wilcoxon test

[a] $P<0.009$; [b] $P<0.04$

		Number	Relative staining intensity			
			–	+	++	+++
Corpus	Regular/mild chronic inflammation	7	3	3	1	0
	Chronic active gastritis	7	0	3	4	0
Antrum	Regular/mild chronic inflammation	13	2	6	5	0[a]
	Chronic active gastritis	12	0	3	3	6[a]
Duodenum	Regular	5	0	0	1	4
	Inflammation	7	0	2	1	4
Ileum	Regular	2	0	0	2	0
	Inflammation	3	0	1	2	0
Colon	Regular	10	0	0	3	7[b]
	Mild inflammation	4	0	0	2	2
	Heavy inflammation	8	0	3	3	2[b]

disease, the squamous epithelium showed a moderate to strong predominantly membranous expression of PrP^c (Fig. 1B). Staining was observed in all layers of the epithelium, although only focally, with decreasing intensity in the superficial layers. Results were largely identical with those of mAbs 3F4, 6H4 and MA1-750.

PrP^c expression in epithelial cells of the stomach

Gastric corpus epithelia of regular mucosa and cases with chronic inactive gastritis were generally negative or only weakly positive for PrP^c (Table 2). PrP^c expression was mostly confined to the neck region of the pits. Gastric corpus glands were considered negative, although they showed a weak focal staining, which, however, was also found in specimens stained with isotype-matched control antibodies. Similarly, focal weak PrP^c expression of the glandular tissue of regular antrum mucosa, and cases with chronic inactive gastritis and chemical gastritis was also confined to the neck region. Furthermore, endocrine cells in the neck regions, that were immunohistologically gastrin-positive on consecutive tissue sections, were strongly PrP^c positive (Fig. 1C). The pyloric glands were negative and the remainder of the pits were mostly negative for PrP^c.

Regenerating epithelia of the neck regions of corpus- (Fig. 1D) and antrum- (Fig. 1E) mucosa with chronic active *Helicobacter pylori* gastritis showed moderate staining for PrP^c. Whereas epithelia of the glands were still negative, the remainder of the pits also showed faint expression for PrP^c. In contrast to mAb 3F4, mAb 6H4 stained epithelia of the stomach only weakly, whereas mAb MA1-750 also stained superficial epithelium of the gastric foveolae in addition to the neck regions. Gastric corpus glands were strongly stained with both mAbs 6H4 and MA1-750 as well as with an isotype control Ab. Therefore, a potentially specific staining could not be discriminated from background staining.

PrP^c expression in epithelial cells of the small bowel

Distinct PrP^c expression in regular duodenal and ileal epithelia was confined to intestinal crypts (Fig. 1F; Table 2). In contrast, epithelium covering the villi showed only weak staining in their lower third. Paneth cells, goblet cells, and Brunner glands were considered negative for PrP^c. There was no upregulation of PrP^c in duodenal epithelia of specimens with celiac disease and ileal mucosa with inflammatory changes due to Crohn's disease. In contrast to these results obtained with mAb 3F4, mAb 6H4 did not convincingly stain epithelia of the small and large bowel. Mab MA1-750 weakly decorated villi, and only focally crypts.

PrP^c expression in epithelial cells of the large bowel

Epithelial cells of the bowel showed a moderate to strong expression of PrP^c (Fig. 1G; Table 2). The staining intensity was stronger in the lower third of the crypts and only weak or negative on the surface. Unlike epithelia of the stomach, colonic crypts showed a focal downregulation of PrP^c in cases with acute inflammation of the lamina propria (Fig. 1H). A similar staining was found in the crypts with mAb MA1-750 when compared with mAb 3F4. In parallel to the findings in the stomach and the small bowel, the superficial epithelium of the colon was partially weakly labeled with this mAb.

PrP^c expression in non-epithelial cells

PrP^c was also detected in nerves and lymphocytes with mAbs 3F4, 6H4, and MA1-750. Therefore, cells of the plexus submucosus, the plexus myentericus (Fig. 2A) and small nerve fibers in the lamina propria, which were found to be strongly positive for PrP^c, as well as lymphocytes served as an internal positive control. In specimens with Crohn's disease of the small bowel and the colon, proliferating PrP^c positive nerves were found in ulcers. These nerve fibers reached the lumen of the bowel (Fig. 2B), which makes them a possible target for prions. Smooth muscle cells were mostly negative or

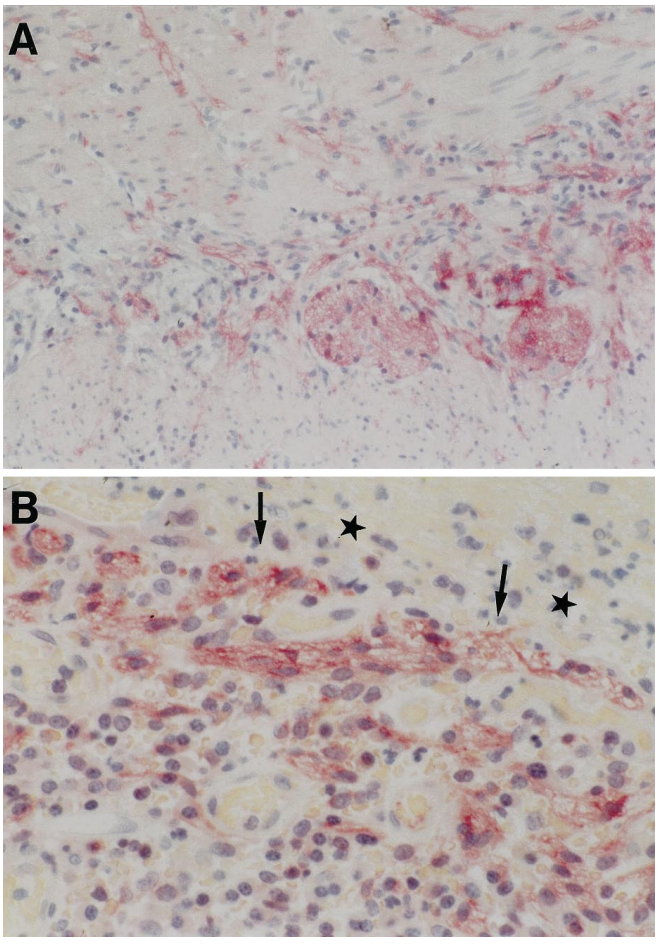

Fig. 2 Expression of cellular prion protein (PrP^c) by nerval tissue of the gastrointestinal tract. Nerves within the muscularis propria (A) and proliferating nerves (*arrows*) adjacent to an ulceration of the colon (B) strongly stain for PrP^c. The *asterisks* mark the lumen of the colon. Original magnifications: **A** 200×, **B** 400×

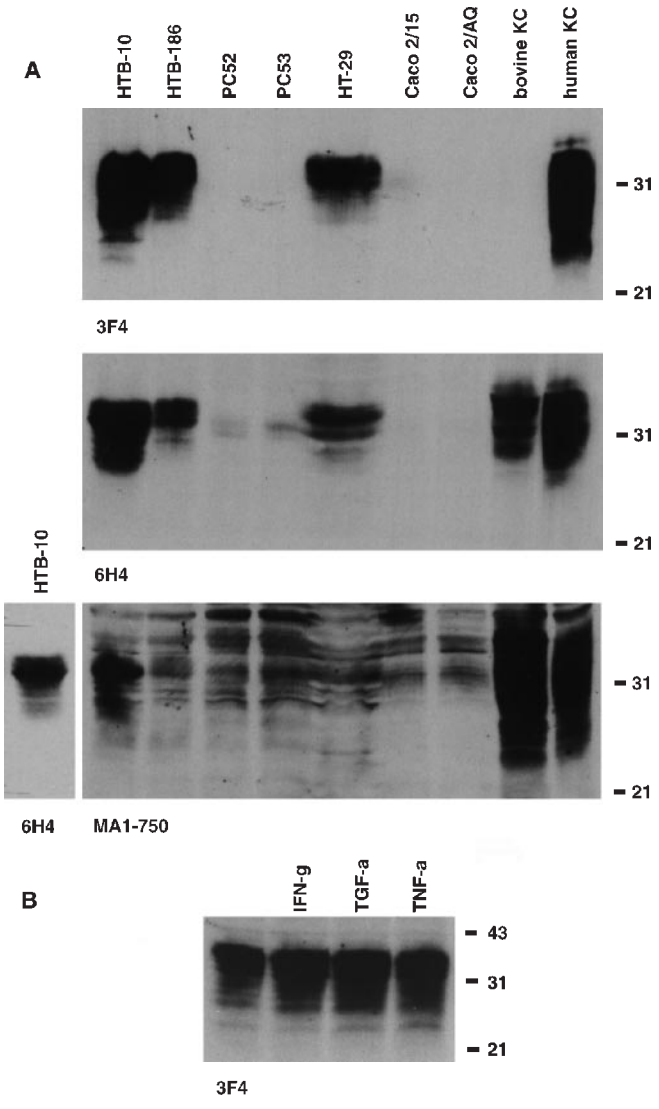

Fig. 3 Expression of cellular prion protein (PrP^c) in colon tumor cell lines. **A** Whereas mAb 3F4 detected PrP^c only in HT-29 cells, PrP^c was also weakly detected by mAb 6H4 in PC52 and PC53 cells and in all five colon tumor cell lines by mAb MA1-750. For a better comparison, a shorter exposure of the immunoblot showing PrP^c in HTB-10 cells using mAb 6H4 is attached to the MA1-750 blot. **B** PrP^c was constitutively expressed in HT-29 cells and slightly upregulated by transforming growth factor (TGF)-α (*lane 2*) and interferon (IFN)-γ (*lane 3*)

showed only weak staining for PrP^c adjacent to ulcerations and fistulas of the bowel.

When comparing the staining intensities of glandular epithelial cells with these internal positive controls, epithelial PrP^c staining graded as strong in this study was weaker than that of nerves. This difference was more pronounced in surgical specimens than in biopsy specimens, which might indicate an effect of the fixation on antigen preservation. Comparing the various mAbs used, labeling of epithelial cells by mAb 3F4 was stronger than that of lymphocytes of the lamina propria. In contrast, mAbs 6H4 and MA1-750 generally stained lymphocytes more intensely. Only the staining intensity of neuroendocrine cells of the stomach was similar to that of nerves.

Expression of PrP^c by colon carcinoma cell lines

Since the three mAbs exhibited different staining patterns in the bowel, we investigated their reactivity to five different colon tumor cell lines (HT-29, Caco-2/15, Caco-2/AQ, and the primary cultures PC52 and PC53) in order to demonstrate the specificity of the binding of mAbs to gastrointestinal epithelial cells. Lysates of two brain tumor cell lines and human and bovine keratinocytes were used as internal controls. Labeling of HT-29 cells with mAb 3F4 revealed a specific pattern of bands in immunoblots, whereas no distinct reactivity for PrP^c was found in the other four tumor cell lines. By contrast, PrP^c was also weakly detected by mAb 6H4 in PC52 and PC53 cells and in all five colon tumor cell lines by mAb MA1-750 (Fig. 3A). Notably, this immunoblot also shows staining of proteins larger than PrP^c, most proba-

bly due to cross reactions. Thus, the specificity of PrP^c staining of the five colon carcinoma cell lines with mAb MA1-750 was further confirmed by an immunoblot using mAb SAF70, which also detected PrP^c in these five cell lines (data not shown). Since mAbs 6H4 and MA1-750 are directed against bovine PrP^c, we included primary keratinocytes as a positive control.

PrP^c was slightly upregulated in HT-29 cells by TGF-α and IFN-γ (Fig. 3B) using mAb 3F4. No upregulation was found in PC52 and 53 cells using mAb MA1-750.

Discussion

In the present study, we demonstrate that PrP^c is constitutively expressed in both squamous and glandular epithelia of the gastrointestinal tract. Whereas staining with both 3F4 and 6H4 mAbs was essentially identical in squamous epithelia of the esophagus, the latter was virtually negative on glandular epithelia of the gastrointestinal tract. This finding mirrors results of other studies showing that mAb 3F4 is able to decorate cells that are negative with other anti-PrP^c antibodies [12]. It was suggested that masking of antigens by other proteins or their modification by tissue fixation may lead to false-negative results with some anti-PrP^c antibodies [12]. Also, the occurrence of several post-translational modifications of PrP^c which were recently observed in various non-neural tissues [20] might explain the differing performance of the three mAbs.

In order to exclude false-positive staining elicited by a cross reaction with another antigen, Western-blot analysis both with tissue homogenates of gastrointestinal mucosa and colon carcinoma cell lines was performed. These blots showed specific bands supporting the notion that the mAbs used in this study indeed recognize PrP^c in the gastrointestinal mucosa. Notably, the relative staining intensities of the five colon tumor cell lines as well as the intensity of the medulloblastoma cell line HTB-186 in comparison with these five cell lines varied strongly in immunoblots (Fig. 3A). Therefore, the staining intensity was not only dependent on the mAb used and its species specificity but also on the cell line investigated. We suggest that post-translational modifications of the protein may differ in various cell lines and cell types. Antibodies recognizing different epitopes might therefore show a different staining pattern or staining intensity. This could account for the observed differences in the results obtained with the three mAbs in the gastrointestinal tract.

The function of PrP^c in epithelial cells and in other tissues is still unknown. In lymphocytes, expression of PrP^c is associated with cell activation [10] and proliferation [23]. We have detected upregulation of PrP^c expression by keratinocytes in lesions with enhanced proliferation, such as wounds and psoriasis. Such an overexpression was inducible by TGF-α in vitro [26], paralleling findings of the present study in which expression of PrP^c was also shown to be most prominent in regions with proliferative activity, i.e., basal cells of squamous epithelia of the esophagus, neck regions of glandular epithelia of the stomach, and crypts of the small and the large bowel. PrP^c acts as an anti-apoptotic protein in immortalized neuronal precursor cells [22], and it might have a similar function in proliferating and activated gastrointestinal epithelial cells, i.e., enhancing cell survival.

The demonstration of PrP^c expression in gastrointestinal epithelia is of interest in view of the hypothesis that nvCJD is linked to BSE through oral exposure to the infectious agent. Similarly, kuru may also have been transmitted through oral ingestion [13]. Therefore, PrP^c expressed by gastrointestinal epithelia may serve as a receptor for prions [18], representing their port of entry. From there, the infectious agent could spread to lymphoid tissue [16, 17, 31] and peripheral nerves [15, 24]. Both have been found to contain PrP^{Sc} in prion diseases.

However, prions could also infect epithelial cells expressing PrP^c. A recent experimental study, in which lemurs were infected with BSE, demonstrated PrP^{Sc} in epithelia of the tonsils and throughout the digestive tract, confirming an oral route of transmission [5, 6]. In this study, pretreatment of sections with formic acid was performed before immunohistochemistry using mAb 3F4 to discern protease-resistant and possibly infectious PrP^{Sc} from physiological PrP^c. The authors reported reactivity for PrP^{Sc} in both basal cells and a number of flattened superficial cells of the stratified epithelium of the tonsils and the esophagus. PrP^{Sc} was also detected in glands of the stomach, whereas columnar epithelium of the gastric pits was either negative [6] or positive at the luminal surface only [5]. In the small intestine, PrP^{Sc} reactivity was found in epithelial cells of the villi. In the colon, PrP^{Sc} was located in the superficial columnar epithelial cells but not in the crypts [6]. This pattern found in lemurs differs in some aspects from the PrP^c expression observed in the human gastrointestinal tract in this study using the same mAb. In particular, we found PrP^c mainly in proliferating epithelial cells of the human gastrointestinal mucosa, whereas Bons et al. observed PrP^{Sc} in differentiated cells. This may be due to slow synthesis and degradation of PrP^{Sc} in contrast to the fast turnover of PrP^c [7], resulting in an accumulation of PrP^{Sc} to levels exceeding that of PrP^c in differentiated epithelial cells as opposed to progenitor cells.

In conclusion, we have investigated the expression pattern of PrP^c in the mucosa of the gastrointestinal tract for the first time. Mucosal epithelial cells express PrP^c and may therefore represent a first target for PrP^{Sc} after oral inoculation. Acute inflammation of the gastric mucosa resulting in an upregulation and possibly a secretion of PrP^c by epithelial cells [12] could account for an individual predisposition to infections by prions. The fact that primates [6, 14], cattle [32], sheep, goats [11], rodents [3], and other animals are susceptible to prion diseases through oral exposure should stimulate further research in the involvement of epithelial cells in the pathogenesis and transmission of prion diseases.

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