Cytokeratin 18 is a specific marker of bovine intestinal M cell

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Hondo T, Kanaya T, Takakura I, Watanabe H, Takahashi Y, Nagasawa Y, Terada S, Ohwada S, Watanabe K, Kitazawa H, Rose MT, Yamaguchi T, Aso H. Cytokeratin 18 is a specific marker of bovine intestinal M cell. Am J Physiol Gastrointest Liver Physiol 300: G442–G453, 2011. First published December 30, 2010; doi:10.1152/ajpgi.00345.2010.—Microfold (M) cells in the follicle-associated epithelium (FAE) of Peyer’s patches have an important role in mucosal immune responses. A primary difficulty for investigations of bovine M cells is the lack of a specific molecular marker. To identify such a marker, we investigated the expression of several kinds of intermediate filament proteins using cell Peyer’s patches. The expression patterns of cytokeratin (CK) 18 in jejunal and ileal FAE were very similar to the localization pattern of M cells recognized by scanning electron microscopy. Mirror sections revealed that jejunal CK18-positive cells had irregular and sparse microvilli, as well as pocket-like structures containing lymphocytes, typical morphological characteristics of M cells. However, CK18-negative cells had regular and dense microvilli on their surface, typical of the morphology of enterocytes. In contrast, CK20 immunoreactivity was detected in almost all villous epithelial cells and CK18-negative cells in the FAE. CK18-positive proliferating transit-amplifying cells in the crypt exchanged CK18 for CK20 above the mouth of the crypt and after moving to the villi; however, CK18-positive M cells in the crypt continued their expression of CK18 during movement to the FAE region. Terminal deoxynucleotidyl-transferase-mediated deoxyuridine-triphosphate-biotin nick-end labeling-positive apoptotic cells were specifically detected at the apical region of villi and FAE in the jejunum and ileum, and all were also stained for CK20. These data indicate that CK18 may be a molecular marker for bovine M cells in FAE and that M cells may transdifferentiate to CK20-positive enterocytes and die by apoptosis in the apex of the FAE.

bovine M cell; cytokeratin 18; apoptosis; Peyer’s patch; follicle-associated epithelium

IN THE SMALL INTESTINE, pluripotential stem cells reside deep within the crypts and give rise to various progressively differentiating epithelial cells through the commitment and proliferation of lineage (31, 44). These cells migrate up toward the crypt mouth and along the villi to be shed from the tip (18). On the other hand, the follicle-associated crypts supply enterocytes and M (membranous or microfold) cells on to the follicle-associated epithelium (FAE) (11). M cells are specialized cells within the FAE overlying the gut-associated lymphoid tissue (41). M cells play an important role in the inception of the mucosal immune response as delivering microorganisms and macromolecules by the active transepithelial vesicular transport from lumen directly to intraepithelial lymphoid cells and subepithelial lymphoid tissue (37). Therefore, antigen-presenting cells in the mucosal immune system encounter various antigens entering the body through the gut mucosa (19).

Typically, M cells exhibit unique morphology that differs from the surrounding absorptive enterocytes (47). The basolateral membrane of M cells is deeply invaginated and forms a pocket-like structure that holds lymphocytes (2, 10, 36). Additionally, it is well known that M cells in the small intestine possess irregular and short microvilli in addition to the microfold (4). Despite the important role of M cells in mucosal immune systems, the detailed characterization and life cycle of M cells remains unclear because of difficulties in identifying and isolating this minor cell population.

With respect to both their size and development, previous studies have reported that the ileal Peyer’s patches of ruminants are unique. Furthermore, it has been reported that they make up ~15% of the length of the small intestine (26, 48). The Peyer’s patches of ruminants are also thought to be histologically mature before birth and involute at a young age, in a similar way to the thymus gland (1, 48). There are fairly substantial differences in the distribution, structure, and development of ileal Peyer’s patches in ruminants relative to other mammalian species. However, the jejunal Peyer’s patches of ruminants resemble those of other mammalian species, as judged by light and electron microscopy (26). Despite the highly characteristic features of M cells and Peyer’s patches in ruminants, relatively few investigations have been published.

Studies of the FAE in bovine Peyer’s patches have reported the morphological characteristics of M cells using electron microscopy and enzyme histochemistry (23–25, 27, 28, 35, 42, 43, 54). The specific detection of M cells has been established only in the last few years. For most M cell markers, however, detection is restricted to a single species, and a universal M cell-specific marker is lacking. For example, the specific marker for M cells in BALB/c mice is α-L-fucose, which can be detected by lectin Ulex europaeus type I (7, 14, 52), and M cells in rats, pigs, and rabbits can be detected by intermediate filament proteins, such as cytokeratin (CK) 8 (22, 46), CK18 (13), and vimentin (16), respectively. It has recently been reported that the staining pattern for actin and villin distinguished bovine M cells in the FAE (17). However, the specific markers for bovine M cells and the expression of intermediate filament proteins in intestinal epithelium has not yet been reported.
In this study, we investigated the expression of intermediate filament proteins for epithelial cells in bovine Peyer’s patches. We reveal the specific expression of CK18 in bovine M cells and CK20 for other enterocytes and have observed apoptotic cells in FAE of Peyer’s patches expressing CK20.

MATERIALS AND METHODS

Animals and tissue preparation. The duodenum, jejunum, ileum, and colon were obtained from 6-, 8-, and 10-wk-old male Holstein calves. All animals were clinically healthy and free of infectious diseases. All experimentation was approved by the Institutional Office of Tohoku University and was conducted in accordance with the Institution’s guidelines for animal experimentation. These have been sanctioned by the relevant Japanese Government Committee.

Primary antibodies. Mouse anti-pan CK monoclonal antibody (MAb) (clone C-11, diluted to 1:2,000; Sigma, St. Louis, MO), mouse anti-CK7 MAb (clone RCK105, 1:1,000; abcam, Cambridge, UK), mouse anti-CK8 MAb (clone 35BH11, Ready-to-use; Dako, Carpinteria, CA), mouse anti-CK18 MAb (clone CY-90, 1:1,000; Sigma), mouse anti-CK18 MAb (clone KS-B17, 1:1,000; Sigma), mouse anti-CK18 MAb (clone CK5, 1:500; Sigma), mouse anti-CK19 MAb (clone A53-B/A2, 1:100; abcam), mouse anti-CK20 MAb (clone KS20.8, 1:2; Nichirei, Tokyo, Japan), and mouse anti-vimentin MAb (clone VIM 3B4, Ready-to-use; Progen, Heidelberg, Deutschland), and mouse anti-Ki-67 MAb (clone MIB-1, 1:100; Dako) were used for the immunohistochemistry.

Scanning electron microscopy. The samples from 6-wk-old calves were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 24 h at 4°C. After fixation, intestinal tissues were immediately cut into about 2 mm × 10 mm specimens. For mirror sections, the paraffin sections from 6-wk-old calves were mounted on silane-coated glass slides, dewaxed in xylene, rehydrated through a series of graded ethanol solutions, and transferred to PBS (pH 7.4). The sections were fixed with 2.5% glutaraldehyde in 0.1 M PB and treated with 0.5% tannic acid in 0.1 M PB for 10 min and 1% tannin acid in 0.1 M PB for 1 h at room temperature. After being washed with 0.1 M PB, the specimens and the sections were dehydrated through a series of graded ethanol solutions, substituted with r-butylalcohol, freeze-dried, and coated with platinum-palladium. The samples were examined by scanning electron microscopy (SEM) (S4200; Hitachi, Tokyo, Japan).

Immunohistochemistry. The samples from 6- or 8-wk-old calves were fixed with 4% paraformaldehyde solution in 0.1 M PB for 24 h at 4°C. Tissues were dehydrated through a series of graded ethanol solutions and embedded in paraffin. The sections (4 µm thick) were mounted on silane-coated glass slides, dewaxed in xylene, rehydrated through a series of graded ethanol solutions, and transferred to PBS. For staining of Ki-67, the sections were heated in an autoclave in Dako Target Retrieval Solution (Dako) for 5 min to facilitate antigen retrieval. After being blocked with 3% normal goat serum in PBS for 20 min, the sections were incubated with primary antibodies for 14 h at 4°C in a moist chamber. For staining using the other primary antibodies, the sections were incubated with primary antibodies for 14 h at 4°C in a moist chamber. These specialized cells were characterized as M cells from their surface characteristics, which have been reported previously (23, 54). These data show that there is a notable difference in the distribution patterns of M cells between the FAE of the bovine jejunum and ileum. Two consecutive sections were mounted on silane-coated slide glasses with the common cut surface facing upward, so that the same surface of sectioned cells could be stained by two different antibodies. To determine the colocalization of Ki-67-positive cells and CK18-positive cells, we used the mirror section technique. In addition, a couple of mirror sections were used to examine the ultrastructure of CK18-positive cells. One section was immunostained with mouse anti-CK18 (clone CY-90) MAb, and the other was examined with SEM.

Localization of M cells in villi and FAE of jejunum and ileum. To observe the localization of bovine M cells, we examined the ultrastructure of epithelium in villi and FAE of jejunum and ileum by SEM. Each villus was covered by absorptive enterocytes with regular and dense microvilli (Fig. 1, A and B). Jejunal FAE had not only absorptive enterocytes but also specialized cells with irregular and sparse microvilli, which presented randomly among the absorptive enterocytes (Fig. 1, C and D). The ileal FAE was almost filled up with the specialized cells having irregular microvilli, but not with absorptive enterocytes (Fig. 1, E and F). These specialized cells were characterized as M cells from their surface characteristics, which have been reported previously (23, 54). These data show that there is a notable difference in the distribution patterns of M cells between the FAE of the bovine jejunum and ileum.

Expression of CK18 in jejunal and ileal M cells. We investigated the expressions of intermediate filament proteins in jejunal and ileal Peyer’s patches. In jejunal Peyer’s patches, pan-CK was strongly stained in all epithelia of the villi, FAE,
and crypt (Fig. 2A). CY-90, Ks-B17.2, and CK5 clones of CK18 were specifically stained in the FAE and crypt, but not in the villi (Fig. 2D–F). CK20 was detected strongly in the villous epithelium and discontinuously in the FAE, but not the crypt (Fig. 2H). In contrast, CK7, CK8, and CK19 could not be detected in FAE and villous epithelium of the jejunum (Fig. 2B, C, and G). The subepithelial area was strongly positive for vimentin, but not epithelial cells in the villi and FAE (Fig. 2I).

Pan-CK was also stained in all of the ileal epithelia containing Peyer’s patches (Fig. 3A). Three kinds of anti-CK18 MAb clones specially reacted to the FAE and crypt, but not the villous epithelium (Fig. 3D–F). CK20 was detected on all villous epithelia and a few epitheliocytes of the FAE (Fig. 3H). CK7, CK8, and CK19 were not detected in any ileal Peyer’s patches (Fig. 3B, C, and G), and vimentin was only stained in the subepithelial area of the ileum (Fig. 3I), where staining properties were the same as in the jejunum. These results suggest that CK18 stains in the same locations as where M cells are clearly defined by the SEM analysis in Fig. 1.

Location of CK18-positive cells. In jejunal and ileal FAE, the expression patterns of CK18 were very similar to the localization pattern of M cells recognized by SEM in Fig. 1 (Fig. 4A, B, E, and F). In addition, CK18 was also detected in all crypt epithelium of jejunal and ileal Peyer’s patches (Fig. 4C and G). Intestinal epithelial cells are known to originate from common ancestral stem cells located in the lower part of the intestinal crypt (47). We investigated the proliferative ability of CK18-positive cells in the crypt using a mirror section technique. A couple of mirror sections were immunostained with CK18 or Ki-67 MAb, a marker for proliferative cells. All Ki-67-positive cells in the crypt were positive for CK18 (Fig. 4C, D, G, and H). These data indicate that CK18 is already expressed in proliferating cells of the crypt.

Ultrastructure of CK18-positive cells in the FAE of the jejunum and ileum. Figure 1 shows that M cells have irregular and sparse microvilli and differ clearly from absorptive enterocytes with their regular and dense microvilli. Furthermore, it is well known that the basolateral plasma membrane of M cells is deeply invaginated and forms a pocket-like structure containing lymphocytes (2, 10, 36). Therefore, we investigated the ultrastructure of CK18-positive cells in the FAE using several mirror sections. One section was stained with anti-CK18 MAb, and the other was examined by SEM. In jejunal FAE, CK18-positive cells had irregular and sparse microvilli and a few epitheliocytes of the FAE (Fig. 5A–C). The FAE of the ileum were almost entirely covered with CK18-positive cells, and all of them had the morphological characteristics of M cells with irregular and sparse microvilli (data not shown). To further clarify the ultrastructure of ileal CK18-positive cells, we subjected part of the FAE containing CK18-negative cells to SEM analysis. We clearly observed that ileal CK18-positive cells had irregular and sparse microvilli on their apical surface, as for absorptive enterocytes (Fig. 5D–F).
Fate of CK20-expressing intestinal epitheliocytes. To clarify the relationship between the expression of CK18 and CK20 in the bovine intestine, we stained sections with both anti-CK18 and anti-CK20 MAbs. CK18 was stained in jejunal FAE with high frequency and consistently in the ileal FAE (Fig. 6, A and E). CK20 immunoreactivity was detected in almost all villous epithelial cells (Fig. 6, B and F) and observed in CK18-negative cells in the FAE (Fig. 6, C and G). It is well known that epitheliocytes generate from the stem cell region of the crypt and move to the villi or FAE (13, 18). Photographs of the crypts (Fig. 6, D and H) indicated that crypt epitheliocytes may exchange CK18 for CK20 once above the mouth of crypt and when they have moved to the villi, whereas M cells appear to continue the expression of CK18 during their movement from the crypt to the FAE region.

It has been reported that M cells might transdifferentiate to enterocytes and finally be excluded near the FAE apex by apoptosis in the porcine intestine (32). To investigate the apoptotic process of bovine intestinal M cells, we performed a triplicate staining using anti-CK18 and anti-CK20 MAbs and the TUNEL method. The TUNEL assay showed that DNA fragmentation in the nuclei of epithelial cells is specifically detected at the apical region of the villi and FAE of both the jejunum and ileum (Fig. 7). In jejunal FAE, CK18-positive M cells and CK20-positive enterocytes were distributed randomly (Figs. 4B and 6C); however, TUNEL-positive cells were observed in the CK20-positive cells (Fig. 7, C and D). Whereas almost all of the ileal FAE was covered with M cells expressing CK18 (Figs. 4F and 6G), all TUNEL-positive cells were also stained for CK20 (Fig. 7, E and F). We prepared a large number of serial sections and searched for CK20-negative M cells and CK20-positive enterocytes, but we were not able to find any dual stained with TUNEL and CK18.

Configurational comparison of CK18- and CK20-positive cells in jejunal and ileal FAE. To clarify whether M cells transdifferentiate to enterocytes, we carefully counted the num-

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**Fig. 2.** Expressions of various cytokeratins and vimentin in jejunal Peyer’s patches. The sections were immunostained with anti-pan cytokeratin (CK) (C-11) (A), anti-CK7 (B), anti-CK8 (C), anti-CK18 (CY-90) (D), anti-CK18 (Ks-B17.2) (E), anti-CK18 (CK5) (F), anti-CK19 (G), anti-CK20 (H), and anti-vimentin (I) monoclonal antibodies. Bars = 100 μm.
ber of CK18- and CK20-positive cells in the jejunal and ileal FAE (Table 1). The sections containing TUNEL-positive cells were selected, and the distance from the mouth of the crypt to the apex of half of the FAE was divided into thirds: lower, peripheral, and apical regions. The staining of the cells in each region was counted. TUNEL-positive cells were only observed in CK20-positive cells at the apical region. The proportions of CK18-positive cells in the lower region were 45 and 96% in the jejunal and the ileal FAE, respectively. However, the number of CK20-positive cells gradually increased from the lower region to the apical region. The rate of CK18-positive cells in the apical region decreased to 21% in the jejunal FAE and 57% in the ileal FAE. These data strongly suggest that bovine intestinal M cells, positive for CK18, transdifferentiate to CK20-positive enterocytes and that possibly they may die by apoptosis at the apex of the FAE.

Expression patterns of CK18 and CK20 in duodenum and colon. We investigated the expression patterns of CK18 and CK20 in the bovine duodenum and colon using dual immunostaining. In the duodenum, CK18 was detected in the crypt and Brunner’s gland, and the staining density of CK18 was stronger in the Brunner’s gland than in the crypt (Fig. 8, A and B). In contrast, because duodenal chorioepithelium expressed CK20, CK18-positive epitheliocytes in the crypt moved to the villi and gradually changed CK18 for CK20 at the mouth of the crypt. In the colon, CK18-positive cells were observed in almost all normal crypt cells, and this was changed for the expression of CK20 at the mouth of the crypt. Finally, colonic absorptive epithelium expressed CK20 in the flat surface facing the lumen (Fig. 8, C and D). It is reported that CK20 is absent from the bottom of the crypt and that its expression level gradually increases as epithelial cells progress to the top of the crypt.
Fig. 4. Localization of CK18-positive cells in Peyer’s patches of the jejunum and ileum. The jejunal sections and the ileal sections containing FAE were immunostained with anti-CK18 (CY-90) monoclonal antibodies. B and F are higher magnification of the boxes in A and E, respectively. A couple of mirror sections of crypt continuing with villous epithelium (V) and FAE (F) were immunostained with anti-CK18 monoclonal antibody (C and G) or anti-Ki-67 monoclonal antibody, a proliferative cell marker (D and H). Arrows show Ki-67-positive cells. Bars = 200 μm (A and E) and 10 μm (B–D and F–H).
crypt (34). However, CK20 was only detected in the upper part of the crypt and the absorptive epithelium in bovine colon in the present experiment. These results show that the expression patterns of CK18 and CK20 in the duodenum and colon are similar to those of the jejunum and ileum.

DISCUSSION

It is well known that different types of cells and tissues are characterized by the specific composition of their intermediate filaments. The composition of intermediate filaments of the epithelia of the small intestine has been reported for the human, pig, and rodent, and these are generally composed of CK8, CK18, CK19, and CK20 (8, 13, 21). Moreover, CK8 was reported to be arranged in pairs together with CK18 or CK19 in intestinal epithelium (9, 33). Although intermediate filament proteins have been little researched in the intestinal epithelium of ruminants, in this study, we observed the expression of CK18 and CK20 in bovine intestinal epithelial cells.

In the FAE of bovine Peyer’s patches, CK18-positive cells showed morphological characteristics of M cells, with irregular and sparse microvilli (Fig. 5). In contrast, other enterocytes in the villi and CK18-negative cells in FAE expressed CK20, but not CK18. These data indicate that CK18 is a useful histochemical marker of bovine intestinal M cells in the FAE. Some investigators have determined several M cell-specific intermediate filament markers, such as CK8 for the rat (46), CK18 for the pig (13), and vimentin for the rabbit (16). These markers are useful tools for investigating a large number of questions regarding M cell proliferation, differentiation, migration, and mechanisms of antigen transport. CK18 as a marker for bovine intestinal M cells might provide advantages for further investigations relevant to these questions. Moreover, in the porcine intestinal epithelium, it is already known that CK18 is expressed in M cells as well as in transit-amplifying cells in only the FAE side of the crypt area (13, 32). In contrast, the expression of CK18 also identified transit-amplifying cells in all of the crypt area of the bovine jejunum and ileum. CK18-positive cells were also detected in the crypt and Brunner’s gland of the duodenum and almost all of the normal colonic crypt cells. Recent reports show that bovine epithelial intestinal

Fig. 5. Ultrastructure of CK18-positive cells in jejunal and ileal FAE. A couple of mirror sections were used. One section was stained with anti-CK18 (CY-90) monoclonal antibody (A and D). The other was fixed with glutaraldehyde, treated with tannic acid, and coated with platinum-palladium for SEM analysis (B and E). C and F are higher magnification of B and E, respectively. Arrows show identical cells. Bars = 10 μm.
Fig. 6. Localization of CK18- and CK20-expressing cells in the jejunum and ileum. Sections were dual immunostained with anti-CK18 (IgG1) and anti-CK20 (IgG2) monoclonal antibodies. CK18 and CK20 were visualized by Alexa Fluor-labeled goat anti-mouse IgG1 (green) and goat anti-mouse IgG2 (red). Next, the sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). F, FAE; V, villous epithelium. Bars = 10 μm.
primoculture and bovine jejuncyte and colonocyte cell lines express CK18 (30, 49). Considering these data, immature bovine intestinal epithelial cells without contact with lumen contents also express CK18.

We observed CK18-positive epitheliocytes in the crypt. These appear to move to the top of the villi and gradually exchange CK18 for CK20 as they progress. CK20 was expressed in enterocytes other than M cells in the villi and FAE (Fig. 6). Previous reports show that CK20 is a differentiation marker of intestinal epithelium in the human and other animals (5, 6, 45). Our results indicate that CK20 is expressed exclusively in differentiated bovine intestinal epithelial cells. In contrast, we show that CK18 is detected in several kinds of mature intestinal epithelial cells (Brunner’s gland in the duodenum, endocrine cells in colonic normal crypt, and M cells in jejunal and ileal FAE). It is known that the intermediate filament proteins participate in a wide range of important cellular functions, including maintenance of cellular mechanical integrity, vesicle transport, and the protection of cells from stress (20, 38). Our observation of distinct CK types may be connected to these different cell functions and their processes, as well as the phase of epithelial differentiation in the intestine.

In the small intestine, stem cells are located deep within the crypts and give rise progressively to various differentiating
epithelial cells through the commitment and proliferation of lineage precursors (31, 44). These cells migrate up toward the crypt mouth and along the villi to be shed from the tip (15). On the other hand, the follicle-associated crypts supply enterocytes and M cells on to the sides of the FAE (11). Investigators have reported two hypotheses with respect to the differentiation of intestinal epitheliocytes. One is that M cells arise and segregate directly from stem cells of the crypt in the same way as other

Table 1. Configurational comparison of CK18 and CK20-positive cells in jejunal and ileal FAE

<table>
<thead>
<tr>
<th>Regions</th>
<th>Marker</th>
<th>Cell No.</th>
<th>TUNEL⁺ Cell</th>
<th>CK18 (%)</th>
<th>CK20 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum (n = 13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cell no./half FAE</td>
<td>CK18</td>
<td>91.5 ± 11.9</td>
<td>0</td>
<td>45.2/54.8</td>
<td></td>
</tr>
<tr>
<td>Lower</td>
<td>CK18</td>
<td>13.9 ± 2.3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CK20</td>
<td>16.8 ± 2.4</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral</td>
<td>CK18</td>
<td>12.1 ± 3.0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CK20</td>
<td>18.4 ± 2.2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apical</td>
<td>CK18</td>
<td>6.5 ± 2.3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CK20</td>
<td>23.8 ± 3.1</td>
<td>1.9 ± 0.8</td>
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<td></td>
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<tr>
<td>Ileum (n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cell no./half FAE</td>
<td>CK18</td>
<td>58.7 ± 6.2</td>
<td>0</td>
<td>96.1/3.9</td>
<td></td>
</tr>
<tr>
<td>Lower</td>
<td>CK18</td>
<td>19.1 ± 1.8</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CK20</td>
<td>0.8 ± 1.0</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>Peripheral</td>
<td>CK18</td>
<td>18.3 ± 1.8</td>
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<td>CK20</td>
<td>1.3 ± 1.7</td>
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<tr>
<td>Apical</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>CK20</td>
<td>8.2 ± 2.5</td>
<td>3.1 ± 0.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results for cell no. of cytokeratin (CK) 18, CK20, or terminal deoxynucleotidyl-transferase-mediated deoxyuridine-triphosphate-biotin nick-end labeling (TUNEL)-positive cells are expressed as means ± SD; n, no. of tissue samples. The sections from jejunal and ileal follicle-associated epithelium (FAE) were stained with anti-CK18 and anti-CK20 monoclonal antibodies and TUNEL. The sections containing TUNEL-positive cells were selected. One-half of the FAE was divided into thirds (lower region, from the mouth of crypt to the peripheral region; peripheral region, middle third of the FAE; and apical region, upper third of the FAE).

![Fig. 8](http://ajpgi.physiology.org/)  
**Fig. 8.** CK18 and CK20 expression in the duodenum and colon. Sections of duodenum (A and B) and colon (C and D) were dual immunostained with anti-CK18 (IgG₁) and anti-CK20 (IgG₂a) monoclonal antibodies. CK18 and CK20 were visualized by Alexa Fluor-labeled goat anti-mouse IgG₁ (green) and anti-mouse IgG₂a (red) antibodies, respectively. Following this, the sections were counterstained with DAPI. B and D are higher magnification of the boxes in A and C, respectively. Bars = 200 μm (A and C) and 10 μm (B and D).
epithelial cells (12, 29, 32). The other is that mature enterocytes switch to the M cell phenotype under the influence of lymphocytes (18, 50) or microorganisms (3, 51). In this study, M cells in the FAE, such as CK18-positive proliferating cells, were located immediately above the crypt mouth (Fig. 4). This result indicates that the hypothesis that M cells may differentiate directly from stem cells may be the correct one.

The fate of M cells in the FAE and their apoptotic processes is still unclear. Underlying the decrease in the number of M cells in the apical area of the FAE (7, 29, 50), M cells may die at the periphery of the FAE or be shed from the FAE periphery. However, apoptotic enterocytes are restricted to the very top of the FAE in the Peyer’s patches of the mouse (53). In the pig, CK18-positive M cells other than in the FAE periphery were only weakly stained for alkaline phosphatase, if at all. However, CK18/alkaline phosphatase-positive cells near to the FAE apex had a columnar shape, similar to that for adjacent enterocytes (32). These data reveal that committed M cells may differentiate to mature M cells through contact with lymphocytes at the FAE periphery, transdifferentiate to enterocytes, and finally excluding cells near to the FAE apex. A recent transmission electron microscope study of M cells in Peyer’s patches of the rat noted that the morphology of M cells gradually changed in mature microvillous epithelial cells and that there was no morphological sign of cell death in M cells in any FAE (39). In the present study, whereas M cells expressing CK18 were scattered in jejunal FAE and covered almost all ileal FAE, the rate of CK18-positive cells decreased in the apical region, and all of the TUNEL-positive cells we could find expressed CK20 (Figs. 6 and 7 and Table 1). These data suggest that CK18-positive M cells might transdifferentiate to enterocytes expressing CK20, migrate to the tip of the FAE, and finally be excluded from the FAE apex by apoptosis. Members of the CK family provide structural support for the cell and are also involved in apoptosis (38). We consider that the difference of CK subtype between M cells and other enterocytes may be because of differences in function and morphology, as well as the final apoptotic processes. Additionally, the fate of M cells may be controlled by the influence of lymphocytes invaginating under the M cells.

GRANTS
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DISCLOSURES
No conflicts of interest are declared by the authors.

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