Mouse Intestinal Goblet Cells Expressing SV40 T Antigen Directed by the MUC2 Mucin Gene Promoter Undergo Apoptosis upon Migration to the Villi

James. R. Gum, Jr., James. W. Hicks, Anne Marie Gillespie, Jose L. Rius, Patrick A. Treseler, Scott C. Kogan, Elaine J. Carlson, Charles J. Epstein, and Young S. Kim

Department of Veterans Affairs Medical Center, San Francisco, California 94121 [J. R. G., J. W. H., J. L. R., Y. S. K.], and Departments of Anatomy [P. A. T., Y. S. K.], Pediatrics [A. M. G., E. J. C., J. J. E.], Medicine [Y. S. K.], and Laboratory Medicine [S. C. K.], University of California at San Francisco School of Medicine, San Francisco, California 94443

ABSTRACT

Mucinous colorectal cancers exhibit a characteristic set of molecular genetic alterations and may be derived from progenitor cells committed to the goblet cell lineage. Previously, we demonstrated that the MUC2 mucin gene promoter drives transgene reporter expression with high specificity in small intestinal goblet cells of transgenic mice. On the basis of these experiments, we reasoned that the MUC2 promoter could be used to drive SV40 T antigen (Tag) expression in the same cell type, decoupling them from their normal antiproliferative controls. A line of mice was established (MUCTag6) that expressed Tag in intestinal goblet cells as determined by RNA blot and immunohistochemical analysis. These goblet cells were markedly involuted however, most notably in the villi. Endogenous intestinal MUC2 message levels were reduced to about one third the normal level in these mice. However, absorptive cell lineage markers were comparable with nontransgenics. Bromodeoxyuridine-positive S-phase cells are limited to crypts in nontransgenic intestine but are present in both crypts and villi in MUCTag6. In contrast, mitotic cells were not present in the villi, indicating that MUCTag6 villi goblet cells do not progress into M phase. Apoptotic cells positive for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end-labeling were increased more than fourfold in MUCTag6 villi (P < 0.0001), and apoptotic goblet cells were evident. Electron microscopic examination of MUCTag6 intestinal villi revealed the presence of degraded cell remnants containing mucin goblets together with other cell debris, further indicating apoptosis of the goblet cell lineage. Thus, the expression of Tag in intestinal goblet cells releases them from normal antiproliferative controls, causing their inappropriate entry into S phase even after they transverse the crypt/villus junction. They do not, however, progress to M phase. Instead, they undergo apoptosis with a high degree of efficiency in S or G2 phase. These experiments demonstrate that apoptosis effectively blocks inappropriate goblet cell proliferation in the intestine, supporting its proposed role as an antineoplastic mechanism.

INTRODUCTION

Colorectal cancers arise from stem or progenitor cells via several pathways involving both genetic alterations as well as epigenetic events (1, 2). These cancers manifest several historical subtypes that exhibit different molecular and clinical features. Mucinous CRCs3 are characterized by their high content (>50% of tumor volume) of extracellular mucus (3, 4), almost always present as a consequence of MUC2 gene expression (5). Compared with nonmucinous CRC, mucinous CRC is more likely to exhibit microsatellite instability and K-ras gene mutations and is less likely to contain p53 gene mutations and to express detectable levels of P53 protein (6–8). Thus, mucinous and nonmucinous CRCs appear to follow different pathways to malignancy.

Several mouse models are available for the genetic analysis of tumorigenesis in the colon (9). The first to be described were Min mice, which have a truncating mutation in codon 850 of the Apc gene (10, 11). These mice rarely if ever develop invasive intestinal cancer. Instead tumor progression is limited to adenoma formation. Moreover, tumorigenesis in Min mice occurs predominantly in the small intestine as opposed to the colon. This seemingly detracts from the attractiveness of these mice as a model for CRC, although it has the advantage that the crypt/villus junction in the small intestine is an unmistakable landmark for studies involving histological analysis. Because of its importance in human CRC cancer, targeted mutagenesis of Apc has been performed as well (9). Notably, mice bearing a more distal truncating mutation at codon 1638 produce early stage carcinomas of the intestine at reasonable frequency (12). Other mouse models of CRC involve disruption of DNA mismatch repair genes (9); interestingly, these mice produce nonintestinal tumors as well as early stage carcinomas and adenomas throughout the gastrointestinal tract.

In previous studies we and others have cloned and characterized the MUC2 goblet cell mucin gene and its promoter both in vitro and in vivo (13–16). Experiments examining the expression of a −2864 to +17 bp MUC2 promoter/hGH reporter construct in transgenic mice have demonstrated highly specific expression in goblet cells of the distal small intestine (16). On the basis of these experiments, we reasoned that the MUC2 promoter could be used to drive SV40 Tag expression in these same cells in transgenic mice. Because of the ability of Tag to decouple the cell cycle from its normal antiproliferative controls (17, 18), we hypothesized that intestinal goblet cells in such mice would experience uncontrolled growth. Depending on other antineoplastic mechanisms in force in this tissue and cell type, mice bearing a MUC2 promoter/Tag hybrid oncogene could manifest several possible phenotypes up to and including the development of malignant, mucinous tumors of the small intestine.

This article describes the effects of expressing Tag under the control of the MUC2 promoter in mice. In particular, a line of mice was established that continuously express Tag in intestinal goblet cells. Unlike intestinal epithelial cells in normal mice, these Tag-expressing goblet cells continue in the cell cycle and proceed into S phase even after they migrate past the crypt/villus junction. They do not, however, undergo mitosis, and intestinal neoplasms do not develop. Rather, these cells are deleted from the intestinal epithelium by a very efficient apoptotic process. Thus, this study provides direct experimental evidence for the antiproliferative effects of apoptosis in the intestinal epithelium. Moreover, the mice provide a model for future studies of apoptosis in the intestine in a highly efficient, genetically modifiable in vivo system.
MATERIALS AND METHODS

Preparation of MUC2 Promoter/Tag Hybrid Oncogene and Transgenic Mice. SV40 DNA was obtained from Life Technologies, Inc., digested with BglII, treated with S1 nuclease, and digested with BamHI, and the SV40 early region fragment encoding the large and small Tags was isolated for cloning into BamHI/EcoRV-digested pBluescript (SK−). A fragment containing bases −2864 to +19 of the MUC2 promoter was retrieved from the previously described pGL2-basic construct (15) by digesting with HindIII, Klenow treating, and digesting with XhoI. This fragment was then cloned into the Tag/ pBluescript (SK−) construct that had been digested with Clal, Klenow treated, and digested with XhoI, allowing the MUC2 promoter/Tag hybrid oncogene to be retrieved by digesting with Xbal/XhoI. This procedure resulted in linkage between the MUC2 promoter and the SV40 Tag gene with the following sequence: 5′-GCCCCTTGCAAGTCGATAAGCCTTGTACC GGCTCTTACAG, where the MUC2 5′-untranslated region is underscored, the linker is plain text, and the Tag 5′-untranslated region is boldface. The linkage region and the regions near the ends of the construct were confirmed by sequence analysis.

Transgenic mice were prepared using C57BL/6J × DBA/2J F1 hybrid zygotes as previously described (16). Transgene was detected in BamHI-digested mouse tail DNA using bases −2864 to +19 of the MUC2 promoter as a probe for blot analysis. Transgene copy number was estimated from the intensity of the 331-bp BamHI fragment internal to the MUC2 promoter using NIH Image software (16).

RNA Blot Analysis. RNA was extracted from mouse tissues using TRI reagent (Molecular Research Center, Cincinnati, OH), and 10-μg aliquots were subjected to electrophoresis and transferred to nylon membranes as previously described (16). A probe for Tag was obtained by digesting the initial Tag-pBluescript (SK−) plasmid described above with BamHI and SalI. A probe for intestinal fatty acid binding protein spanning bases 343–543 (19) was prepared by digesting with XhoI and BamHI, and the SV40 early region fragment encoding the large and small Tags was isolated for cloning into the Tag-pBluescript (SK−) construct that had been digested with Clal, Klenow treated, and digested with XhoI, allowing the MUC2 promoter/Tag hybrid oncogene to be retrieved by digesting with Xbal/XhoI. This procedure resulted in linkage between the MUC2 promoter and the SV40 Tag gene with the following sequence: 5′-GCCCCTTGCAAGTCGATAAGCCTTGTACC GGCTCTTACAG, where the MUC2 5′-untranslated region is underscored, the linker is plain text, and the Tag 5′-untranslated region is boldface. The linkage region and the regions near the ends of the construct were confirmed by sequence analysis.

Transgenic mice were prepared using C57BL/6J × DBA/2J F1 hybrid zygotes as previously described (16). Transgene was detected in BamHI-digested mouse tail DNA using bases −2864 to +19 of the MUC2 promoter as a probe for blot analysis. Transgene copy number was estimated from the intensity of the 331-bp BamHI fragment internal to the MUC2 promoter using NIH Image software (16).

RNA Blot Analysis. RNA was extracted from mouse tissues using TRI reagent (Molecular Research Center, Cincinnati, OH), and 10-μg aliquots were subjected to electrophoresis and transferred to nylon membranes as previously described (16). A probe for Tag was obtained by digesting the initial Tag-pBluescript (SK−) plasmid described above with BamHI and SalI. A probe for intestinal fatty acid binding protein spanning bases 343–543 (19) was prepared by PCR of mouse intestinal cDNA. Probes for mouse Muc2 (16) and glyceraldehyde-3-phosphate dehydrogenase and dipeptidylpeptidase IV (20) were also used to assess apoptosis in tissue sections (24, 25).

Goblet Cell Involution and Apoptosis. Goblet cells were visualized using periodic acid-Schiff/Acan blue/hematoyxlin staining. Depth of mucin goblets was measured using a Zeiss Universal Research Microscope fitted with a Hitachi video camera using Scion Image software. Apoptosis was visualized after H&E staining using standard morphological criteria (22) and using the TUNEL assay (23). (For qualitative analysis of TUNEL-positive cells per villi, a cell was scored TUNEL positive only if it occupied at least 50% of a ×400 microscopic field, ensuring reasonable longitudinal orientation. Immunohistochemical staining with monoclonal antibody against single-stranded DNA was also used to assess apoptosis in tissue sections (24, 25). Monoclonal antibody F7–26 was obtained from Chemicon International Inc., and single-stranded DNA was stained using the protocol suggested by the manufacturer.

Immunohistochemical Staining. For SV40 Tag detection, antigen retrieval was performed by microwaving rehydrated sections in 10 mm citric acid (pH 6.0) four times for 5 min each with 1 min between heatings. Monoclonal antibody against SV40 (Pab 108; Santa Cruz Biotechnology, Santa Cruz, CA) was applied at a 1:40 dilution at 4°C overnight. Histochem-Plus kits were used to detect bound primary antibody. Lysozyme immunohistochemistry was performed by the Moffitt Pathology laboratory using polyclonal antibody obtained from Cell Marque.

Electron Microscopy. Samples were fixed in 2.7% glutaraldehyde, 0.8% paraformaldehyde in 0.1 m cacodylate buffer (pH 7.4) for 2 h at room temperature. They were rinsed in distilled water and postfixed in 2% osmium tetroxide for 1 h, dehydrated through graded alcohols to 100% ethanol, and embedded in Epomate resin (Polysciences, Inc., Warrington, PA). Seventy-nanometer sections were cut and stained with uranyl acetate (10% in 50% ethanol) and Reynold’s lead citrate (microwave method). A Philips Bioscan Techni 10 was used at 80 kV for photography.

RESULTS

Production and Characteristics of Initial Hybrid Oncogene Recipient Mice. A total of five mice were obtained that had one to approximately eight copies of the MUC2 promoter/Tag hybrid oncogene incorporated into their genome. These mice all died within 26 weeks (Table 1). A mucinous occlusion occurred in the lumen of the distal colon in mouse 28. This mass contained mucin-producing (periodic acid-Schiff-positive) cells but did not appear to be firmly attached to the bowel wall. Mouse 1 developed intraepithelial neoplasia in its cervix as well as intraepithelial neoplasia in its submandibular gland and a sarcoma in its thorax. Mouse 17 developed high grade carcinoma in situ in its cervix as well as intraepithelial neoplasia in its submandibular gland that had a small foci of adenocarcinoma. Mouse 6 developed splenic and cecal tumors, which will be described in the next section. Mouse 4 developed a splenic tumor as well but died unexpectedly, compromising detailed histological examination. In several cases RNA was extracted from these tumors, and all tested samples expressed high levels of Tag (data not shown). An attempt was made to propagate these mice by breeding to the C57BL/6 strain but was only successful in the case of mouse 6. Offspring of this mouse were designated line MUCTag6 and were characterized in detail as described below. The mice used in this study were from generations F1 to F3.

Description of the MUCTag6 Line. MUCTag6 mice develop to adulthood normally and resemble their nontransgenic littermates in appearance and behavior. Both males and females are fertile and propagate the hybrid oncogene in an autosomal dominant fashion. As shown in Fig. 1A, these mice express high levels of Tag in the middle and distal portions of their small intestine and very low to undetectable levels in other tissues, including their colons. Thus, the tissue-specific pattern of MUCTag6 transgene (Tag) expression mimics the pattern observed with multiple pedigrees of MUC2 promoter/hGH

Table 1 Tumor formation in hybrid oncogene recipient mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Transgene copy no.</th>
<th>Sex</th>
<th>Tumor site</th>
<th>Tumor type</th>
<th>Age at death (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>F</td>
<td>Salivary gland</td>
<td>Intraepithelial neoplasia</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>F</td>
<td>Spleen</td>
<td>Sarcoma</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>F</td>
<td>Spleen/Cecum</td>
<td>Histiocytic sarcoma</td>
<td>26</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>F</td>
<td>Cervix</td>
<td>Carcinoma in situ</td>
<td>13</td>
</tr>
<tr>
<td>28</td>
<td>2</td>
<td>M</td>
<td>Salivary gland/Colon</td>
<td>Intraepithelial neoplasia</td>
<td>12</td>
</tr>
</tbody>
</table>

* Mouse 4 died unexpectedly without overt symptoms, which precluded detailed histological examination.

* Histiocytic sarcoma located in unencapsulated lymphoid tissue in cecum.
The sizes of the spleens and lymph nodes are given. Non-TG). The spleens and lymph nodes of young nontransgenic littermates (B, hyde-3-phosphate dehydrogenase (GAPDH) was used to ensure that all samples contained GAPDH Muc2. A separate blot was also probed for endogenous from an individual mouse are indicated by a line drawn under the GAPDH autorad.

Fig. 1. RNA blot analysis of Tag expression in MUCTag6 mice. A, RNA was extracted from the normal tissues of a F1, male 7 weeks of age and subjected to blot analysis. PSI, MSI, DSI, proximal, middle, and distal small intestine, respectively. Stom, Liv, Kid, stomach, liver, and kidney. Probe used is given left of the autorad. Probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to ensure that all samples contained RNA. B, RNA was extracted from the tissues of various MUCTag6 mice (TG) or their nontransgenic littermates (Non-TG). The sizes of the spleens and lymph nodes are given. A separate blot was also probed for endogenous Muc2 gene expression. All samples taken from an individual mouse are indicated by a line drawn under the GAPDH autorad.

reporter transgenic mice (16). The spleens and lymph nodes of young MUCTag6 mice appear normal. However, they become enlarged with aging in most animals. Six of seven of the spleens examined from 18- to 27-week-old MUCTag6 mice expressed detectable levels of Tag message, but the spleen of a 6-week-old mouse did not (Fig. 1B). In general, the level of Tag expressed increased with the size of the spleens. The enlarged lymph nodes found in MUCTag6 mice also express Tag, whereas the nontransgenic spleens used as controls were negative for expression. The salivary gland of a 27-week-old MUC-Tag6 mouse also failed to express detectable Tag. In contrast to expressing Tag, the enlarged spleens and lymph nodes of MUCTag6 did not express endogenous Muc2 (Fig. 1B). On histological examination, the enlarged spleens and lymph nodes contained areas of tumor cells that exhibited the typical morphology of histiocytic sarcoma. Two cases of histiocytic sarcoma in liver were also noted. Although histiocytic sarcoma is a rare tumor in humans, it commonly occurs in some strains of mice (26). The tumor cells were immunoreactive for lysozyme, confirming their histiocytic lineage. Moreover, immunostaining also revealed Tag expression in these tumor cells (not shown). As is commonly the case, the histiocytic tumor cells were accompanied by extramedullary hematopoiesis. MUCTag6 mouse become moribund with enlarged spleens as they reach 5–7 months of age.

S-Phase Cells and Goblet Cell Involvement in MUCTag6 Small Intestinal Villi. Intestinal epithelial cells in the normal mouse proliferate strictly in the crypts of Lieberkühn; i.e., cell cycle activity ceases before the cells migrate past the crypt/villus junction (27, 28). This withdrawal from the cell cycle appears to be caused by marked decreases in cyclin D1 levels, resulting in the accumulation of unphosphorylated Rb, which binds and inactivates the E2F family of transcription factors required for entry into S phase (17, 29). Tag, however, inactivates Rb (17, 18). Thus, the expression of Tag in intestinal goblet cell tumors could cause abnormal proliferation of this cell lineage. To test this possibility, we used BrdUrd labeling to identify S-phase cells in the intestinal epithelium of MUCTag6 mice and their nontransgenic littermates. As expected, S-phase cells were confined to the crypts of nontransgenic mouse small intestine (Fig. 2A). In MUCTag6, however, S-phase cells were present in the crypts as well as in the lower part of the villi (Fig. 2B). Some of the S-phase villi cells could be clearly identified as goblet cells. Moreover, MUCTag6 goblet cells express Tag, which is detectable by immunohistochemistry (Fig. 2, C and D). These villi goblet cells are frequently misshapen, however, appearing involuted with small nuclei displaced apically and with markedly diminished mucin vacuole content. Some Tag-expressing nuclei appear not to be goblet cell associated in Fig. 2D. This could be caused by both goblet involution and by nuclei and goblets lying in different planes of the section. Goblet cell involution is clearly visualized with periodic acid-Schiff/Aldcan blue/hematoxylin staining (Fig. 2, E and F). Although this aberrant goblet cell entry into S phase and morphology was readily apparent, the MUCTag6 intestinal epithelium did not develop tumors. No gross alterations were detected in MUCTag6 epithelial structure, and dysplastic features such as branched crypts or villi were not detected (30).

MUCTag6 Intestinal Villi Goblet Cells Are Deleted by Apoptosis. The experimentation described above indicates that MUCTag6 intestinal villi goblet cells continue in the cell cycle, freely entering S phase. Mitotic cells, however, although common in intestinal crypts, could not be detected in MUCTag6 villi. This and the fact that what appeared to be involved goblet cells were common in MUCTag6 villi suggested the hypothesis that these cells were removed from the epithelium by apoptosis. Microscopic examination of H&E-stained MUCTag6 intestine supported this hypothesis. The MUCTag6 villi epithelium was laden with what appeared to be the remnants of cells in various stages of apoptosis. Nuclei that were apically displaced, condensed, distorted, blebbing, and/or fragmented were common (Fig. 3, A–E). In some cases, remnants of mucus goblets can be seen to be associated with these nuclei (Fig. 3, A–C). We also used the TUNEL assay (23), which detects the fragmented DNA characteristic of apoptosis, to characterize the MUCTag6 intestinal epithelium (Fig. 3, G–K). TUNEL-positive nuclei were common, exhibiting a spectrum of apoptotic morphology similar to that observed using H&E staining. Antibody to single-stranded DNA, which binds as a manifestation of nuclear protein degradation (24, 25), confirmed that these structures were undergoing apoptosis (Fig. 3, L–P). In contrast to the high rate of apoptosis observed in the MUCTag6 villi epithelium, apoptotic cells were rare in the intestinal crypts of both MUCTag6 and nontransgenic mice.

Because of the alterations observed in MUCTag6 villi goblet cells, we sought to determine whether absorptive cells, the major cell type found in villi (27, 28), were also affected. Microscopic examination did not reveal any alterations in absorptive cell morphology, and, by all appearances they remained the major epithelial cell type of MUCTag6 villi (Figs. 2 and 3). To examine this in more detail, we performed RNA blot analysis of cell lineage markers in MUCTag6 and nontransgenic mouse intestine (Fig. 4). Here, the steady state mRNA levels of two absorptive cell markers, dipeptidyl peptidase IV (20) and intestinal fatty acid binding protein (19), were compared. The blots detected no differences in the level of these markers in MUCTag6 intestine versus nontransgenic. Hence, there was no discernable affect of the hybrid oncogene on the absorptive cell lineage. Also in Fig. 4, the expression of endogenous Muc2 (mouse) message was examined. Levels of this goblet cell marker in MUCTag6 small intestine were about one third that found in the nontransgenic control.
Although this is difficult to quantitate precisely because of the polydisperse nature of Muc2 mucin mRNA isolated using conventional methods (31), it is consistent with the goblet cell involution observed in histological sections. It is also possible that down-regulation of Muc2 expression by Tag contributes to this effect. The absence of Tag expression in nontransgenic mouse intestine is also shown in Fig. 4.

Quantitative analysis of goblet cell involution and apoptosis in MUCTag6 villi was also performed. The total number of periodic acid-Schiff-positive goblet cells per MUCTag6 villi was approximately the same as in nontransgenic villi (12.2 ± 4.2 versus 10.4 ± 3.3, n = 25 villi; data not shown). The slight increase in goblet cell number observed in MUCTag6 could reflect an expected increase in goblet cell proliferation in the crypts attributable to Tag expression. Many of the MUCTag6 mucus goblets counted were very small, however, as described above. Therefore, we measured both the mean size of the mucus goblets and the average number of “normal-sized”

Fig. 2. Goblet cell alterations in the MUCTag6 small intestinal epithelium. All samples were removed from the distal third of the small intestine of MUCTag6 mice (B, D, and F) or a nontransgenic littermate (A, C, and E). Bars, 100 (A, B, E, and F) or 25 μm (C and D). S-phase cells identified by BrdUrd labeling in nontransgenic mice are confined to the crypts (A); in MUCTag6 mice, however, S-phase cells persist into the villi (B). Inset in B, S-phase cells with clearly identifiable goblet cell morphology. Tag expression in the nuclear region of MUCTag6 goblet cells is shown in D, whereas nontransgenic goblet cells fail to stain (C). Mucin goblets are stained blue in C and D with Alcian blue. E and F, periodic acid-Schiff/Alcian blue/hematoxylin-stained sections from nontransgenic and MUCTag6 intestine, respectively; insets, enlarged views of goblet cells. D and F, involution of the goblet cells in MUCTag6, compared with nontransgenic (C and E).
mucus goblets per villi in MUCTag6 and nontransgenic intestine. Here, normal-sized mucus goblets were defined as goblets equal to or larger than the mean minus SD of goblets found in nontransgenic intestine (Fig. 5). When counted using this criteria, the number of normal-sized goblet cells per villi in MUCTag6 was less than one fifth that of nontransgenic (Fig. 5). Moreover, almost all of the normal-sized goblet cells counted in MUCTag6 were located near the crypt/villus junction. Also shown in Fig. 5, the mean size of MUCTag6 goblets was approximately one half that of nontransgenic (measured in length from the apical part to the basal part of the goblet). Because living goblet cells are three-dimensional objects, opposed to the two-dimensional measurements necessitated by histological sectioning, the actual difference is greater. We also counted the number of TUNEL-positive apoptotic cells per villi and found approximately fivefold more in the MUCTag6 villi versus nontransgenic (Fig. 5). Thus, almost all MUCTag6 intestinal villi goblet cells are involuted, and the epithelium contains dramatically elevated levels of cells undergoing apoptosis.

Finally, electron microscopy was used to gain further insight into cells undergoing apoptosis in MUCTag6 villi. Goblet cells in nontransgenic mouse epithelium are packed with apically located, tightly packed mucus vacuoles (Fig. 6A). These cells also have well-developed, rough endoplasmic reticulum and Golgi, necessary for the production and packaging of large quantities of secretory mucus. Normal-appearing goblet cells were seldom encountered in MUC-
Tag6 villi. Instead, apoptotic fragments and the remnants of involuted goblet cells were common (Fig. 6, B–D). Figure 6B shows examples of an involuted mucus goblet and a condensed vacuole containing cell debris. Figure 6C shows two examples of degraded cell remnants, both containing vacuoles associated with other condensed cell debris. Also very common were the remnants of mucin goblets together with other cell debris, including rough endoplasmic reticulum located near the cusp of the epithelium (Fig. 6D). Thus, electron microscopic examination of MUCTag6 intestinal villi revealed the presence of degraded cell remnants including mucin goblets together with other cell debris, further indicating apoptosis of the goblet cell lineage.

DISCUSSION

The intestinal epithelium represents a continuum of cell division, differentiation, migration, and removal that is maintained for the lifetime of an animal. All intestinal epithelial cells are derived from a small number of pluripotent stem cells located near the base of the crypts of Lieberkühn (28, 32, 33). These cells are believed to give rise to both long- and short-lived progenitor cells that retain the capacity to divide but that can be committed to the generation of one of four cell lineages (absorptive cells, goblet cells, enteroendocrine cells, and Paneth cells; Refs. 28, 32, and 33). Paneth cells migrate downward to occupy positions in the base of the crypts, whereas the other cell lineages migrate upward. In the small intestine, all cell cycle activity ceases before the cells reach the crypt/villus junction, with the cells apparently arrested in G1 phase, as judged by their content of cyclin E (29). Goblet cells differentiate while still deep in the crypts, forming...
characteristic apically located mucus-containing goblets (34, 35). They then migrate for 2–3 days until they reach the tip of the villi where they are deleted by a sloughing process that may also involve apoptotic mechanisms and the partial reclamation of cellular material (22). The colonic epithelium experiences a pattern of cell renewal similar to what occurs in the small intestine, although the process is less well studied (36). In colon cancer, genetic and epigenetic events occur that cause the dysregulation of genes involved in controlling various aspects of this cell renewal process (1, 2, 37).

In this study we sought to perturb the regulation of intestinal goblet cell proliferation via the expression of Tag under the control of the MUC2 promoter in transgenic mice. We have previously shown that this MUC2 promoter directs reporter gene expression to goblet cells of the distal portion of the mouse small intestine (16). Reporter expression initiates deep in the crypts, where goblet cells are still actively dividing (34, 35). The principle effect of Tag on the cell cycle is to bind and inactivate the Rb protein, thus releasing E2F transcription factor and driving the passage of cells from G1 to S phase (17, 29). Our hypothesis was that this would cause the uncontrolled proliferation of goblet cells, leading to the expansion of the goblet cell lineage as well as priming for further mutations that would ultimately lead to the development of mucinous CRC. A further hypothesis was that the expression of Tag in still-dividing, deep crypt cells, as opposed to quiescent villi cells, may be important in the initiation of tumorigenesis. The formation of these hypotheses was guided by previous studies in which intestinal cell lineage-restricted promoters were used to drive Tag expression in mice (21, 38, 39). Use of the Fabpl promoter resulted in Tag expression in the villi of the small intestine (21). This caused reentry of quiescent villi absorptive cells into the cell cycle but had no measurable effect on absorptive cell numbers or villi cell lineage distribution, nor did neoplasms arise. Interestingly, however, an increase in apoptosis was noted in the villi of these mice (29, 30). Mice with SV40 Tag driven by the cryptdin-2 promoter experienced Paneth cell ablation in the small intestine instead of proliferation and surprisingly developed neuroendocrine tumors of the prostate, derived from a second cell type in which the cryptdin-2 promoter is also active (38). A similar attempt using a glucagon promoter resulted in endocrine tumors in the colon and the pancreas, where Tag was expressed in endocrine cells (39).

Forced expression of Tag did release intestinal goblet cells from at least one antiproliferative control. As shown in Fig. 2B, MUCTag6 goblet cells fail to undergo G1 arrest and continue into S phase inappropriately as they migrate up the crypts and into the villi. This alone, however, does not lead to intestinal neoplasia. It appears rather, that these S-phase cells are removed by a very efficient apoptotic process in lieu of entering mitosis. The evidence for this is as follows: (a) the absence of mitotic cells in the MUCTag6 villi despite the presence of S phase goblet cells; (b) the presence of large numbers of TUNEL and anti-single-stranded, DNA-positive, and histologically evident apoptotic cell bodies in MUCTag6 villi, some of which retain morphological traces of their goblet cell origins (Figs. 3 and 6); and (c) the involution of MUCTag6 mucin goblets (visualized by periodic acid-Schiff/Alcian blue staining), resulting in their near-complete removal as they migrate away from the crypt/villus junction (Figs. 2, E and F, and 6). At the final stages of this process, some material from the degraded cells, especially the mucus, appears to be extruded into the intestinal lumen, whereas some apoptotic cellular material appears to be engulfed by neighboring cells (Figs. 2, E and F, and 6). The cellular debris from intestinal apoptosis, at least in this setting, is both endocytosed and sloughed. This suggests the possibility that both processes may be used to delete intestinal epithelial cells from the extrusion zone near the villi tips at the end of their natural life span (22).

The antineoplastic effects of apoptosis in the intestine has been suggested by other studies, including analysis of cells in culture and the observation that the proapoptotic gene BAX is frequently mutated in microsatellite unstable CRCs (40, 41). It is also been observed that both CRCs and adenomas have increased apoptotic indices (42, 43). Furthermore, the colonic epithelium of azoxymethane-treated rats exhibits abundant apoptotic indices (44). This study provides direct experimental evidence for the antiproliferative effects of apoptosis in the intestine in a well-defined, in vivo system. Because goblet cells are morphologically distinct from other intestinal cell lineages, it is clear that complete or near-complete ablation of these cells has occurred by the time of their migration to the upper part of the villi. Thus, intestinal apoptosis can be extremely efficient, and the bulk of the in vivo evidence suggests that it occurs as an adaptive response to the uncontrolled proliferation of intestinal epithelial cells.

The development of histiocytic sarcomas in MUCTag hybrid oncogene-bearing mice was unexpected. MUC2 gene expression is very tightly regulated, and to the best of our knowledge MUC2 has not been reported to be expressed in macrophages, the presumed precursors of histiocytic sarcomas. It is likely that the hybrid oncogene expresses Tag in a population of macrophages and that this leads to expansion of this cell lineage. The correlation of Tag expression with spleen size in MUCTag6 supports this hypothesis (Fig. 1B). Further, evidence of apoptotic and genetic changes may then be required for histiocytic sarcoma formation. The observation that endogenous Muc2 message is not expressed in the histiocytic sarcomas (Fig. 1B) indicates that the hybrid oncogene can be active in a population of cells, albeit a selected population, that does not express endogenous MUC2. The development of histiocytic sarcomas and ensuing limitation of life span in MUCTag6 may preclude the possibility of intestinal neoplasm formation, which could require antiapoptotic genetic changes. The notion that additional genetic changes may be required for neoplasia in Tag-expressing intestinal epithelial cells is supported by the observation that cell lines developed from Tag-expressing intestinal epithelial cell also do not form colonies in soft agar or produce tumors when transplanted into nude mice (45). Furthermore, decreased apoptosis failed to increase intestinal tumor formation in the genetically initiated Apc1638 mouse model (46). It is also possible that in the MUCTag6 model, Tag is not expressed early enough in the intestinal goblet cell differentiation pathway to effect neoplasia formation, although deep crypt goblet cells retain the ability to divide (34).

In addition to Rb inactivation, Tag is known to inactivate P53 (17, 18). Because P53 has proapoptotic activity (47), a cell expressing Tag may be expected to be apoptosis deficient. On the other hand, P53-independent pathways for apoptosis are known to be operative in many cell types (48). Most relevant to this study, intestinal absorptive cells forced to reenter the cell cycle by Tag expression in the villi undergo apoptosis with equal efficiency in P53 wild-type and P53 null mice (29, 30). Thus, it is most likely that the goblet cell apoptosis demonstrated in this study proceeds via a P53-independent pathway.

In summary, this study demonstrates the effectiveness of apoptosis in counterbalancing uncontrolled proliferation in the intestine using a well-defined, in vivo system. MUCTag6 intestinal goblet cells continue into S phase during their migration from the crypts to the villi. Rather than progressing to mitosis, however, these cells undergo apoptosis, resulting in the near complete ablation of the goblet cell lineage in the upper reaches of the villi. Thus, these experiments demonstrate that apoptosis counteracts uncontrolled proliferation in the intestine and support the hypothesis that apoptosis functions as an important antineoplastic mechanism in this tissue. In addition, the MUCTag6 line provides a model that will be useful for the examination of several parameters important in regulating apoptosis in the lower gastrointestinal tract, including the roles of the BAX, APC, and
ACKNOWLEDGMENTS

We thank Sandra Huling and Ivy Hsieh for their aid with electron microscopy.

REFERENCES


Mouse Intestinal Goblet Cells Expressing SV40 T Antigen Directed by the MUC2 Mucin Gene Promoter Undergo Apoptosis upon Migration to the Villi

James. R. Gum, Jr., James. W. Hicks, Anne Marie Gillespie, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/8/3472

Cited articles
This article cites 45 articles, 25 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/8/3472.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/8/3472.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.