Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Altered Epithelial Cell Lineage Allocation and Global Expansion of the Crypt Epithelial Stem Cell Population Are Associated with Ileitis in SAMP1/YitFc Mice

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Crohn's disease is characterized by cycles of mucosal injury and ulceration followed by epithelial regeneration and restoration of normal epithelial function. In this study, we examined whether ileitis in SAMP1/YitFc mice, a recombinant-inbred line that spontaneously develops ileitis resembling human Crohn's disease, was associated with alterations in normal patterns of epithelial differentiation or changes in epithelial regeneration after experimental injury. Increased numbers of Paneth, goblet, and intermediate cells were present focally in the ileum of SAMP1/YitFc mice by 4 weeks of age, before any histological evidence of acute or chronic inflammation. This increase in secretory cells became more pronounced at sites of ileitis with increasing age and inflammation. Additionally, there was mispositioning of Paneth and intermediate cells along the crypt-to-villus unit. A concomitant reduction in the number of absorptive enterocytes was observed. In contrast to the ileal-specific changes in lineage allocation, crypt stem cell numbers began to increase in both the ileum and proximal jejunum at the onset of inflammation in SAMP1/YitFc mice. These data suggest that the alterations in epithelial cell differentiation and increases in the size of the crypt stem cell population observed in SAMP1/YitFc mice are regulated by distinct mechanisms. We speculate that these epithelial alterations may play a role in the pathogenesis of ileitis in this murine model of Crohn's disease. (*Am J Pathol* 2005, 166:1055–1067)

that regulates the uptake and presentation of luminal antigens and bacteria to the mucosal immune system. Recent studies have established that the intestinal epithelium is an important component of the innate immune response, playing an active role in the host defense against luminal bacteria and other pathogens. For example, Paneth and goblet cells elaborate a variety of antimicrobial peptides and other mediators in the normal intestine. Mucins and intestinal trefoil factor, products of mature goblet cells, prevent interactions of the epithelium with luminal bacteria and facilitate epithelial restitution, respectively, thus ensuring that the barrier function of the intestinal epithelium is maintained.^{1–3} On the other hand, Paneth cells have more direct anti-microbial activity, secreting peptides such as defensins/cryptidins, and enzymes, such as lysozyme and phospholipase A₂, that can permeabilize bacterial cell walls and dissolve other components.^{4–6} The spectrum of peptides normally produced by these cells can be modulated by intestinal flora and the inflammatory response. Products such as tumor necrosis factor (TNF)- α and granulocyte macrophage-colony stimulating factor, which are produced by Paneth cells under pathological conditions, can in turn regulate immune function.^{7–9}

Inflammatory bowel disease is characterized by cycles of mucosal injury and ulceration, followed by epithelial regeneration and restoration of normal epithelial architecture and function. In several mouse models of inflammatory bowel disease, alterations of epithelial function have been implicated in the pathogenesis of chronic inflammation.^{10,11} In the normal intestine, patterns of cellular replication and differentiation are tightly coupled and

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The intestinal epithelium is the major interface between the luminal environment and the host, acting as a barrier

spatially regulated along the crypt-to-villus axis. Epithelial cells differentiate into the mature cell lineages found in the intestine as they migrate away from the rapidly proliferating cell zone located in the crypt. Paneth cells differentiate as they localize to the base of the crypt, whereas all of the other cell types differentiate while migrating onto the villus.^{12–14} Expansion of the proliferative zone, increased crypt branching, and changes in the normal patterns of cellular differentiation have been documented in human inflammatory bowel disease,¹⁵ suggesting that the pathways that regulate the relationship between cellular replication and epithelial differentiation have been altered in these diseases. Although it is clear that the intestinal epithelium responds to inflammation and mucosal injury by initiating a reparative response, the specific effects inflammation may have on the *in vivo* turnover of epithelial stem cells or progenitor cells, or the way in which the inflammatory milieu may perturb normal epithelial differentiation and/or function remain obscure.

The SAMP1/Yit mouse, a recombinant-inbred line that spontaneously develops ileitis, was originally derived from AKR mice by Matsumoto and colleagues.¹⁶ The SAMP1/YitFc (SAMP1/Fc) substrain was subsequently derived from the SAMP1/Yit mice after 20 generations of brother-sister mating in our colony at the University of Virginia.¹⁷ This substrain is distinguished from the parental SAMP1/Yit by the emergence of perianal fistulizing disease, a pathological feature that has not been reported in the original SAMP1/Yit colony. SAMP1/Yit and SAMP1/Fc mice develop spontaneous ileitis that shares many features with human Crohn's disease. Development of ileitis in these mice is accelerated by the presence of luminal bacteria, and is characterized by discontinuous segmental inflammation involving the ileum while sparing the proximal small intestine and colon.^{16,17} Histopathological features of ileitis in this model include transmural inflammation, crypt abscesses, and epithelial changes, including loss of villi, crypt elongation, and crypt branching. The spontaneous onset of inflammation in nearly 100% of mice by 10 weeks of age and the segmental nature of the inflammatory response make the SAMP1/Fc mouse a unique model system. These features allow separation of local from systemic effects of inflammation on the fate, proliferation, and differentiation of intestinal epithelial stem cells. In this study, we determined whether specific alterations in epithelial differentiation or in cell lineage allocation occurred at sites of ileal inflammation in SAMP1/Fc mice. We further investigated whether such differences could be accounted for by changes in the regulation of the clonogenic crypt stem cell population.

Materials and Methods

Animals

The SAMP1/YitFc (SAMP1/Fc) mice are a substrain derived from SAMP1/Yit mice provided by S. Matsumoto (Yakult Central Institute for Microbiological Research, Tokyo, Japan). The SAMP1/Fc substrain is separated by

more than 20 generations from the parental SAMP1/Yit strain and is characterized by the unexpected emergence of fistulizing perianal disease in some of the mice. Many of the phenotypic and histological features of ileitis in this substrain have been described in previous studies.^{16,17} C57BL/6j and AKR mice for use as controls were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in autoclaved microisolator cages maintained under positive pressure HEPA-filtered air in a specific pathogen-free facility at the University of Virginia. Mice were maintained on a strict 12-hour light/dark cycle, received autoclaved tap water supplied in individual sipper bottles, and were fed autoclaved standard laboratory mouse chow *ad libitum*. For experimental purposes, some mice were irradiated in a Gammacell 40 cesium irradiator at 0.9 cGy/minute. All mice used in these studies were cared for in accordance with protocols approved by the University of Virginia Institutional Animal Care and Use Committee, using Association for Assessment of Laboratory Animal Care guidelines.

Histology and Immunohistochemistry

Tissue from the indicated regions of the small intestine was rapidly dissected from mice, fixed in either Bouin's fixative or neutral buffered formalin, and embedded in paraffin as previously described.¹⁸ For analysis of inflammation, the distal 15 cm of small intestine was fixed in Bouin's fixative. Deparaffinized 3- μ m sections were stained with hematoxylin and eosin (H&E), then graded for the severity and extent of villus distortion, acute inflammation (granulocyte infiltration), and chronic inflammation (mononuclear cell infiltration). For each parameter, tissue samples were scored from 0 (normal) to 3 (severe), then this severity score was multiplied by a factor representing the extent of inflammation, which ranged from 0.5 (single focus of inflammation) to 4 (>75% involvement of the mucosal surface area). A total inflammatory index was obtained by adding the individual scores for each category. Periodic acid-Schiff (PAS)-Alcian-blue staining identified goblet cells, whereas Paneth and intermediate cells, identified by their characteristic eosinophilic granules, were enumerated in neutral-buffered formalin-fixed tissue sections. Ileal lipid-binding protein (Fabp6/ILBP) was detected using anti-ILBP¹⁹ (1:200 dilution) and visualized with Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA).

Crypt Stem Cell Survival

The number of regenerating crypts was measured 3.5 days after irradiation, using a modification of the microcolony assay as previously described.^{20–22} Briefly, S phase cells were labeled by administering 120 mg/kg of 5-bromo-2'-deoxyuridine (BrdUrd) and 12 mg/kg of 5-fluoro-2'-deoxyuridine to each mouse for 2 hours before euthanasia. Segments of small intestine were rapidly dissected from mice 84 hours after irradiation and fixed in Bouin's fixative. Paraffin sections (5 μ m) were obtained

from intestinal segments oriented such that the sections were cut perpendicular to the long axis of the intestine. Cells incorporating BrdUrd were visualized by immunohistochemistry using goat anti-BrdUrd as described.²³ A surviving crypt was defined as one containing five or more BrdUrd-positive cells, as previously detailed.²² Six complete, well-oriented cross-sections were scored for each mouse. Fractional crypt survival was defined as the ratio of the mean number of surviving crypts in intestinal cross-sections from irradiated mice to the number of crypts per cross-section from the same region of the intestine in unirradiated mice of the same strain and age.

Apoptosis

The jejunum and ileum were obtained from mice 6 hours after irradiation, fixed overnight in 10% neutral-buffered formalin, and embedded in paraffin. Sections prepared from the jejunum were oriented such that the sections were cut perpendicular to the long axis of the intestine. Sections were stained with H&E, and the number of apoptotic cells per crypt was assessed by morphological criteria as previously described.²⁴ Briefly, well-oriented crypts in longitudinal sections containing Paneth cells, a crypt lumen, and an uninterrupted column of epithelial cells extending to the crypt-villus junction were selected. An apoptotic cell was defined as a cell that contains a single large fragment of condensed chromatin or a group of smaller fragments clustered together within an area similar to the size of an adjacent epithelial cell. For each mouse, 20 well-oriented crypts were analyzed in each of eight jejunal cross sections. Apoptotic cells were also detected *in situ* using the terminal dUTP nick-end labeling (TUNEL) assay (Boehringer Mannheim, Indianapolis, IN), following the manufacturer's instructions. The number of apoptotic cells per crypt, as assessed with the terminal dUTP nick-end labeling assay, was ~40% greater than that assessed by morphological criteria. Nonetheless, the distribution pattern of apoptotic cells among the test groups was identical to that seen morphologically.

Repair of Potentially Lethal Damage to Clonogenic Stem Cells

The capacity of clonogenic stem cell populations in SAMP1/Fc mice and age-matched controls to repair potentially lethal radiation damage was assessed using the method described by Roberts and colleagues.²⁵ The number of regenerating crypts was measured in mice after a single dose (14 Gy) of radiation, as described above. Crypt survival was determined in a second group of mice to which the same total dose of radiation was delivered in two smaller fractions, separated by a period of time sufficient for damaged clonogenic stem cells to repair potentially lethal radiation damage (~5 hours). This fractionated dose protocol results in an increase in the number of surviving clonogenic stem cells compared with the number found in mice that receive the total radiation dose as a single exposure. The repair factor is defined as the ratio of the number of regenerating crypts

in mice treated with the fractionated dose protocol to the number of regenerating crypts in mice receiving the 14-Gy radiation in a single exposure.

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Levels of mRNA for fibroblast growth factor (FGF)-2, FGF-7, FGF-10, and interleukin (IL)-11 in mouse ileal segments were quantified using real-time PCR analysis. Total RNA was isolated from each sample using the RNeasy kit from Qiagen (Valencia, CA) according to the manufacturer's directions. cDNA was prepared by reverse transcription of the isolated RNA, and mRNA levels were quantified by real-time PCR using an ABI Prism SDS7000 sequence detection system (Applied Biosystems, Foster City, CA). For reverse transcription, random hexamers (1 μ g) and 10 ng of total RNA were combined with 200 U of Superscript (Invitrogen Life Technologies, Carlsbad, CA) in a final reaction volume of 20 μ l. Primers for PCR were designed using the Primer Express software (Applied Biosystems) and sequence data found in GenBank. Primers for measuring levels of FGF mRNA were: FGF-2 forward primer, 5'-GTCACGGAAATACTCCAGTTGGT-3' and reverse primer, 5'-CCCGTTTTGGATCCGAGTTT-3'; FGF-7, forward primer, 5'-GAAAGGGACCAGGAGATGAA-3' and reverse primer, 5'-CAACTGCCACGGTCCTGATT-3'; and FGF-10, forward primer, 5'-AGCGGGACCAGAATGAAGA-3' and reverse primer, 5'-CAACAACCTCCGATTTCCTACTGA-3'. The PCR reaction was performed in triplicate using 10% of the volume of the first strand synthesis in a total volume of 50 μ l that included 25 μ l of CYBR Green master mix (Applied Biosystems) and primers at a final concentration of 250 nmol/L. Primer concentrations were previously optimized as suggested in User Bulletin no. 2 (Applied Biosystems). The amplification of cDNA was performed for 40 cycles by a preset cycling program that included the generation of a melting curve. Thermocycling conditions were as follows: 1) 50°C for 2 minutes (activation of AmpErase UNG); 2) 95°C for 10 minutes (activation of AmpliTaq Gold enzyme); 3) 95°C for 15 seconds (denaturation) and 60°C for 1 minute (anneal/elongation) for 40 cycles. PCR controls included no RT and no template reactions. The ΔC_T method was used to quantify relative mRNA levels as described in User Bulletin no. 2 (Applied Biosystems), using 18S RNA as the reference and internal standard. TaqMan primer-probe set for 18S RNA with the Fam/Tamra detection system was used to simultaneously measure 18S RNA in replicate samples. IL-11, COX-1, COX-2, TNF- α , trefoil factor 3 (TFF-3), transforming growth factor (TGF)- β 3, IL-1, ileal lipid binding protein (*Fabp6/ilbp*), and ileal bile acid transporter (*asbt*) mRNA levels were quantified using Applied Biosystem's Celera Assay on Demand kit according to the manufacturer's directions.

Statistical Methods

Parametric data were analyzed using pairwise t-tests that used the pooled estimate of variance and Bonferroni's

correction of the *P* values for multiple comparisons. Pair-wise Wilcoxon-Mann-Whitney tests with Bonferroni's adjustment of *P* values for multiple comparisons were used to analyze nonparametric data.

Results

Ileitis in SAMP1/Fc Mice Is Characterized by Epithelial Injury, Regenerative Changes, and Local Alterations in Epithelial Differentiation Pathways

Histopathological examination of the small intestine and colon from SAMP1/Fc mice demonstrated the presence of segmental ileitis, characterized by the presence of a transmural acute and chronic inflammatory infiltrate (Figure 1A). Histopathological evidence of epithelial injury, including villus shortening, cryptitis and formation of crypt abscesses, was present by age 10 weeks. These features of ongoing epithelial injury were accompanied by regenerative changes in the epithelium, including frequent branching crypts and marked crypt elongation in involved areas of the ileum (Figure 1; A to D). To determine whether elongation of crypts in areas of inflammation was associated with expansion of the replicating transit cell population within the crypt, the replicating cells were localized by immunohistochemistry after administration of BrdUrd to SAMP1/Fc and control mice. The size of the replicative zone was increased in involved areas of ileal mucosa compared with both adjacent non-involved segments of ileum and the jejunum of SAMP1/Fc mice (Figure 1, B and C). The number of replicating cells per crypt in jejunum or uninvolved segments of ileum was similar to the number of S phase cells found in the corresponding regions of age-matched AKR or C57BL/6j control animals (data not shown).

Alterations in epithelial differentiation were also prominent in the ilea of SAMP1/Fc mice. Dramatic increases in the number of Paneth, intermediate, and goblet cells were evident within elongated crypts (Figure 1; D, E, and H). Cells with Paneth cell-type granules were found distributed throughout the crypt up to the crypt-villus junction and occasionally on the lower portion of the villus epithelium (Figure 1, D and E). Many of these granule-containing crypt epithelial cells were also positive for staining with PAS-Alcian blue, suggesting that these cells express mucins, consistent with an intermediate cell phenotype (Figure 1H). Increased numbers of Paneth and intermediate cells were evident in focal areas of the ileum in at least 50% of SAMP1/Fc mice as early as 4 weeks of age (Figure 1H and Table 1), a time when there was no histological evidence of either acute or chronic inflammation (total inflammatory index, 0.22 ± 0.22 ; Table 1). By contrast, age-matched AKR mice did not display aberrant numbers of Paneth and intermediate cells (Figure 1G). At older ages (10 and 40 weeks), when ileal inflammation is a prominent histological feature in SAMP1/Fc mice, all mice examined had markedly increased numbers of Paneth and intermediate cells in this region of the

Table 1. Appearance of Abnormal Crypts Precedes Histological Evidence of Inflammation in SAMP1/Fc Mice

Age (weeks)	Total inflammatory score (average $\pm$ SEM)	% Mice with aberrant crypts
4	0.22 ± 0.07	54.5 (6/11)
10	12.1 ± 1.7	100 (10/10)
40	17.9 ± 0.97	100 (10/10)

gut (Table 1). Electron microscopic analysis of crypt epithelial cells in SAMP1/Fc mice revealed that, in areas of ileitis, many cells in the mid and upper crypt contained immature Paneth cell granules characteristic of intermediate cells (Figure 1, F and I).

Expression of Enterocytic Lineage Markers in the Ileum

The significant increase in the number of secretory cells observed in SAMP1/Fc mice suggests that the balance between enterocytic and secretory cell differentiation in areas of inflammation is perturbed to favor differentiation of the secretory cell lineages. To further investigate this possibility, we examined the expression of ileal lipid-binding protein (*Fabp6/ilbp*), a small lipid-binding protein found exclusively in differentiated enterocytes of the ileum. At age 4 weeks, no differences were apparent in the expression of *Fabp6/ilbp* mRNA in the ileum of SAMP1/Fc compared to AKR controls. However, *Fabp6/ilbp* mRNA expression was significantly down-regulated in the SAMP1/Fc ileum at age 10 weeks compared with AKR mice and further reduced by age 40 weeks (Figure 2A). Expression of the ileal-specific bile acid transporter (*asbt*), another marker of differentiated ileal enterocytes, was also reduced in SAMP1/Fc compared with AKR control mice at both 10 and 40 weeks of age, suggesting that there is a progressive and selective reduction in the number of absorptive enterocytes with increasing inflammation. To confirm that the decrease in expression of these enterocytic products is reflected in a selective reduction in the number of absorptive cells, cell-specific expression of *Fabp6/ILBP* was also examined by immunohistochemistry (Figure 2; B to E). At 4 weeks of age, ILBP was expressed abundantly in villus-associated enterocytes above the crypt-villus junction, in both SAMP1/Fc and AKR ilea (Figure 2, B and C). However, by 10 weeks of age there was an overall decrease in the number of ILBP-positive cells in areas of ileal inflammation in the SAMP1/Fc mouse (Figure 2D). Occasional cells that stained strongly for *Fabp6/ILBP* were observed in the upper crypt and villus at this age. In areas of severe inflammation in older SAMP1/Fc mice (age 40 weeks), *Fabp6/ILBP*-positive cells were not observed (Figure 2E).

The Effects of Inflammation on the Crypt Epithelial Stem Cell Population in SAMP1/Fc Mice

Because definitive markers for clonogenic stem cells in the gut have yet to be validated, we used a functional

Figure 1. Histopathological features of ileitis in SAMP1/Fc mice. **A:** Ileitis is characterized by the presence of a marked transmurular acute and chronic inflammatory infiltrate (H&E stain). Crypt abscesses (**arrow**) and features suggestive of epithelial regeneration such as branching crypts (**A**, **arrowhead**) accompany the marked inflammatory infiltrate. Replicating epithelial cells were identified in 10-week-old AKR (**B**) and SAMP1/Fc mice (**C**) by BrdUrd incorporation. Cells incorporating BrdUrd were detected by immunohistochemistry (brown pigment). Note the marked expansion of the replicative zone within ileal crypts in the SAMP1/Fc mice compared with age-matched AKR control mice. **D:** Loss of villi, marked crypt elongation, and increased numbers of Paneth cells and intermediate cells containing eosinophilic granules (**open arrows**) are evident throughout the crypt of SAMP1/Fc mice at 20 weeks of age. In AKR mice, Paneth cells are normally restricted to cell positions 1 to 4 at the crypt base. **E:** Higher magnification of several crypts from an area of inflammation in the ileum of a 20-week-old SAMP1/Fc mouse. Note the eosinophilic granules in numerous cells located from the base of the crypt to the crypt-villus junction. PAS-Alcian blue staining demonstrates increased numbers of goblet cells in areas of inflammation in 4-week-old SAMP1/Fc mice before histological evidence of inflammation (**H**) compared with age-matched AKR mice (**G**). Electron microscopic analysis of crypt epithelium of 10-week-old SAMP1/Fc mice shows cells in the mid and upper crypt containing immature Paneth cell granules characteristic of intermediate cells (**F** and **I**). Original magnifications: $\times 400$ (**D**); $\times 1000$ (**E**, **H**).

assay to investigate the potential effects of inflammation on the crypt stem cell population in the SAMP1/Fc mouse intestine. The response of the crypt stem cell to injury induced by a variety of genotoxic and cytotoxic agents

has been primarily studied using the microcolony formation assay, which is based on the capacity of surviving stem cells (clonogens) to regenerate crypt-like foci of replicating epithelial cells that can be scored histologi-

Figure 2. Expression of mature enterocytic cell lineage markers. Levels of mRNA for intestinal lipid-binding protein (*Fabp6*, **A**) and ileal-specific bile acid transporter (*asbt*, **A**) were measured in total RNA preparations isolated from the ileal segments of SAMP1/Fc and AKR mice at the indicated ages as given in Materials and Methods. SAMP1/Fc mRNA levels are normalized at each age relative to age-matched AKR mRNA expression levels. Data are shown as the mean $\pm$ SEM for four mice per group. *, $P \leq 0.01$ versus AKR; [†], $P \leq 0.001$ versus AKR; and ^{††}, $P \leq 0.05$ versus AKR. Cellular expression of *Fabp6* was examined in ileal tissue sections from 4-, 10-, and 40-week-old SAMP1/Fc (**C-E**) and 10-week-old AKR (**B**) mice using an antibody to *Fabp6*/ILBP as given in Materials and Methods. Note the progressive age-related loss of cellular *Fabp6*/ILBP staining in the SAMP1/Fc mouse ileum compared to AKR.

cally 3 to 4 days after injury.^{26,27} The response of the epithelium to radiation injury has been the most extensively characterized model system of injury repair. After exposure to ionizing radiation, rapidly replicating crypt transit cells either undergo apoptosis or cease replication. However, migration of these cells out of the crypt and onto the villus continues after injury. Thus, in the absence of a surviving clonogenic stem cell the crypt will disappear. If one (or more) clonogenic stem cell survives irradiation, it will proliferate and ultimately give rise to an entire regenerative crypt. The number of microcolonies present in tissue cross-sections can therefore be used as a surrogate measure of the number of clonogenic stem cells surviving injury.

The number of regenerating crypts after radiation injury was examined in SAMP1/Fc mice with well-es-

Figure 3. Regeneration of crypts after radiation injury. Mice were pulse-labeled with BrdUrd 2 hours before tissue harvest. **A** and **B** demonstrate the appearance of regenerating crypts in H&E-stained sections of ileum from 40-week-old SAMP1/Fc mice (**A**) and age-matched AKR (**B**) mice 3.5 days after 14 Gy γ -irradiation. **C** and **D** show immunohistochemical detection of S phase cells in adjacent sections detected with anti-BrdUrd. A regenerative crypt is observed if one or more clonogenic stem cells within the crypt survive radiation injury and are able to subsequently replicate. Note that the number of regenerating crypts is markedly increased in the SAMP1/Fc (**C**) mice compared to the age-matched AKR controls (**D**). **E** and **F** show the number of regenerating crypts per cross-section in the indicated region of small intestine determined in 40-week-old AKR, C57BL/6j (BL/6), and SAMP1/Fc mice as described in Materials and Methods. Crypt survival was increased in both inflamed (ileum, **E**) and in noninflamed (proximal jejunum, **F**) regions of the small intestine in SAMP1/Fc mice. Data are shown as the mean $\pm$ SEM for six mice per group. *, $P < 0.01$.

lished ileitis and control mice. A dramatic increase in the number of regenerating crypts after 14-Gy γ -irradiation was observed in the ileum of 40-week-old SAMP1/Fc mice compared with that of similarly treated age-matched AKR or C57BL/6j control mice (Figure 3). The regenerating crypts observed in histological sections of SAMP1/Fc and control animals contained abundant S phase cells, as assessed by immunohistochemistry for BrdUrd, confirming that the regenerating crypts observed in histological sections were viable (Figure 2, C and D). When the numbers of regenerating crypts per cross-section were counted, we observed an ~ 7.0 -fold increase in SAMP1/Fc ileum compared with AKR mice and an ~ 7.9 -fold increase compared with that observed in the ileum of C57BL/6j mice (Figure 3E). Surprisingly, the number of regenerating crypts was not only increased in the ileum of the SAMP1/Fc mice but also in the proximal jejunum, a region of the gut where inflammation

Figure 4. The effect of radiation dose on crypt regeneration in SAMP1/Fc and control mice. The effect of radiation dose on fractional crypt survival in SAMP1/Fc, AKR, and C57BL/6j (BL6) control mice was determined 3.5 days after a single dose of radiation as described in Figure 3 and Materials and Methods. Data are shown as the mean $\pm$ SEM for six mice per group.

is not observed in this mouse model (Figure 3F). The increase in the number of regenerating crypts in the proximal jejunum of SAMP1/Fc mice compared with AKR and C57BL/6j controls was of similar magnitude to that observed in the ileum (Figure 3, compare E and F).

The effect of radiation dose on the number of regenerating crypts was examined in 40-week-old SAMP1/Fc mice and age-matched controls (Figure 4). There was no significant decrease in crypt survival in SAMP1/Fc, AKR, or C57BL/6j mice at radiation doses up to 10 Gy compared to the number of crypts in jejunal cross-sections of unirradiated mice. Fractional crypt survival decreased rapidly with increasing radiation dose in AKR and C57BL/6j mice. At 16 Gy, the number of surviving crypts was reduced to $\sim 2\%$ in AKR and 2.8% in C57BL/6j mice compared with the number of crypts present in jejunal cross-sections from unirradiated control mice. Crypt survival was significantly enhanced in the jejunum of SAMP1/Fc mice compared with AKR or C57BL/6j control mice at radiation doses greater than 10 Gy. The radiation dose-response curve for SAMP1/Fc mice had a broader shoulder region than observed in AKR and C57BL/6j control mice. At more than 12 Gy, the radiation dose-response curve in SAMP1/Fc mice was approximately parallel to that for AKR and C57BL/6j mice except that it was shifted to higher doses, suggesting that the intrinsic radiosensitivity of the clonogenic stem cells in each strain was similar. The radiation dose response in the ileum was similar to that observed in the jejunum (data not shown).

To determine whether the increase in regenerating crypts after radiation injury was temporally related to the onset of ileitis in SAMP1/Fc mice, crypt survival was compared with inflammatory scores determined histologically at various ages (Figure 5). Before the onset of inflammation, no difference in crypt survival was observed between SAMP1/Fc and control mice (age, 4 weeks to 8 weeks). However, at age 10 weeks, coincident

Figure 5. Crypt survival as a function of age. SAMP1/Fc, AKR, and C57BL/6j mice were analyzed 3.5 days after receiving 14 Gy γ -irradiation. The number of surviving crypts per cross-section was determined as described in Materials and Methods. Data are shown as the mean $\pm$ SEM for six mice per group. *, $P < 0.05$.

with the onset of significant histological evidence of ileitis (total inflammatory index, 12.5 ± 1.75), crypt survival in the SAMP1/Fc mice was increased 2.2-fold ($P < 0.005$) compared with AKR and 2.3-fold ($P < 0.005$) compared with C57BL/6j control mice. Further increases were observed with increasing age, such that by age 40 weeks the number of regenerating crypts in SAMP1/Fc mice was 5.7-fold ($P < 0.01$) and 3.7-fold greater than in AKR and C57BL/6j control mice, respectively.

Increased Crypt Stem Cell Survival Is Not because of Inhibition of Damage-Induced or Spontaneous Apoptosis

The global increase in the number of regenerating crypts observed in SAMP1/Fc mice after radiation injury could result from suppression of radiation-induced programmed cell death (apoptosis) in the lower crypt, where the epithelial stem cell population is thought to reside. We therefore examined crypt epithelial cell apoptosis induced by radiation injury in the small intestine of SAMP1/Fc and AKR mice (Figure 6). No significant difference was observed in the number of apoptotic cells per crypt induced by 8-Gy γ -irradiation in ilea from 10-week-old SAMP1/Fc

Figure 6. Radiation-induced apoptosis in AKR and SAMP1/Fc mice. Crypt epithelial apoptosis was quantified using histological criteria and confirmed by *in situ* terminal dUTP nick-end labeling staining in 10-week-old SAMP1/Fc and AKR mice 6 hours after receiving 8 Gy γ -irradiation (**A** and **B**) or in untreated mice (**C** and **D**). In SAMP1/Fc mice the frequency of radiation-induced or spontaneous apoptosis in either the proximal jejunum (**A** and **C**) or in histologically uninvolved areas of ileum (**B** and **D**) did not differ significantly from that of the corresponding region of intestine from AKR controls. Data are shown as the mean $\pm$ SEM for six mice per group.

mice (2.7 ± 0.53 apoptotic cells/crypt) compared with age-matched AKR mice (2.4 ± 0.32 apoptotic cells/crypt). Similarly, there was no significant difference in crypt epithelial apoptosis induced by radiation injury in 40-week-old SAMP1/Fc mice compared with control mice (data not shown). In the absence of experimental radiation injury the rate of spontaneously occurring apoptosis in proximal jejunum (0.12 ± 0.09 apoptotic cells/crypt) of SAMP1/Fc mice was low and similar to that observed in the jejunum of AKR controls (0.36 ± 0.17 apoptotic cells/crypt) (Figure 6C). Similarly, noninflamed areas of ileum of SAMP1/Fc mice manifested very low rates of spontaneous apoptosis (0.14 ± 0.01 apoptotic cells/crypt), which also did not differ significantly from rates observed in the ileum of age-matched AKR controls (0.12 ± 0.06 apoptotic cells/crypt) (Figure 6D).

The Capacity of Stem Cells to Repair Potentially Lethal Radiation Damage Is Not Altered in SAMP1/Fc Mice with Ileal Inflammation

The global increase in crypt regeneration observed in SAMP1/Fc mice could also result from an increased ability of crypt stem cells to repair potentially lethal radiation-induced damage. To investigate this possibility, we measured the capacity of the clonogenic stem cell population in SAMP1/Fc and age-matched control mice to repair potentially lethal radiation damage using the method described by Roberts and colleagues²⁵ (Figure 7). The number of regenerating crypts was determined in mice after either a single dose of radiation or the same total

Figure 7. Repair of potentially lethal radiation damage in crypt stem cells. SAMP1/Fc, AKR, and C57BL/6j mice (40 weeks old) were irradiated with a single dose of 14 Gy or with two doses of 7 Gy each, separated by a 5-hour recovery period. Fractional crypt survival (**A**) was determined at 84 hours after irradiation. The ability of stem cells to repair potentially lethal radiation-induced damage (repair factor, **B**) was assessed by determining the ratio of survival after two doses of radiation separated by an intervening recovery period (allowing sufficient time for the clonogenic stem cells to repair radiation-induced damage) with survival after radiation administered as a single dose. Note that there was a significant enhancement in crypt survival in all of the mouse strains when mice were treated with the fractionated dose protocol compared to crypt survival in mice receiving 14 Gy as a single dose. Differences in the repair factors between SAMP1/Fc, AKR, and C57BL/6j were not significant. Data are shown as the mean $\pm$ SEM for five mice per group.

dose of radiation delivered in two fractions separated by a period of time sufficient for the surviving clonogens to repair potentially lethal radiation damage (~ 5 hours). As expected, there was significant enhancement in crypt survival in both the SAMP1/Fc mice and controls receiving a cumulative radiation dose of 14 Gy given as two fractions compared with that in mice receiving a single dose of 14 Gy (Figure 7A). However, the overall enhancement of crypt survival resulting from fractionating the radiation dose (repair factor) was not significantly different among the three mouse strains examined (Figure 7B).

Expression of Inflammatory Mediators and Growth Factors Associated with Altered Crypt Stem Cell Survival or Proliferation

A number of studies have suggested that peptide growth factors, prostaglandins, cytokines, and other regulatory peptides can affect cell survival, both *in vivo* and *in vitro*. Our observation that there is global enhancement of intestinal crypt survival in SAMP1/Fc mice suggests that a circulating factor or factors produced as a consequence of the inflammatory state of the intestine affects crypt survival and stem cell regeneration. To determine the identity of such a factor or factors, the tissue expression of IL-1 and IL-11, FGF-1, FGF-2, FGF-7 and FGF-10, intestinal TFF-3, TGF- β 3, tumor necrosis factor α (TNF- α), and cyclooxygenases 1 and 2 (COX-1, COX-2), was examined by real-time PCR in total RNA preparations of ilea from 4-, 10- and 40-week-old SAMP1/Fc and age-matched AKR control mice. No strain-specific differences in the expression of any of these effector molecules were observed at 4 weeks of age, ie, before the onset of ileal inflammation. Levels of FGF-2, FGF-7, FGF-10, and IL-11 mRNA remained constant as a function of age in AKR mice. However, in SAMP1/Fc mice expression of these mediators increased significantly by age 10 weeks (Figure 8). FGF-7 and IL-11

Figure 8. Expression of mediators known to affect crypt stem cell survival after irradiation. Levels of mRNA for FGF-2, FGF-7, FGF-10, IL-11, TNF- α , and IL-1 were measured in total RNA preparations isolated from ileal segments at the indicated ages of SAMP1/Fc and AKR mice as given in Materials and Methods. mRNA levels are expressed relative to 18S RNA ($\times 10^{-5}$). Data are shown as the mean $\pm$ SD for four to six mice per group. **, $P \leq 0.01$ versus AKR; *, $P \leq 0.05$ versus AKR.

showed the greatest increase in mRNA levels compared to age-matched AKR mice, (~ 4.4 -fold and ~ 13 -fold, respectively). FGF-2 and FGF-10 mRNA levels showed a more modest increase (~ 3.4 -fold greater than those of age-matched AKR mice). The marked increase in expression of these mediators observed in the ileum of SAMP1/Fc mice persisted through at least age 40 weeks (Figure 8). By contrast, expression of IL-1 β and TNF (Figure 8) as well as COX1, COX2, and TGF- β 3 (data not shown) did not change in the ileum of SAMP1/Fc mice as a function of age. Interestingly, although we observed a substantial expansion of the goblet cell population, there was no concomitant increase in the expression of TFF-3, a goblet cell-specific product known to be up-regulated at sites of mucosal injury in some model systems and to enhance crypt stem cell survival after irradiation (data not shown).^{28–30}

Discussion

In this study, we used the SAMP1/Fc mouse as a model system for determining the local and systemic effects of

inflammation on both lineage-specific epithelial differentiation pathways and the size of the replicating crypt epithelial cell population. We found increased numbers of Paneth, intermediate, and goblet cells associated with ileal inflammation in SAMP1/Fc mice, which is consistent with the recent appreciation that these cells play a central role in inflammatory bowel disease and innate immunity.^{7,31–33} Paneth cells are normally most abundant in the terminal ileum;³⁴ however, in patients with Crohn's disease, Paneth cell metaplasia is also found in the colon and rectum,³⁵ where they may participate in the chronic phase of the disease. Increased numbers of Paneth, intermediate, and goblet cells have also been observed in mice infected with *Trichinella spiralis*. Infection with this parasite results in a Th2-mediated, T-cell-dependent enteropathy.³⁶ Paneth cells produce multiple antimicrobial peptides, defensins/cryptidins, lysozyme, phospholipase A₂, and angiogenins. Angiogenins produced by Paneth cells may play a dual role in host defense because they have specific microbicidal activity for *Candida albicans* and *Streptococcus pneumoniae*, in addition to promoting regeneration of the intestinal microvasculature.³³ More recently, it has been appreciated that these cells also produce peptides with immune-regulatory functions, such as TNF- α and granulocyte macrophage-colony stimulating factor.^{8,9} Indeed, human Paneth cells only express TNF- α under pathological conditions, further implicating this cell type in the pathogenesis of Crohn's disease.³⁷ Supporting evidence for this conclusion comes from studies in mice that demonstrate that deletion of the regulatory region of the TNF- α gene leads to the production of TNF- α protein and concomitant development of terminal ileitis.³⁸ Additionally, Paneth cells are a major cellular site of NOD2 expression in Crohn's disease,³⁷ mutations in which are associated with ileitis.^{39–41} Increased expression of NOD2 has also been observed in metaplastic Paneth cells found *de novo* in the colon of Crohn's disease patients. These observations led Lala and colleagues³⁷ to speculate that, in some patients with Crohn's disease, mutations in NOD2 may result in an inappropriate response of Paneth cells to luminal microflora, leading to intestinal inflammation and epithelial injury.

In this study, we observed a progressive expansion of the secretory cell lineages, including Paneth, goblet and intermediate cells, and a concomitant decrease in the number of differentiated enterocytes in the ileum of SAMP1/Fc mice. The changes were evident in the SAMP1/Fc ileum before any histological evidence of inflammation and occurred as early as age 4 weeks. These findings suggest that the abnormalities in lineage allocation observed in the SAMP1/Fc ileum are not merely the sequelae of local inflammation, but reflect a primary alteration of epithelial function in these mice. Whether such an alteration in epithelial function is one of the initiating events leading to intestinal inflammation in this model remains to be determined.

Goblet and Paneth cells differentiate from a common secretory cell lineage progenitor that is distinct from the long-lived progenitor that gives rise to the enterocytic cell lineage. However, both the enterocytic and secretory cell

lineage progenitors are derived from one or more pleuripotent epithelial stem cells located in the lower portion of the crypt. Thus, one explanation for our findings is that there is an increased probability that the stem cell daughter will become committed to the secretory as opposed to the enterocytic cell lineage. Recent studies have indicated that the activities of the Math 1 and Hes-1 transcription factors are crucial in the progenitor cell population for regulating this deterministic point. In the absence of Math 1 activity, as seen in Math 1-null mice, only enterocytic cell progenitors are derived, while all secretory lineages are absent.⁴² In normal mice, Hes-1 acts as a transcriptional repressor to modulate the activity of Math 1, ensuring that the correct number of secretory and absorptive cells are produced in the intestine.⁴³ It is therefore possible that the increase in Paneth/goblet cell numbers results from dysregulation of Math 1 and/or Hes-1 activity at an early age in the SAMP1/Fc mouse. However, further investigation will be required to determine whether changes in the activity of Math 1, Hes-1, or some other factor does in fact account for the alterations in epithelial lineage allocation observed in this study.

Paneth cells in the ileum are normally restricted to cell positions 1 to 3 at the crypt base. However, we observed that Paneth and intermediate cells in the SAMP1/Fc ileum are found throughout the crypt up to the crypt-villus junction, rather than being restricted to the crypt base. This suggests that SAMP1/Fc mice also manifest a defect or alteration in the normal topographic sorting along the crypt-to-villus axis of differentiated cells from the undifferentiated replicating cells and progenitors. A recent study by Battle and colleagues⁴⁴ has defined some of the molecular mechanisms that regulate this phenomenon. Their work demonstrates that cell positioning in the intestine is mediated by the interaction of ephrins and their Eph receptors, which are localized to the surface of intestinal epithelial cells. The normal downward migration of Paneth cells is thought to be because of the high levels of EphB3 receptor expression and the fact that they lack any ephrin ligand expression. Similar to our observation in SAMP1/Fc mice, EphB3-null mice were found to have Paneth cells distributed randomly throughout the crypt-to-villus axis.⁴⁴ This finding suggests that a possible mechanism which could account for our observation is altered expression and/or localization of Eph receptors and ephrin ligands, induced by components of the localized inflammatory response. Whether such a mechanism operates in the SAMP1/Fc mouse remains to be seen, and the specific mediators of such alterations will need to be defined.

We also observed expansion of the replicative zone and crypt branching or fission that were restricted to areas of ileal inflammation. These histological features of epithelial regeneration are similar to those observed in human inflammatory bowel disease.¹⁵ Epithelial regeneration leading to the restoration of normal mucosal barrier in areas of ulceration is a complex, multistep process. First, the continuity of the epithelial layer is re-established by migration of adjacent epithelial cells over the wound or ulceration. Stem cells within surviving crypts proliferate to give rise to progenitors of the more rapidly proliferating

transit cell population. The transit cell population then quickly expands to form a regenerative crypt. If the injury has completely destroyed some crypts, the surviving crypt stem cells can divide to increase their numbers and subsequently, restore sufficient numbers of crypts by crypt fission, to maintain epithelial homeostasis.²⁶ Because the epithelial stem cells within a crypt segregate to daughter crypts when crypt fission occurs, an expansion of the stem cell number must accompany this reparative response. However, the factors that coordinate this complex process in inflammatory bowel disease have not been fully elucidated.

Co-incident with the onset of inflammation in SAMP1/Fc mice, we found a marked increase in the number of regenerating crypts after experimental injury. This not only occurred in areas of inflammation, but also throughout the entire small intestine. These regenerating crypts, or microcolonies, arise after irradiation from the survival of one or more clonogenic stem cells that are subsequently able to proliferate.²¹ Several different mechanisms could account for the observed increase in the number of surviving stem cells and subsequent regenerating crypts after injury in SAMP1/Fc as compared with control mice. These include inhibition of radiation-induced apoptosis, increased repair of potentially lethal damage, or an increase in the crypt clonogen content before experimental injury. We observed no difference in radiation-induced apoptosis between SAMP1/Fc and control mice. The broadened shoulder in the radiation dose versus stem cell survival curve (Figure 4) suggests that either the number of clonogens per crypt is increased or that the capacity of clonogenic stem cells to repair potentially lethal damage is enhanced in SAMP1/Fc mice after the onset of ileitis.²⁶ However, our experiments demonstrate that the ability of clonogenic stem cells to repair potentially lethal radiation damage was not significantly different in SAMP1/Fc mice from that observed in AKR or C57BL/6j control mice. Thus, the most likely explanation for the increase in crypt stem cell survival observed in the SAMP1/Fc mice is that there is a global increase in the number of clonogenic stem cells per crypt that is associated with the onset of localized ileal inflammation in these mice.

The global increase in the number of regenerating crypts after experimental injury was first observed at age 10 weeks, coincident with the onset of localized ileal inflammation in SAMP1/Fc mice, suggesting that these two processes are related. Little is known about the biological consequences of chronic intestinal inflammation on the fate or proliferation of the stem cell population *per se*. The global increase in the number of potential clonogenic stem cells observed in SAMP1/Fc mice could represent a generalized protective response that enhances the capacity of the epithelium to rapidly restore the mucosal barrier after subsequent injury. Although our data demonstrate that SAMP1/Fc mice acquire an increased regenerative response to a radiation-induced global injury, it is not clear whether the enhancement of epithelial regeneration would occur after injury induced by other etiological agents. It is possible that the increase in the number of stem cells may be a by-product of the

sustained production of circulating cytokines and/or growth factors at sites of ileal inflammation in these mice.

Our data demonstrate that the number of replicating transit cells is increased only within crypts in areas of ileal inflammation. However, the number of stem cells per crypt is globally expanded, even in intestinal segments that show no histological evidence of an inflammatory response. The molecular mechanisms that regulate the size of the rapidly replicating transit cell population must therefore be distinct from the signals that mediate proliferation of clonogenic stem cells in the context of ileitis in the SAMP1/Fc mouse. Although the identity of these distinct signaling pathways remains to be determined, we have begun to identify some potential mediators of these pathways.

A number of cytokines, growth factors, and other mediators associated with intestinal inflammation or injury can induce increases in crypt stem cell survival when exogenously administered before radiation. These include TNF- α , TGF- β 3, TFF-3, IL-1, IL-11, COX-1, COX-2, FGF-2, FGF-7, and FGF-10.^{22,45–54} In this study we observed that expression of FGF-2, FGF-7, FGF-10, and IL-11 increased significantly coincident with the onset of inflammation in SAMP1/Fc mice. FGFs are a large family of heparin-binding growth factors with pleotropic effects on a wide variety of cell types that have previously been implicated in epithelial regeneration. However, relatively little is known about the relationship(s) between FGFs and chronic intestinal inflammation. Previous studies have suggested that FGF-2, FGF-7, and FGF-10 may each play a role in regulating repair of injury to the intestinal epithelium.^{51,53,55–61} For example, serum levels of FGF-2 were significantly increased in patients with active inflammatory bowel disease compared with controls and in a study of pediatric patients with Crohn's disease serum FGF-2 levels showed a strong correlation with disease activity.^{60,61} We have previously demonstrated that FGF-2 is induced in the small intestine by radiation injury and that exogenously administered FGF-2 enhances mucosal regeneration after injury.⁵³ Several reports also suggest that FGF-7 and its receptor are expressed at higher levels in the colonic mucosa of patients with inflammatory bowel disease.^{55,56,58} Administration of FGF-10 accelerated healing of small bowel ulceration in an indomethacin-induced injury model and increased the number of regenerating crypts after injury.⁵¹ However, data such as these are difficult to interpret from a mechanistic standpoint, since inflammation, mucosal injury, and repair all occur simultaneously.

The data demonstrating an increase in IL-11 is interesting in light of recent reports that show that administration of recombinant IL-11 before irradiation can enhance intestinal stem cell survival.⁶² IL-11 is produced by epithelial cells in response to various cytokines that regulate the inflammatory response, such as IL-18. Recent studies have shown that administration of IL-11 reduced the severity of colitis in HLA-B27 rats with established colonic disease.^{63,64} In other studies, IL-11 also increased survival in a model of severe cytoablative therapy. This occurred in conjunction with increased villus length, preserved crypt/villus ratio, and reduced hepatic bacterial

foci, as well as decreased apoptosis of intestinal epithelial crypt cells.⁶⁵ These data have led to the hypothesis that IL-11 may exert its beneficial effects by simultaneously affecting stem cell production and/or survival and immune system modulation including reduction of proinflammatory cytokines. These studies suggest that endogenous production of FGF-2, FGF-7, FGF-10, and/or IL-11 promote repair of mucosal injury in the ileum of SAMP1/Fc mice. However, determining whether enhanced expression of one or more of these peptides as a consequence of ileal inflammation directly mediates the effects observed on epithelial stem cells and differentiation of progenitor cells, will require further study.

In this study, we have demonstrated that localized perturbations in lineage-specific epithelial differentiation occur in very young SAMP1/Fc mice, well before any histological evidence of acute or chronic inflammation. This suggests an as yet unidentified early underlying abnormality of epithelial function in these mice. These changes in epithelial differentiation persist into adulthood and are further amplified with the development of a local inflammatory response. This raises the possibility that reciprocal interactions between inflammation and epithelial differentiation also occur in these mice. We further found significant effects on the dynamics of crypt stem cell turnover and the size of the rapidly replicating transit cell population in response to inflammation in SAMP1/Fc mice. However, distinct mechanisms appear to regulate these disparate phenomena, suggesting that different components of the inflammatory response are involved, including both systemic and localized effects. Such profound alterations in both epithelial differentiation and the dynamics of the stem cell population are likely to have important implications for understanding the pathogenesis of inflammatory bowel disease and innate immunity.

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