Abbreviations: UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel disease; 6-TG, 6- thioguanine; Spi, sensitive to P2 interference; MI, microsatellite instability; TGF-β1, transforming growth factor -β1

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ABSTRACT

Individuals with inflammatory bowel disease are at increased risk of developing gastrointestinal cancer. Here, we have tested the possibility that chronic inflammation could trigger mutations. For this, we have used IL-10 deficient (*IL-10*$^{-/-}$) mice, which spontaneously develop intestinal inflammation, in combination with a transgenic gpt gene and *red/gam* gene (*gpt*$^{+}$*IL-10*$^{-/-}$) which is a well-characterized mutation reporter locus.

The total mutation frequency in the colon of *gpt*$^{+}$*IL-10*$^{-/-}$ mice was about 5 times higher than that in normal *gpt*$^{+}$*IL-10*$^{+/+}$ mice. In the particular case of G:C to A:T transitions, the frequency of mutations in *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 4.1 times higher than in control mice. Interestingly, the frequency of small deletions and insertions was also strikingly increased(about 10 times). The majority of the deletion or insertion mutations were observed in the monotonous base runs or adjacent repeats of short tandem sequences. In contrast, the frequency of large deletions, detected by loss of the Spi marker present in the *red/gam* transgene were similar between the mouse strains.

Finally, as a control, the mutation frequency in non-inflamed tissues,

such as the liver, were similar between *gpt*$^{+}$*IL-10*$^{-/-}$ mice and *gpt*$^{+}$*IL-10*$^{+/+}$ mice.

Our data demonstrate that the chronic inflammatory environment in the colon promotes the generation of mutations.

Introduction

Ulcerative colitis (UC) and Crohn's disease are chronic inflammatory bowel diseases (IBD) associated with a high risk of gastrointestinal cancer. This risk begins to increase approximately 10 years after the onset of the disease and increases with the extent and duration of the inflammatory process [1]. Gastrointestinal cancer in individuals with IBD appears to develop through a multistep process involving genomic instability and the progressive accumulation of genomic alterations [2-4]. However, it has not been fully elucidated what kinds of genomic mutations are critical for tumorigenesis.

It has been reported that interleukin-10 knockout (*IL-10*$^{-/-}$) mice spontaneously develop intestinal inflammation characterized by discontinuous transmural lesions affecting the small and large intestine and by the dysregulated production of proinflammatory cytokines [5]. Inflammatory changes first appear in the cecum and ascending and transverse colon of such 3-weeks old mice, and thereafter spread to the remainder of the colon and rectum [5]. Prolonged disease with transmural lesions and a high incidence of colorectal adenocarcinomas are also

observed. However, in germ free conditions, *IL-10*$^{-/-}$ mice never develop inflammation nor adenocarcinomas [5].

Recently, a new transgenic mouse line, *gpt* delta (*gpt*$^{+}$), was established to facilitate the detection and analysis of mutations *in vivo* [6]. The striking feature of *gpt*$^{+}$ mice is their ability to reveal deletions and point mutations. About 80 copies of lambda EG10 shuttle vector DNA carrying the *red/gam* gene of lambda phage and the *gpt* gene of *Escherichia coli* are integrated in chromosome 17. Relatively large deletions in the *red/gam* gene are individually identified by sensitive to P2 interference (Spi)$^{-}$ selection, and base substitutions or small flameshifts in the *gpt* gene are individually identified by 6-thioguanine (6-TG) selection, respectively [6,7].

IL-10$^{-/-}$ mice and *gpt*$^{+}$ mice are C57BL/6J background, although the vendors of those mice were different each. Therefore, the recombinant mice, *gpt*$^{+}$*IL-10*$^{-/-}$, are much like *IL-10*$^{-/-}$ mice or *gpt*$^{+}$ mice. In this paper, to elucidate the role of inflammation on the accumulation of mutations in colonic DNA, we analyzed *gpt*$^{+}$*IL-10*$^{-/-}$ and *gpt*$^{+}$*IL-10*$^{+/+}$ mice by 6-TG selection, Spi^{-} selection, and direct sequencing method. Then we

compared the patterns and frequencies of mutations in colonic DNA.

Material and Methods

Mice.

The experimental protocol was approved by the committee of animal research of the Tohoku University School of Medicine, Sendai, Japan. *IL-10*$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and *gpt*$^{+}$ mice were obtained from SLC (Hamamatsu, Japan). To investigate the role of inflammation in the mutagenicity, the recombinant mice, *gpt*$^{+}$*IL-10*$^{-/-}$, were established by crossing *gpt*$^{+}$ with *IL-10*$^{-/-}$ mice. Mice were housed in plastic cages in an environmentally controlled room (24°C, 12-h/12-h light/dark cycle). Chow (Nippon Nosan, Yokohama, Japan) and tap water were given ad libitum during the experiment. At 15-weeks or 40-weeks of age, eight *gpt*$^{+}$*IL-10*$^{-/-}$ mice and eight *gpt*$^{+}$*IL-10*$^{+/+}$ mice (four 15-weeks mice and four 40-weeks mice, for each type, all siblings) were weighed and sacrificed by cervical dislocation. The colon was removed and divided into proximal and distal portions.

DNA Isolation and *in vitro* Packaging.

Genomic DNA was extracted from the colon using RecoverEase™ DNA Isolation Kit (Stratagene, La Jolla, CA) according to the manufacture's recommendations. Lambda EG10 phages were rescued from genomic DNA by the *in vitro* packaging method using Transpack® Packaging Extract (Stratagene, La Jolla, CA) following the instructions.

Mutation Assay and Sequencing Analysis

The 6-TG selection was carried out as described previously [6]. DNA sequencing of the *gpt* gene was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The PCR primers of the *gpt* gene were primer-1 (5'-TACCACTTTATCCCGCGTCAGG-3') and primer-2 (5'-ACAGGGTTTCGCTCAGGTTTGC-3') [6].

The sequencing primers were primer-A (5'-GAGGCAGTGCGTAAAAAGAC-3') and primer-B (5'-CTATTGTAACCCGCCTGAAG-3').

The Spi⁻ selection was perfomed as described previously [7]. Phage lysates of the recovered Spi⁻ mutants were used as templates for PCR

analysis. The PCR primers were primer-001 (5'-CTCTCCTTTGATGCGAATGCCAGC-3') primer-002 (5'-GGAGTAATTATGCGGAACAGAATCATGCCAGC-3') primer-005 (5'-CGTGGTCTGAGTGTGTTACAGAGG-3') primer-006 (5'-GTTATGCGTTGTTCCATACAACCTCC-3') primer-012 (5'-CGGTCGAGGGACCTAATAACTTCG-3'). The appropriate primers for DNA sequencing were selected based on the results of the aforementioned PCR analysis [7].

Statistical Analysis

Data were expressed as mean ± standard error (SE). Differences between two groups were tested for statistical significance using Student's t-test. A P value < 0.05 denoted the presence of a statistically significant difference.

Results

At 15-weeks of age, the average weight of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 23.7 ± 3.1g, and that of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice was 28.1 ± 2.1g ($P < 0.05$). Two of the four *gpt*$^{+}$*IL-10*$^{-/-}$ mice and none of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice showed bloody stools or prolapse of the anus. At 40-weeks of age, the average weight of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 26.2 ± 4.7g, and that of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice was 30.6 ± 3.0g ($P < 0.05$). One of the four *gpt*$^{+}$*IL-10*$^{-/-}$ mice and none of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice showed bloody stools or prolapse of the anus. The excised colons from the *gpt*$^{+}$*IL-10*$^{-/-}$ mice were slightly thick and edematous compared with those of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice. As reported previously [5], the *gpt*$^{+}$*IL-10*$^{-/-}$ mice developed inflammation in SPF conditions.

The 6-TG mutant frequency in the total colon of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 13.4×10^{-6}, which was about 5 times higher than that of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice (2.8×10^{-6}) (Fig. 1). In both the proximal and distal colon of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice, the 6-TG mutant frequencies were significantly higher than those of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice (11.8×10^{-6} vs $3.3 \times$

10^{-6}, P=0.004, 15.0×10^{-6} vs 2.3×10^{-6}, P=0.01 respectively).

At 15-weeks of age, the 6-TG mutant frequency of the total colon in the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 11.6×10^{-6}, which was about 5 times higher than that of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice (2.3×10^{-6}) (Fig. 2). In the sequencing analysis of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice, 51.5% of the mutants were single base substitutions (G:C to A:T transition, 14.7%; A:T to G:C transition, 1.5%; G:C to T:A transversion, 7.4%; G:C to C:G transversion, 14.7%; A:T to T:A transversion, 8.8%; A:T to C:G transversion, 4.4%), 35.3% were 1bp deletions, and 13.2% were 1bp-3bp insertions. In contrast, 91.6% of the mutants in the *gpt*$^{+}$*IL-10*$^{+/+}$ mice were single base substitutions (G:C to A:T transition, 33.3%; A:T to G:C transition, 16.7%; G:C to T:A transversion, 33.3%; G:C to C:G transversion, 8.3%), 8.3% were 1bp deletions, and none were insertions or complex mutants (Table 1). The frequency of transition mutations in the colitis mice was 1.7 times higher than that of the control mice, the transversion was 4.4 times higher, and the 1bp deletion was 21.6 times higher (Table 1). Furthermore, 13.2% of the mutants in the colitis mice were insertions, in marked contrast with the result that insertions were not observed in the control mice. In the *gpt*$^{+}$*IL-10*$^{-/-}$ mice, 93.8% of the

1bp deletions and insertions occurred in the monotonous base runs or adjacent repeats of short tandem sequences (Table 2). In both the proximal and distal colon of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice, the 6-TG mutant frequencies were higher than those of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice (11.1×10^{-6} vs 2.8×10^{-6}, 12.1×10^{-6} vs 1.7×10^{-6}, respectively), but the differences did not reach significance.

At 40-weeks of age , the 6-TG mutant frequency of the total colon in the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 15.2×10^{-6}, which was about 5 times higher than that of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice (3.3×10^{-6}) (Fig. 2). In the sequencing analysis of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice, 84.1% of the mutants were single base substitutions (G:C to A:T transition, 46%; A:T to G:C transition, 11.1%; G:C to T:A transversion, 19%; G:C to C:G transversion, 1.6%; A:T to T:A transversion, 3.2%; A:T to C:G transversion, 3.2%), 9.5% were 1-3 bp deletions, and 6.4% were 1-2 bp insertions. In contrast, 93.4% of the mutants in the *gpt*$^{+}$*IL-10*$^{+/+}$ mice were single base substitutions (G:C to A:T transition, 52.2%; A:T to G:C transition, 2.2%; G:C to T:A transversion, 32.6%; G:C to C:G transversion, 6.5%), 4.3% were 1bp deletions, and 2.2% were 2bp insertions (Table 3). The frequency of transition

mutations in the colitis mice was 1.8 times higher than that of the control mice, the transversions was 1.3 times higher. In the transitions, the frequency of G:C to A:T in the *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 4.1 times higher than that of the control mice, and 4.1 times higher than that of the 15-weeks *gpt*$^{+}$*IL-10*$^{-/-}$ mice. Furthermore, the small deletions of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice were 10.3 times higher (Table 3), and the small insertions were 13.4 times higher than those of the control mice. In the *gpt*$^{+}$*IL-10*$^{-/-}$ mice, 90% of the deletions and insertions occurred in the monotonous base runs or adjacent repeats of short tandem sequences (Table 4). In both the proximal and distal colon of the *gpt*$^{+}$*IL-10*$^{-/-}$ mice, the 6-TG mutant frequencies were higher than those of the *gpt*$^{+}$*IL-10*$^{+/+}$ mice (12.4×10^{-6} vs 3.9×10^{-6}, 17.9×10^{-6} vs 2.8×10^{-6}, respectively), but the differences did not reach significance.

The Spi^{-} mutant frequency of the total colon in the *gpt*$^{+}$*IL10*$^{-/-}$ mice was not significantly different from the *gpt*$^{+}$*IL10*$^{+/+}$ mice (15 and 40 weeks; 1.5×10^{-6} vs 1.4×10^{-6}, P=0.9, 15 weeks; 1.1×10^{-6} vs 0.8×10^{-6}, P=0.4, 40 weeks; 1.8×10^{-6} vs 2.0×10^{-6}, P=0.8) (Fig. 3, 4). In sequencing analysis, the pattern of the mutations was identical in both types of mice (Table 5, 6).

Discussion

Assaying mutations using transgenic mice is a powerful tool for obtaining information about the pattern and frequency of inflammation-induced mutations. A feature of the assay using *gpt*$^{+}$ mice is the incorporation of two distinct selections for detecting different types of mutations: Spi^{-} selection for relatively large deletions and 6-TG selection for base substitutions and small frameshifts [6,7]. In this study, we examined the *in vivo* mutation spectrum induced by chronic inflammation by comparing *gpt*$^{+}$*IL-10*$^{-/-}$ mice with *gpt*$^{+}$*IL-10*$^{+/+}$ mice.

The *APC* gene is a tumor suppressor gene, and carcinomas from familial adenomatous polyposis (FAP) patients or non-FAP patients exhibit a high frequency of mutations in the *APC* gene. In FAP tumors or sporadic tumors, small deletions and insertions of the *APC* gene are most frequent [8]. In the present study, small deletions and insertions strikingly increased in the *gpt*$^{+}$*IL-10*$^{-/-}$ mice. Regarding the point mutations of the *APC* gene in FAP tumors or sporadic tumors, G:C to A:T transitions were most prevalent [8], which was similar to the *gpt*$^{+}$*IL-10*$^{-/-}$ mice. The

frequency of G:C to A:T transitions in the 40-weeks *gpt*$^{+}$*IL-10*$^{-/-}$ mice were 4.1 times higher than those of the 40-weeks control mice, and 4.1 times higher than those of the 15-weeks *gpt*$^{+}$*IL-10*$^{-/-}$ mice. It is suggested that G:C to A:T transitions in the inflamed colon accumulate with time. Furthermore, point mutations and allelic loss of the *APC* gene have been reported in UC-related dysplasia and cancer, although there is a controversy about the frequencies [9]. In that report, five of the 7 *APC* mutations were frameshifts and 2 were point mutations. Of the five frameshifts, 4 were deletions, and 3 of these occurred within homocopolymer tracts and 1 was a 4-base pair direct repeat (AAGA). On this point, the mutation spectrum of our result was similar to that of *APC* mutations.

The *p53* gene is a member of a family of tumor suppressor genes, and inactivation of this protein plays a crucial role in the emergence and further progression of a multitude of human malignancies including carcinoma of the colon and rectum. It was reported that the *p53* mutation can be detected in early colitic cancer and dysplasia of UC patients' colon, in contrast to sporadic colon cancer [10]. Previous studies demonstrated that over 50% of ulcerative colitis samples had increased frequency of G:C

to A:T transition mutations of the *p53* gene [10-14]. In our study, it is suggested that G:C to A:T transitions in the inflamed colon accumulate with time. Therefore, our data may reflect some mechanisms responsible for the p53 mutations of ulcerative colitis.

Microsatellite instability (MI) has been reported not only in colitic cancers but also in dysplasias and even in nondysplastic inflamed mucosa, though the frequencies were not so great [4]. It seems that MI is related to insufficient repair of replication errors. Transforming growth factor (TGF)-β1 inhibits the differentiation of some cells of mesodermal origin and potently inhibits the proliferation of epithelial cells. Conversely, cells that lose responsiveness to TGF-β1 may show uncontrolled growth and become tumorigenic. Previous studies showed that mutational inactivation of the polydeoxyadenine (poly A) microsatellite tract within *TGF-β1 receptor type II* (*TGF-β1RII*) occurs early and in a subset of UC neoplasms, and that the majority of reported mutations were 1- or 2- base deletions or insertions [15]. In our study, small deletions and insertions had greatly increased and about 90 % of the 1bp deletions and insertions occurred, just like microsatellite sequences and poly A tract, in the

monotonous base runs or adjacent repeats of short tandem sequences. In a previous study on DNA polymerase δ, John and his colleagues suggested that strand slippage during replication may be a primary source of insertion and deletion mutagenesis in eukaryotic genomes [16]. Therefore, it may be suggested that the 1bp deletions and insertions in the *gpt*$^{+}$*IL-10*$^{-/-}$ mice increased because of a replication error following repeated mucosal injury and regeneration in the chronic inflammation.

Shin reported that the predominant spontaneous events observed in a mouse kidney epithelial cell line (K435) were G:C to C:G transversion mutations and small events observed in mutant cells isolated from the hydrogen peroxide and ionizing radiation exposed cells were also predominantly G:C to C:G transversions. They suggested that the mechanism did not include a classical deficiency in mismatch repair and the initial formation of C:C or G:G mispairs provided the most plausible explanation [17]. At 15-weeks of age, the frequency of G:C to C:G in the colitis mice was most frequent and 9 times higher than that of the control mice. The mutation mechanism which did not include a classical deficiency in mismatch repair in the report on K435 may partially

contribute to our data in *gpt*$^{+}$*IL-10*$^{-/-}$ mice.

UC is a chronic inflammatory disease that produces reactive oxygen and nitrogen species and increases the risk of colorectal cancer. Reactive oxygen and nitrogen species produced by inflammatory cells can interact with key genes involved in carcinogenic pathways such as *p53*, DNA mismatch repair genes, and even DNA base excision-repair genes [18, 19]. In previous studies, a positive correlation was observed between higher inducible nitric oxide synthase (iNOS) activity and increased *p53* G:C to A:T transitions in inflamed colon and colon cancer [11, 14]. The deamination of 5-methylcytosine has been argued to be a major mechanism for the induction of G:C to A:T transitions at CpG dinucleotides in DNA [20]. Nitric oxide produced during inflammation may cause both deamination and oxidative damage to DNA. It has been reported that *IL-10*$^{-/-}$ mice had increased damage scores and granulocyte infiltration concurrent with increased mRNA and protein synthesis for iNOS in intestinal tissues [21]. These data suggest that oxidative stress and DNA adducts may drive the accumulation of mutations in the colon of the *gpt*$^{+}$*IL10*$^{-/-}$ mice. In a previous paper on chronic *Helicobacter pylori*

infections, it was suggested that the Helicobacter-infected mice exhibited severe gastritis and a high level of iNOS messenger RNA expression and A:T to C:G and G:C to T:A transversions had greatly increased [22]. In our study, the frequency of G:C to T:A transversions in the 40-weeks *gpt*$^{+}$*IL-10*$^{-/-}$ mice was 2.6 times higher than that of the 40-weeks control mice, and 3.3 times higher than that of the 15-weeks *gpt*$^{+}$*IL-10*$^{-/-}$ mice. On this point, the mutation spectrum of our result was similar to that of the paper on chronic *Helicobacter pylori* infections. On the contrary, our data suggested that G:C to A:T transitions in the inflamed colon accumulate with time unlike the paper on chronic *Helicobacter pylori* infections. We think that it is due to differences of organs, duration of inflammation, or methods of mutation assay.

It was necessary to confirm that the mutant frequency in non-inflamed organs of *IL10*$^{-/-}$ mice did not increase. We analyzed livers in the 40-weeks mice as non-inflamed organ in order to clear whether the effect observed is associated to inflammation or IL-10 deficiency. At 40-weeks of age, the 6-TG mutant frequency in the liver of the *gpt*$^{+}$*IL10*$^{-/-}$ mice was 2.4×10^{-6}, which was not significantly different from the

gpt^{+}*IL10*$^{+/+}$ mice (1.7×10^{-6}). The livers of the *gpt*$^{+}$*IL10*$^{-/-}$ mice were not inflamed, macroscopically. In consequence, it was suggested that the effect observed was associated to inflammation.

In our data, several types of mutations increased, and it is suggested that multiple mechanisms have a role in carcinogenesis of inflamed colon. In the mutations of the *gpt*$^{+}$*IL10*$^{-/-}$ mice, short deletions or insertions in the monotonous base runs or adjacent repeats of short tandem sequences and G:C to A:T transitions were striking mutations. Therefore, biochemical support may be useful for proving that replication error and oxidative stress mainly play a role in carcinogenesis in the inflamed colon.