The initiation of colon cancer in a chronic inflammatory setting

Ru Chen, Peter S. Rabinovitch, David A. Crispin, Mary J. Emond, Mary P. Bronner and Teresa A. Brentnall

Department of Medicine, Department of Pathology and Department of Biostatistics, University of Washington, Seattle, WA 98195, USA and Department of Anatomic Pathology, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

To whom correspondence should be addressed at: Department of Medicine, Box 356424, University of Washington, Seattle, WA 98195, USA. Tel: +1 206 543 3280; Fax: +1 206 685 9478; Email: teribr@u.washington.edu

Chronic inflammation predisposes to cancer. We used an inflammation-induced human model of tumorigenesis to explore how populations of mutated cells expand and initiate the earliest stages of cancer. Ulcerative colitis (UC) is a chronic inflammatory disease of the colon associated with an increased risk of colorectal cancer mediated through a process of genomic instability. In order to characterize the process of clonal expansion, arbitrary primed (AP) and inter-simple sequence repeat (ISSR) PCR DNA fingerprint mutation profiles of single crypts were compared with the mutational profiles from clusters of crypts and whole biopsies within the same individual. To provide information at the earliest steps of neoplastic progression, we examined histologically negative crypts, as well as dysplastic crypts. Crypts from UC dysplasia/cancer show alterations in 10–20% of DNA fingerprint sites, regardless of whether the crypts were dysplastic or non-dysplastic and whether the DNA came from one crypt or thousands of crypts. Of the mutational changes in single crypts, almost half are clonally expanded to adjacent crypts and to the thousands of crypts in a single biopsy. Using fluorescent in-situ hybridization to examine p53 alterations in individual crypt cells, we demonstrate that the mechanism of clonal expansion can occur through crypt fission. DNA alterations are initiated in colonic crypts and expand to adjacent crypts through crypt fission. Our data suggest that a continuous process of DNA mutations, clonal expansion through crypt fission and clonal succession initiates the development of inflammatory-associated colon cancer; this mutational process is moderated by crypt cell turnover and cell death. This paradigm may apply to other inflammatory-induced cancers.

Introduction

Many chronic inflammatory diseases are associated with an elevated risk to cancer. Examples include hepatitis/hepatoma (1), gastritis/gastric cancer (2,3) and ulcerative colitis/colon cancer (4,5). How cancer is initiated in the setting of chronic inflammation is unknown. We have studied ulcerative colitis (UC) as a model of an inflammatory disease that follows this cancer paradigm (6,7). Using molecular methods, we have demonstrated that widespread genomic instability is present in the entire colons of UC patients with cancer/precancer (UC progressors), while instability is absent in the colons of UC patients who are cancer/dysplasia-free (UC non-progressors) (8). There is evidence that the genetic abnormalities accumulate early, before histological changes can be detected, and are probably the soil in which cancer grows (8–10).

How and when do mutated cells expand into dysplasia (precancer) and cancer? Does the mutation load increase as cells become increasingly dysplastic? There are hypotheses about how genetic abnormalities contribute to the mutator phenotype (11) and how clonal succession could be associated with tumor progression (12). However, little is known at the level of the crypt of how clonal succession and expansion occurs. The purpose of this study was to characterize mutational evolution and expansion at the level of single crypts in the setting of chronic inflammation.

To study mutational evolution, we used two PCR-based DNA fingerprinting techniques that use random primers to amplify the genome, AP–PCR (arbitrarily primed PCR) (13,14) and ISSR–PCR (inter-simple-sequence-repeat PCR) (15–17). These two methods were used to assess the fingerprint from each sample, so that each method could cross-validate the other. AP–PCR uses arbitrarily chosen primers and low stringency PCR to generate a linear display of PCR products that represent a DNA fingerprint. Comparison of the normal constitutional tissue (blood or muscle) fingerprint to the target crypt fingerprint can reveal mutational changes in the target DNA.

The second method, ISSR–PCR, amplifies the DNA segments of the genome using primers of inverted abundant repetitive elements. Again, the approach is to use a random cocktail of primers to generate a DNA fingerprint of a given sample. While these two DNA fingerprinting techniques use different random amplification approaches, both of them can compare the DNA sequences from normal tissue (control) with the sequences from colonic crypts (target) allowing the assessment of mutations/alterations in the target DNA. The detection of fingerprint band gains (including appearance of new band and gain of band intensity) and band losses (including reduction of band intensity and complete loss of the band) can reveal allelic gains or losses, deletion or addition of a DNA segment, as well as base-pair mutations that occur in the fingerprint primer regions (8,17,18).

In many cancers associated with chronic inflammation such as UC, genomic instability underlies the process of
tumorigenesis. The histologically negative tissue in UC patients with cancer/precancer, already contain a high mutational load (6,9,19-21) and is the soil in which dysplasia and cancer develop. To understand the changes that occur at the earliest stages of tumorigenesis, we analyzed DNA fingerprint profiles for mutational changes from non-dysplastic biopsies of UC patients with cancer/dysplasia elsewhere in the colon. Clonal expansion and clonal succession were assessed in individual crypts (crypt to crypt changes), in clusters of crypts (100 adjacent crypts combined) and in whole biopsies representing thousands of crypts. Finally, to explore the mechanism of clonal expansion, we used dual-color FISH (fluorescence in situ hybridization) to detect and track the specific mutational alterations in individual crypt cells. FISH can identify chromosome alterations in single interphase cells (9,22), allowing us to discover the mechanism of mutational spread from crypt to crypt before clonal expansion occurs.

Materials and methods

Specimens

Three UC patients with high-grade dysplasia were included in this study; both dysplastic and non-dysplastic crypts were analyzed. In addition, four non-UC control specimens were used to validate the methodology used. Biopsies were obtained at colonoscopy or colectomy in accordance with approved Human Subject’s guidelines at the University of Washington. All biopsies were frozen at -70°C in Minimal Essential Medium with 10% DMSO until use.

Single colonic crypt microdissection

Frozen biopsies were placed mucosa side up into a Petri dish containing enough phosphate buffered saline to cover the biopsy. Under a dissecting microscope, intact single crypts were separated from the perimeter of the biopsy using a 25-gauge needle and then the individual crypts were deposited on a tube for DNA fingerprinting or slide for FISH procedure (Figure 1).

Crypt clusters

Crypt clusters were DNA samples prepared by combining ~100 adjacent single crypts that had been gathered from the same colonic biopsy. The cluster of crypts was used to evaluate the clonal expansion of DNA abnormalities identified from individual crypts from the same biopsy.

Whole biopsies

Colon epithelial cells were isolated from whole biopsies by EDTA shake-off; this provides >90% purity of epithelial cells, as previously described (9). Residual stroma (microscopically free of epithelium) was used as normal controls.

ISSR-PCR and AP-PCR

ISSR-PCR and AP-PCR were performed as previously described (8,15,23). Primer MCG and blue were used for AP-PCR. Primers CG8RG and CG8RY were used for ISSR-PCR. The PCR products were analyzed in 6% denaturing polyacrylamide gel. Dried gels were then scanned using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Details regarding primer sequences and PCR recipes can be found in previous work (8).

Quantification of fingerprints

Band intensity was measured by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For each sample, the fingerprint bands from colonic crypt DNA were compared with fingerprint bands of stroma DNA (normal) from the same patient. Any band with ≥2-fold intensity change compared with the normal counterpart was scored as a change, alteration or mutation. The total number of band alterations divided by the total number of bands examined was defined as percent alteration or genomic instability. A widespread alteration (clonal alteration) was defined as an alteration that occurred in more than half of the single crypt examined. Conversely, alterations that occurred in less than half of the single crypt examined were considered non-widespread.

FISH

FISH was performed as described previously (9). Briefly, microdissected single crypts were dropped on plain glass slides and fixed with 3:1 methanol:acetic acid, followed by 1% paraformaldehyde. Slides were hybridized to paired FITC-labeled chromosome 17 centromere and TRITC-labeled p53 probes (Vysis, Downers Grove, IL). Slides were dehydrated in 70, 85 and 100% ethanol at 4°C; dehydrated in 70% formamide at 72°C; dehydrated in 70, 85 and 100% ethanol at -20°C and incubated overnight with the probe pairs. Slides were then washed in 2× SSC at 72°C and 2× SSC with 0.03% Tween (pH 5.3). Cells were covered with Anti-fade (Oncor, Gaithersberg, MD) containing 0.25 ng/μl diamindino-2-phenyl indole (Sigma, St Louis, MD) and slides were examined by microscopy at 100× under oil.

Results

Reproducibility of DNA fingerprinting

Studies were undertaken to determine the reproducibility of the assay in single crypts and in whole biopsies using normal non-UC colons. Intra-crypt variability was examined by comparing DNA fingerprints from each half of 14 single crypts from normal colons: 7 each by ISSR-PCR and AP-PCR, respectively. The intra-crypt variation (percent of PCR bands with 2-fold intensity difference between samples, see Materials and methods) was 0.7 ± 0.6% for ISSR-PCR and 1.8 ± 1.1% for AP-PCR. Thus, the DNA fingerprint technique is highly reproducible and one-half of a normal single crypt is sufficient to generate reproducible PCR product bands.

Inter-crypt variability was assessed by comparing the DNA fingerprint of one single crypt with another derived from the same normal biopsy. The inter-crypt variation for normal colons was 3.6 ± 0.6% for ISSR-PCR (n = 21) and 3.6 ± 0.8% for AP-PCR (n = 24). These data were consistent with the previous study of normal crypt using ISSR-PCR analysis (24). We also assessed fingerprint variation between normal biopsies, which proved to be 1.5 ± 0.6% (n = 12) for ISSR-PCR and 2.5 ± 0.41% for AP-PCR (n = 24). These data suggest that inter-crypt and normal biopsy variability is low; however, not surprisingly, intra-crypt variability is the lowest.

Fingerprinting of single UC crypts and crypt clusters that are histologically negative

In order to assess the mutational load in histologically negative colonic mucosa, we quantified and compared the amount of variability in individual crypts compared with crypt clusters (100 crypts) and whole biopsies (1000 crypts). Eighteen single crypts and a cluster of 100 crypts were microdissected from a single histologically negative biopsy from a patient with high-grade dysplasia elsewhere in the colon (case 1). The single crypts and the crypt cluster were then subjected to ISSR-PCR (n = 8 crypts) and AP-PCR (n = 10 crypts). DNA
fingerprinting was also performed on the same whole biopsy (histologically negative biopsy, approximately thousands of crypts) and on whole biopsies of indefinite for dysplasia, low-grade dysplasia, and high-grade dysplasia from the same patient. Examples of DNA fingerprint alterations of a single crypt are shown in Figure 2. The upper panels are the densitometry tracings of each lane of the gel, shown in the same order. By comparing the fingerprints from the normal DNA to a single crypt DNA in the same individual, it is possible to detect alterations in the single crypt fingerprint profiles. This single crypt was microdissected from a negative biopsy from a UC patient who had dysplasia elsewhere in the colon.

Analysis by ISSR-PCR fingerprinting revealed an average of 18.5 ± 1.5% bands altered in single crypts (n = 8) (Table I), a proportion similar to that of alterations in crypt clusters (14.3%) and the average of whole biopsies (19.1 ± 2.4%). The difference between the average alterations of single crypts and whole biopsies (thousands of crypts) is not statistically different (P = 0.70). Similar results were obtained from another 10 crypts from the same biopsy analyzed by AP-PCR (Table I). An average of 10.4 ± 1.3% alteration were present in ten single crypts, while the alterations for crypt clusters and whole biopsies were 11.1 and 10.2 ± 3.5%, respectively. There is no statistical difference (P = 0.47) between the alterations of single crypts and thousands of crypts (whole biopsies).

In order to trace clonal expansion and clonal succession, we studied the pattern of changes in the DNA fingerprints to the clonal changes from a single crypt to thousands of crypts (whole biopsies). Detailed maps showing the band alterations obtained by ISSR-PCR in single crypts, crypt clusters and the whole negative diploid biopsy from case 1 are presented in Figure 3. Many of the alterations in single crypts were present in more than half of the single crypts (widespread alterations), and of these widespread alterations, the majority was also present in the crypt cluster. However, some of the band alterations were limited to only one or a few single crypts and these alterations were infrequently present in the crypt cluster or whole biopsy. For example, loss of bands C, D and G were widespread alterations, which were also common in both individual crypts and whole biopsy; however, alterations of band O, V and W occurred in only one or a few of the individual crypts (non-widespread alteration) and were not present in the crypt cluster or whole biopsy. Thus, some DNA alterations were clonally expanded across multiple crypts, while many alterations were limited to a single crypt or a few crypts (data not shown).

Similar results were obtained when we performed AP-PCR on 10 single negative crypts from another patient with high-grade dysplasia elsewhere in the colon (case 2). Some alterations appeared to be clonally expanded in the whole biopsy and were present in both the whole biopsy analysis and the single crypts analyses; however, the majority of alterations were found in only one crypt or a few crypts and were not detected in the whole biopsy analysis. Finally, we analyzed the frequency of band gains and band losses and observed that band gains occur more frequently—for a single crypt from a negative biopsy, band gains represent 58% of the alterations compared with band losses at 42%.

### Table I. Summary of average band alteration in single crypts, crypt clusters and biopsies

<table>
<thead>
<tr>
<th>Case</th>
<th>Fingerprint method</th>
<th>Single crypts (%)</th>
<th>Crypt cluster (%)</th>
<th>Biopsies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>ISSR-PCR</td>
<td>18.5 ± 1.5</td>
<td>14.3</td>
<td>19.1 ± 2.4</td>
</tr>
<tr>
<td>(NEG crypts)</td>
<td>AP-PCR</td>
<td>10.4 ± 1.3</td>
<td>11.1</td>
<td>10.2 ± 2.4</td>
</tr>
<tr>
<td>Case 2</td>
<td>AP-PCR</td>
<td>12.6 ± 1.7</td>
<td>-</td>
<td>7.7 ± 2.6</td>
</tr>
<tr>
<td>(NEG crypts)</td>
<td>ISSR-PCR</td>
<td>17.4 ± 2.3</td>
<td>9.1</td>
<td>16.5 ± 4.3</td>
</tr>
<tr>
<td>Case 3</td>
<td>AP-PCR</td>
<td>12.1 ± 1.6</td>
<td>13.2</td>
<td>7.8 ± 1.7</td>
</tr>
<tr>
<td>(RGD crypts)</td>
<td>ISSR-PCR</td>
<td>12.4 ± 1.8</td>
<td>10.2</td>
<td>8.9 ± 2.1</td>
</tr>
<tr>
<td>(ALT crypts)</td>
<td>ISSR-PCR</td>
<td>12.6 ± 1.7</td>
<td>11.1</td>
<td>10.2 ± 2.4</td>
</tr>
</tbody>
</table>

Percent band alteration was calculated by dividing the total number of band alteration over the total number of band surveyed in particular sample (single crypt, crypt cluster or whole biopsies). The percent band alterations were then averaged for each category ± SEM. (SEM: standard error.)

Fingerprinting of single high-grade dysplasia crypts

In order to evaluate the mutational load in dysplastic mucosa, we microdissected 18 single crypts and a crypt cluster from a high-grade dysplasia biopsy (case 3) and performed DNA fingerprinting (ISSR-PCR: n = 10 and AP-PCR: n = 8). In addition, whole biopsies from the complete spectrum of histology (including negative for dysplasia, indefinite for dysplasia, low-grade dysplasia and high-grade dysplasia) were also analyzed by DNA fingerprinting. The results, summarized in
Table I and Figure 4, reveal a pattern that is remarkably similar to the mutational profiles described above in the nondysplastic samples. On average, 17.4 ± 2.3% of bands by ISSR–PCR or 12.1 ± 1.6% by AP–PCR were altered in the single crypts (Table I). Over half of the band alterations were present in 5 or more of the 10 single high-grade dysplasia crypts. Band alterations that were present in most of the single crypts were widespread, e.g. clonally expanded. These widespread alterations detected in the single crypts were also frequently found in the crypt clusters or whole high-grade dysplasia biopsies. Band alterations that were uncommon in the crypts were absent in the crypt cluster or whole biopsy. For example, alterations of bands A, B, C and D (Figure 4) were common in both individual crypts and the crypt cluster, while alterations of bands O, P and Q were present in a few of the individual crypts and not present in the crypt cluster or the whole biopsy. As for the frequency of band gains and band losses, band gains occurred more frequently in a single crypt from an HGD biopsy—band gains occurred in 76% of DNA alterations and band losses in 24%.

**Clonal expansion patterns of single negative crypts and single high-grade dysplasia crypts**

We compared and contrasted the mutation profiles of negative crypts and dysplastic crypts in order to understand whether there are differences in the quantity and extent of the mutational load as neoplastic progression occurs. For the histologically negative biopsies examined from cases 1 and 2, the overall frequency of altered fingerprint bands in single crypts was 13.0 ± 1.0% for all 28 single crypts examined (Table II).

To understand the extent of clonal expansion of these DNA alterations, we analyzed the distribution of all of the abnormal bands. Using the combined data from the DNA fingerprinting methods, a total of 90 bands were surveyed in all 18 single crypts from a negative biopsy from case 1: of these bands, 33 had alterations in one or more single crypts. Of these 33 alterations, 47.8% were clonally expanded into more than half of the single crypts (widespread alterations); however, 52.2% were present in only one or a few crypts (non-widespread alterations). Thus, nearly half of the abnormal DNA mutations were shared among 18 crypts. Of the widespread alterations in
crypts, the majority (90.9%) had also clonally expanded into the crypt cluster. Of the non-widespread alterations in the crypts, the majority (66.7%) was not present in the crypt cluster. These results are summarized in Table II.

For the single high-grade dysplasia crypts examined from case 3, the mean alteration for all 18 single crypts was 15.8% ± 1.5% (Table II). In order to understand whether the mutations were clonally expanded among the single crypts studied, we surveyed all the bands (a total of 111 bands) from all 18 single crypts. Thirty-three mutated bands were present in one or more single crypt(s). Of these 33 alterations, 56.5% were widespread (e.g. occurring in more than half of the single crypts); and 43.5% were non-widespread alterations. Of the widespread alterations, more than two-thirds (69.2%) also occurred in the crypt cluster. Of the non-widespread alterations, the majority (90.9%) had also clonally expanded into the crypt cluster. Thus, the pattern of fingerprint changes in the high-grade dysplasia crypts is similar to those observed in the analyses of histologically negative crypts.

Mechanism of clonal expansion

How do crypts come to share the same complex mutational patterns? We hypothesized that crypt fission—the process of crypt duplication whereby a single crypt splits or branches into two crypts—is a natural way to duplicate the heterogeneous changes that are apparent in individual UC crypts. To test this hypothesis, we microdissected and examined a crypt in fission (a branched crypt). To track and compare the mutational changes in the individual cells from each side (each branch) of the fissioning crypt, we performed dual-color FISH, probing a commonly altered gene in UC tumorigenesis, p53. Fluorescent p53 probe and a chromosome 17 centromere probe were used to stain individual branched crypt cells and assess for p53 locus and centromere losses and gains (Figure 5). Three surrounding crypts were also assessed for p53 locus and centromere losses and gains (Table III). Many of the p53 alterations were shared by all five crypts (fissioning and non-fissioning); however, the diversity of p53 alterations were most completely mirrored in the two branches of the fissioning crypt. In addition, cells in the two arms of this branched crypt shared a unique mutation category: gain of one p53 allele and one chromosome 17 centromere, was only seen in the arms of this branched crypt, but not in surrounding crypts.

![Fig. 5. FISH results on a branched crypt using p53 and chromosome 17 centromere probes. Each circle represent a cell that can be clearly assessed for FISH count. White color represents normal FISH count. All other colored circles represent different abnormal FISH counts (copy number changes). Note that the red color, representing gains of one p53 allele and one chromosome 17 centromere, was only seen in the arms of this branched crypt, but not in surrounding crypts.](image)

| Table II. Clonal expansion of fingerprint alterations in single crypts and crypt clustersa |
|-----------------------------|-----------------------------|
| Alteration                  | NEG single crypts (n = 28 crypts) | HGD single crypts (n = 18 crypts) |
| Average % alteration per single crypt | 13.0 ± 1.0% | 15.8 ± 1.5% |
| Total number of different band alterationsb | 33 | 33 |
| % Widespread                | 47.8 | 56.5 |
| % Non-widespread            | 52.2 | 43.5 |
| Of widespread alterationsb | Also occurring in crypt cluster (%) | 90.9 | 69.2 |
| Not occurring in crypt cluster (%) | 9.1 | 30.8 |
| Of non-widespread alterationsb | Also occurring in crypt cluster (%) | 33.3 | 20.0 |
| Not occurring in crypt cluster (%) | 66.7 | 80.0 |

*aCrypt clusters having ~100 crypts.

*bSince fingerprinting data were not available for crypt cluster in case 2, only data in cases 1 and 3 were quantified.

| Table III. Assessment of p53 arm and centromere FISH counts in cells of crypts |
|-----------------------------|-----------------------------|
| Karyotype                   | Branch arm 1 | Branch arm 2 | Surrounding crypt 1 | Surrounding crypt 2 | Surrounding crypt 3 |
| 2a2c*                       | 47 | 58 | 65 | 42 | 102 |
| 2a3c                        | 14 | 10 | 5  | 9  | 31  |
| 1a2c                        | 4  | 3  | 3  | 7  | 10  |
| 3a2c                        | 6  | 2  | 6  | 6  | 18  |
| 2a1c                        | 2  | 3  | 6  | 9  | 5   |
| 3a3c                        | 9  | 3  | 0  | 0  | 0   |
| Total                       | 82 | 79 | 85 | 73 | 166 |

*a is p53 arm probe, c is chromosome 17 centromere probe. 2a2c represents diploid.
**Discussion**

In this study, we applied fingerprinting methods to understand the expansion of mutations as crypts undergo neoplastic progression. Fingerprinting allows the comparison of DNA profiles of individual crypts to the profile of normal DNA from the same patient. Band changes in the crypt profiles identify altered DNA sequences in the crypt(s). It has been previously shown that DNA fingerprinting gains and losses represent sequence changes in DNA—band changes can represent the insertion or deletion (novel band gain) (8,18), loss of DNA sequence (band loss) (17) and/or amplification of a DNA sequence (increase in band intensity) (17). We have previously cloned and sequenced an altered band (a novel band) from one of the fingerprints and verified that the altered band resulted from a deletion of 1015 bp DNA sequence (8).

In a previous study, we showed that genomic instability is low in the epithelium from UC non-progressors. In contrast, levels of instability are high in the epithelium from UC progressors (8); additionally, we verified that the DNA fingerprinting technique is highly accurate, that the band changes in the fingerprint truly represent changes in DNA sequence and that repetitive fingerprinting of the same sample of DNA provides highly reproducible results. In this report, we extended the DNA fingerprinting technique to analyze single colonic crypts and discovered that the colonic crypts of UC progressors have 10–20% of DNA mutated at any given time. In contrast, the background DNA alteration in normal colon biopsies is >5%. In crypts from UC progressors, the mutational load does not differ between the non-dysplastic and the dysplastic crypts. Moreover, the spectrum of changes (DNA gain and loss, DNA duplication) is similar between the non-dysplastic and dysplastic crypts. These findings are a clear illustration of the mutator phenotype occurring at a steady state in both the normal and dysplastic crypts of UC patients with cancer/dysplasia elsewhere in the colon. In validation of this estimate of mutational load, we have used the Affymetrix Centurion 100 k SNP chip to study copy number alterations in UC-associated neoplasia (unpublished data). In epithelium isolated from a biopsy negative for dysplasia biopsy from a UC patient with colon cancer elsewhere, there are 9380 LOH (loss of heterozygosity) events out of 34090 polymorphic SNPs (27%). In contrast, the frequency of LOH events for a UC non-progression is <1%. These preliminary data from a different platform validates the DNA fingerprinting findings described in this manuscript—that ~20% genomic DNA is altered in the colonocytes of UC patients with dysplasia while genomic DNA is unaltered in UC patients who are dysplasia-free.

It is interesting that while ~20% of DNA sequences in normal appearing crypts of UC progressors appear mutated and half of these changes are clonally expanding, yet there is no apparent change in phenotype—the cells look normal. One possibility is that these mutations may passively accumulate having no impact on cell growth or control, but remaining silently present in the single crypt through the process of clonal succession. Indeed, there is likely to be a mixture of DNA damage, some of which gives a growth advantage and some is insignificant and occult.

How does this mutator phenotype initiate colon cancer? Chronic inflammation in colon has been associated with elevated levels of reactive oxygen species and reduced oxidative defenses, both of which might contribute to the genetic damage that can be detected by DNA fingerprinting (26–30).

We postulate that at the initiation of UC tumorigenesis, DNA alterations caused by oxidative damage accumulate through clonal succession in individual crypts. Some of these mutated cells will clonally succeed and those with a selective growth advantage will expand to even larger fields and eventually drive the progression to cancer. If this hypothesis is true, one would anticipate that some of the more widespread abnormalities would take place in genes of functional consequence. Indeed, we have previously mapped out a specific p53 gene mutation and p53 allelic loss in UC progressor colonos and discovered that the areas of mutation or the areas of loss can be large, covering up to one quarter of the colon (19). In addition, we have sequenced several of the most widespread (clonally expanded) bands described here and discovered that they include deletions in putative tumor suppressor regions (unpublished data).

What is the mechanism that leads DNA sequence alterations to spread from one crypt to another? How do mutations clonally expand? We hypothesized that crypt fission (duplication of crypts by branching) is a natural way to duplicate the heterogeneous changes that are apparent in individual UC crypts. Crypt fission is believed to be the underlying factor in the development of adenomas, and is a histological hallmark feature in normal appearing mucosa of chronic colitis (31–33). The p53 gene has long been known to be frequently mutated in UC-associated colorectal cancer (34,35). In this study, we tracked specific p53 alterations in the single cells of several crypts, including one crypt that was in the process of fission. Our results demonstrated that the majority of the p53 FISH abnormalities are shared by all of the crypts (fissioning and the surrounding crypts), and supported the view that multiple fission events can lead to a clonal patch with branched crypts surrounded by crypts containing the same mutation. Our results also illustrated that the p53 alterations that were present in the cells on one side of the crypt branch were clearly mirrored in the cells of the opposite side branch (Figure 5). More importantly, a unique crypt mutation in the two arms of the branching crypt was absent in other surrounding non-branching crypts. The individual cells of the branching crypt are significantly different from those of adjacent non-branching crypts (P = 0.00004), while the cells from the two arms of the branching crypt are statistically homogeneous. These findings suggest that a possible mechanism of mutational expansion could occur through crypt fission, which can clonally extend the heterogeneous changes from one crypt to the adjacent crypt.

What prevents the mutational load from accumulating in colonic crypts to become increasingly dysplastic? Something must balance the continually occurring mutations. We observed similar frequencies of alterations in the single crypts compared with crypt clusters or whole biopsies. It is likely that the DNA abnormalities are in a state of flux but remain at a constant frequency. For instance, while some genomic changes clonally expand (present in clusters and whole biopsies), other changes become extinct or limited. In fact, there seems to be a limit to the amount of mutations that can accumulate, as even in the dysplastic tissue, the amount of instability did not exceed 20%. Multiple protective mechanisms within cells may prevent damage from exceeding a catastrophic level, including both p53 dependent and independent apoptosis. Lastly, mutated cells are lost as daughter cells in the crypts that migrate to the lumen and are sloughed off. This process is accelerated in chronic inflammation because of the rapid cell turnover. Thus, continuous mutation may be balanced by cell loss and death.
In summary, our data reflect the on-going genetic damage in the life of a single crypt superimposed on the dynamics of colonic epithelial renewal. Oxidative injury causing widespread DNA damage leads to the inception of cancer. Our data suggest that it is possible that crypt fission propagates these heterogeneous DNA changes from one crypt to the next. While some of the mutations lead to advantageous functional changes, the majority of mutations are probably functionally silent. Importantly, there appears to be an upper limit of mutational load that a cell can withstand, regardless of whether the cell is dysplastic or not; the maximum mutational load appears to be \( \approx 20\% \) of the DNA sequences assayed by AP-PCR and ISSR-PCR. The continuous and competing mutational alterations among crypts are possibly limited by cell turnover and apoptosis. Eventually, some of these changes may induce a growth advantage and clonal expansion, as evidenced by our findings that some of the mutational changes in one crypt can be found in thousands of adjacent crypts. The final stages of tumorigenesis in the chronic inflammatory setting occur when an accumulation of select mutations allows clonal progression to overtake the balancing forces of cell death and loss.

Acknowledgements

We thank Dr Larry Loeb for his critical review and comments on this manuscript. This work was funded by a grant from the NIH R01CA68124.

Conflict of Interest Statement: None declared.

References