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Perspective

Clonal Expansion in the Human Gut

Mitochondrial DNA Mutations Show Us the Way

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mitochondrial, intestine, clonal, stem cell

ABBREVIATIONS

mtDNA	mitochondrial DNA
SDH	succinate dehydrogenase

ABSTRACT

The mechanisms of how DNA mutations are fixed within the human gastrointestinal tract and how they spread are poorly understood and are hotly debated. It has been well documented that human colonic crypts are clonal units; one epithelial stem cell within the crypt becoming dominant and taking over the crypts' entire stem cell population—so called *monoclonal conversion*. Studies have revealed that crypts can exist as families and develop into patches. The questions have been how do such patches in the human colon develop? Does this have implications on how DNA mutations spread? We have previously shown that mitochondrial DNA (mtDNA) mutations, which result in the deficiency of cytochrome *c* oxidase, are established within a single colonic crypt stem cell, resulting in a crypt with a mixed phenotype. Over time that mutated stem cell can take over the entire stem cell population resulting in a wholly-mutated crypt. We have furthered this research by showing that entirely cytochrome *c* oxidase–deficient crypts are able to divide by a process called *crypt fission*, to form two cytochrome *c* oxidase-deficient daughter crypts, each sharing the exact parental mtDNA mutation. Furthermore, patches of these crypts also possess a founder mtDNA mutation suggesting that fission repeats itself to form patches, which increase in size with age. Here, we hypothesize that this can be expanded into other areas of the gastrointestinal tract, especially the stomach, where there is a paucity of data regarding clonality and the spread of DNA mutations. We ask if these mutated crypts expand at a different rate to wild type ones. We also discuss the implications for the spread of potential carcinogenic mutations within the gut.

INTRODUCTION

Mitochondria are the primary generators of ATP within the cell. They possess their own genome which is approximately 16.6 Kb, self-replicating, and encodes 13 essential proteins of the mitochondrial oxidative phosphorylation complexes, 2 rRNA and 22 tRNA genes. There are multiple genomes within an individual cell. Mitochondrial DNA (mtDNA) appears to be more susceptible to mutation than genomic DNA due to having limited repair mechanisms, a lack of protective histones and the fact they exist within an oxidative environment in the presence of free-radical-generating enzymes. MtDNA mutations appear to be random and increase in frequency with age.[1,2] These mutations can affect all copies of the mitochondrial genome (homoplasmy) or a proportion (heteroplasmy) and for a mutated cellular phenotype to be observed homoplasmy or a high degree of heteroplasmy must be present. Clonal expansion of mtDNA mutations within a single stem cell is a complex issue which may involve a random process.[3] In some cells there is a high level of mutation leading to deficiency of cytochrome *c* oxidase; we have been using histochemical methods to detect cytochrome *c* oxidase (primarily encoded by mtDNA) and succinate dehydrogenase (SDH, entirely encoded by genomic DNA, and used to highlight cytochrome c oxidase deficiency) activity with the specific aim of identifying a marker of clonal expansion of colonic crypt stem cells.[4,5]

INTESTINAL STEM CELLS AND THE CLONAL ORIGINS OF COLONIC CRYPTS

The intestinal epithelial stem cell is thought to be located at, or towards, the bottom of the colonic crypt.[6] The number of stem cells present in an individual crypt is unknown, but we do know that there are multiple stem cells; Taylor et al,[4] have shown that some human crypts histochemically stained for cytochrome *c* oxidase and SDH, show a mixed phenotype with ribands of cytochrome *c* oxidase-deficient epithelial cells, extending from the base mixed in with wild type epithelial cells. This suggests that there are at least two

stem cells, one with normal cytochrome *c* oxidase activity and one deficient in cytochrome *c* oxidase.

Can one stem cell dominate the entire stem population within a crypt? Studies in mice that have received an injection of the mutagen ethylnitrosaurea (that can cause mutations to occur in the glucose-6-phosphate dehydrogenase gene, *G6PD*) initially show a G6PD deficiency in only some crypt cells but over time the entire crypt can convert to being wholly deficient.[7] Later studies have shown similar results with the induction of crypt-restricted metallothionein by ethylnitrosaurea.[8] This process has been termed 'monoclonal conversion' and we believe that partially cytochrome *c* oxidase mutated crypts within the human colonic crypt will, over time, become wholly mutated.

Novelli and colleagues in 1996[9] observed that human colonic crypts were clonal structures by showing that crypts from an XO/XY patient either contained a Y-chromosome or did not; furthermore, the same group also showed that, in a population of Sardinian women who had X-inactivation of the G6PD gene after Lyonization, large patches of mutated crypts were present and could number in excess of 400 crypts.[10] We have also observed that the number of wholly-cytochrome c oxidase-deficient crypts increases with age[4,5] suggesting that these crypts are able to expand into patches. The overall conclusion is that intestinal crypts become clonal in nature and these seem able to expand into patches.

How do these patches develop? The most obvious method of clonal crypt expansion is that crypts themselves divide. This has previously been shown to be an active process in mice and crypt budding has been recognised for some time.[7] We have shown that identical mtDNA mutations are found in separate crypts within a cytochrome *c* oxidase-deficient patch. Furthermore, we have recently shown that crypt fission is the mechanism by which human colonic crypts divide by showing that both arms of a bifurcating, cytochrome *c* oxidase-deficient crypt contains an identical mtDNA mutation. The odds of these two arms receiving an identical mutation independently are incredibly small and therefore rules out the possibility that 2 separate crypts, with the same mutation, have collided and are starting to fuse. These data tell us not only how crypts are able to maintain their numbers but also show how mutations spread within the human intestine.

DO CYTOCHROME *C* OXIDASE-DEFICIENT CLONES WITHIN THE HUMAN COLON EXPAND AT A DIFFERENTIAL RATE TO WILD TYPE CLONES?

Bjerknes in 1996[11] proposed a mathematical model for calculating the relative expansion rate of mutated stem cell populations in the human colon. He predicted that if mutated crypts expanded at the same rate as normal crypts then, with age, one-half of mutant crypt clusters should contain only a single crypt. When his model was applied to data of aberrant crypt foci accumulated by Roncucci et al,[12,13] and Pretlow et al,[14] he showed that crypts within such foci expand at an increased rate in comparison to normal crypts with the majority of clusters containing greater than 1 mutated crypt. When we applied the same model to our data of patch size analysis of cytochrome *c* oxidase-deficient crypts in *morphologically-normal* human colons we found that the ratio of singletons to patches of crypts numbering 2 or more was 0.46 (unpublished observations). When adjusted for age we discovered that cytochrome *c* oxidase-deficient crypts were expanding only 1.15 times faster than the cytochrome *c* oxidase-normal crypts (unpublished observations). Bjerknes qualified his model by saying that although the mutated crypts in his study were expanding >40 times faster than normal crypts this did not mean that the cells within these crypts were cycling at 40 times the rate of normal cells. Nevertheless it does suggest that mutated crypts that are wholly-deficient for cytochrome *c* oxidase are expanding at a comparable rate to cytochrome *c* oxidase-normal crypts within our recent study.

However, a paper by Payne et al,[15] have suggested that there is reduced cytochrome *c* oxidase protein expression to a greater degree in morphologically-normal human colonic crypts from patients with colorectal cancer compared with those from patients with diverticulitis or other undefined noncancer resections. Furthermore, they have suggested that these normal cytochrome *c* oxidase-negative crypts from cancer patients are less apoptotic than those from their control patients, suggesting that cytochrome c oxidase-deficient crypts may expand at a faster rate. Although it is of interest that morphologically-normal cytochrome *c* oxidase-deficient cells maybe more resistant to apoptosis from patients with cancer, we have the major criticism that they have not accounted for patient age, which is paramount in determining the statistical outcome of their study. Although they do not reveal the ages of their patients, it is likely that those with a lower apoptotic index were also from the older patients. We have shown an age-dependent increase in the number of cytochrome c oxidase-deficient crypts in our recent study (found in the online supplemental information)[5] and this includes morphologically-normal specimens from patients with colorectal adenocarcinoma, diverticular disease and one patient that had a cecal stricture. We do not find any statistical difference between these groups. We would therefore suggest that any alteration in apoptotic indices should be normalised with age.

CAN mtDNA MUTATIONS TELL US ANYTHING ABOUT CLONAL ARCHITECTURE OF OTHER REGIONS OF THE GUT?

We have expanded our investigation of clonality and DNA mutation spread from colon to the stomach. Little is known about stem cell biology within the human gastric gland. It is thought that the stem cell region is somewhere in the neck/isthmus; in particular, there is debate as to whether or not these glands are monoclonal or polyclonal units. Where it is becoming clear that gastric glands in the mouse are monoclonal,[16] in the humans the issue is more complex. Some groups have used detection of x-linked inactivation of the human androgen receptor (HUMARA) genes within the stomach and shown that while pyloric glands are homotypic and therefore monoclonal, 50% of the body-type glands appear to be heterotypic and therefore polyclonal.[17] These results suggest that either there is regional variation in the clonality of gastric glands or HUMARA analysis is not reliable. We have some preliminary, unpublished evidence to suggest that human gastric glands (from along the greater curve of the body region) are monoclonal in nature. We have used cytochrome *c* oxidase/SDH histochemistry to show that cytochrome *c* oxidase-deficient glands are present in the human stomach (Fig. 1, manuscript in preparation). We have also evidence to suggest that entire glands can be deficient in cytochrome c oxidase. This suggests that one cytochrome *c* oxidase-deficient stem cell has taken over the entire stem cell population within that gland so that all the progeny are cytochrome c oxidase- deficient. This is very good evidence to suggest that human gastric glands become monoclonal.

We also believe that glands are able to divide themselves by fission and have shown the presence of patches of wholly mutated glands.

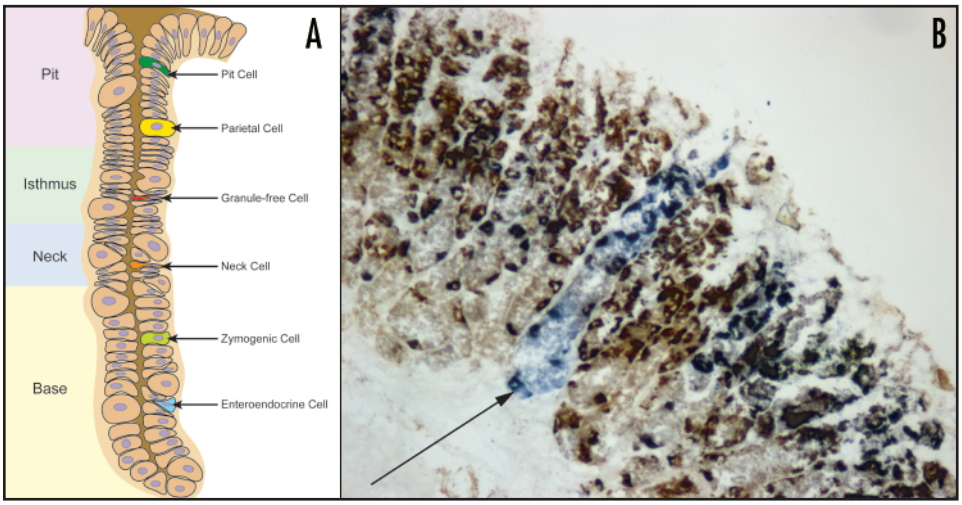

Figure 1. (A) A stylized view of the human body-type gastric gland indicating the various differentiated epithelial lineages contained within. The stem cell is thought to reside somewhere in the isthmus/neck region. (B) Monoclonal conversion in the human gastric gland. A cytochrome *c* oxidase deficient gland (arrow) from the stomach of a 67 year-old male taken from the body region along the greater curve. We predict that initially one stem cell within this gland would have mutated and, by a stochastic process, taken over the entire stem cell population of this gland.

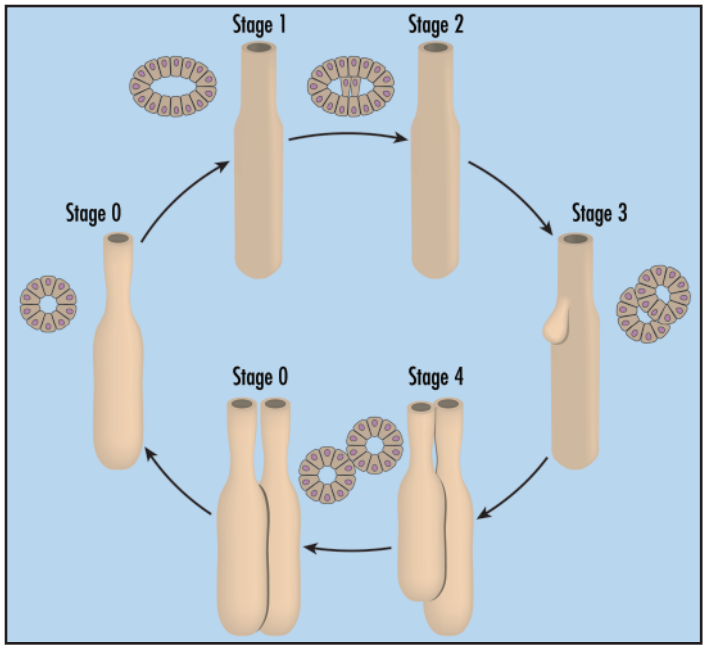

Figure 2. The gland fission cycle. This has been adapted from Hattori and Fjuita.[18] Initially the gland begins to widen at the neck/isthmus region (stage 1), and is bisected (stage 2). At stage 3, budding of the neck region occurs and this leads to the growth of a new gland (stage 4). There is no evidence in the literature to indicate how long this process takes but work in our laboratories is ongoing to answer this question.

When cells are laser-capture microdissected from each of these cytochrome *c* oxidase-deficient glands and compared their mtDNA sequence with the mtDNA sequence from cytochrome *c* oxidase-normal glands we find that all the cytochrome *c* oxidase-deficient glands share the same mtDNA mutations whereas the normal glands have a wild type phenotype. The chances of these mutated glands sharing the exact same mutation by chance are so small to make that theory implausible. This then suggests that at some point one wholly-mutated gland has divided to form two mutated daughter glands—*gland fission*. As it is thought that gastric gland stem cells are located in the isthmus/neck region we propose that at the commencement of fission, a bud develops from the neck and a new gland develops as shown in Figure 2.[18]

mtDNA MUTATIONS AND THE SPREAD OF COLORECTAL CANCER

The subject of the role of mtDNA mutations and the development of colorectal cancer is controversial with no evidence to date that they contribute to the development of the tumor, however, the group of Bert Vogelstein in 1998[19] demonstrated that mtDNA mutations were present in 7 out of 10 colorectal cancer cell lines so there is a possibility that they may play some role. This however, does not stop us from hypothesising that such mtDNA mutations can be used as clonal markers in colorectal cancer. Our recent study was able to demonstrate how morphologically-normal human colonic crypts were able to divide into clonal patches and therefore provides a strong indication for how mutations spread in the human colon. What happens then in colorectal cancer? If crypt fission is the mechanism by which normal crypts divide, dysplastic ones would surely follow the same process, possibly at a higher rate. Previous data from our laboratory has shown that crypts extracted from patients with familial adenomatous polyposis have a significantly higher rate of fission compared to those extracted from normal mucosa.[20,21] This work was based on morphological analysis and it would be of great interest to use the cytochrome c oxidase model to see if each crypt within an aberrant crypt foci contain the same mtDNA mutation(s). This could be extended to fit in with the 'bottom-up' theory of dysplastic spread[22] where one dysplastic crypt could divide from the base up resulting in two identical dysplastic crypts.

SUMMARY

It is becoming clear that all epithelial units of the gastrointestinal tract are clonal or are striving to be clonal through the process of monoclonal conversion. Furthermore we believe that DNA mutations spread in the gut by crypt or gland fission and that this is the mechanism by which aberrant crypt foci develop from a single monocryptal or monoglandular. We would also hypothesize that in conditions of high oxidative stress such as in inflammatory bowel diseases the number and sizes of patches of cytochrome *c* oxidase-deficient crypts would be higher (and therefore increasing at a higher rate) and this work is currently being carried out in our laboratories. We would also anticipate to be able to calculate, by mathematical modelling, the crypt fission index of the normal human colon, which could provide insights into how the human gut grows, and maintains its crypt numbers.

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