A TEST OF THE STOCHASTIC THEORY OF STEM CELL DIFFERENTIATION

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ABSTRACT Stochastic theories of stem cell renewal are shown to predict turnover of intestinal crypts. While I found ample evidence of production of new crypts from direct in vivo studies in adult mice, I failed to find evidence of crypt loss. Thus, it would appear that the simple stochastic models may not provide an adequate theory of control of intestinal stem cell function.

INTRODUCTION

Extensive cell production and loss is a normal component of the function of many tissues (e.g., gut and blood). Central to renewal in these tissues are stem cells that have the potential to divide throughout life and to produce either stem cells or cells that differentiate (Leblond and Cheng, 1976). The nature of the process governing whether, when, and where stem cells differentiate is a matter of long and intensive interest. Among the more widely discussed theories of the control of stem cell behavior are the stochastic theories that assume that stem cell differentiation occurs randomly (Till et al., 1964; Vogel et al., 1969). Here I present evidence that a key prediction of the stochastic theories does not appear to hold in the intestinal epithelium.

In general the stochastic models of stem cell function assume (*a*) stem cells have independent and identically distributed cycle times; (*b*) stem cell mitoses are independent and symmetric (Bjerknes, 1985) in the sense that they produce either two stem cells (with probability α) or two cells that differentiate (with probability $1 - \alpha$), with the expected number of stem cells resulting from a stem cell mitosis $m = 2\alpha$; and (*c*) the stem cell to differentiating cell transition is irreversible.

Under these assumptions, the stochastic models predict that a population of stem cells has a finite probability of eventual extinction through differentiation of all stem cells in the population (these models are age-dependent branching processes; Karlin and Taylor, 1975; Harris, 1963). The probability of extinction will be 1 if $m \leq 1$, and <1 if $m > 1$ (Karlin and Taylor, 1975; Harris, 1963). From this simple statement it seems reasonable to assume that $m > 1$ in normal renewing tissues, otherwise, the tissues would die out with certainty. Even with $m > 1$, the extinction probability of any given stem cell may be quite high. An example makes this obvious. If a stem cell has probability $\alpha = 0.6$ of producing stem cells, then the probability of extinction of that stem cell will be at least 0.4 because it can go extinct on its next cell division with that probability.[1] Stochastic theories of stem cell behavior get around this prediction by reminding us that the stem cell populations contain large numbers of cells, and hence the probability of extinction of all stem cells is very small. However, in a situation where only a small number of stem cells are under consideration, extinction is likely. This prediction forms the basis of the test that I describe here.

The small intestinal epithelium is a renewing tissue containing stem cells in the base of structures known as the crypts of Lieberkuhn (Cheng and Leblond, 1974; Leblond and Cheng, 1976; Bjerknes and Cheng, 1981*b*). Our estimate is that crypts contain an average of ~15 stem cells each (see Discussion and Bjerknes and Cheng, 1981*b*). There do not appear to be any long term resting, that is, noncycling stem cells in the epithelium (Cheng and Leblond, 1974). When all stem cells in a crypt are lost, for example as a result of radiation or drugs, the crypt disappears in a few days. These properties of the intestinal epithelium make it an ideal test system for the prediction made by the stochastic theories that a pool of stem cells has a finite probability of extinction.

If a stem cell (and its offspring) had probability q of eventual extinction, then given the assumption of independence of stem cells from each other, a crypt containing k stem cells would have probability q^k of eventual death through differentiation of all its stem cells. I argued above that $m > 1$. Since $m = 2\alpha$ it follows that $\alpha > 0.5$. I would now argue that in the adult intestine, α must be only slightly >0.5 (also see footnote 2 below). This is because

[1] In fact the probability of eventual extinction of a stem cell and all of its offspring may be shown to be equal to the smallest positive root of $s = \phi(s)$, where $\phi(s)$ is the probability generating function for the offspring of a stem cell mitosis. Under the assumptions of the present model, the probability of eventually extinction, $q = (1 - \alpha)/\alpha$ (Karlin and Taylor, 1975). In this case, $q = (1 - 0.6)/0.6 = 0.67$.

the tissue is in an approximate steady state. The tissue as a whole is growing slowly (Cheng and Bjerknes, 1985), but the rate of growth is low relative to the stem cell cycle time. If α was significantly >0.5, say 0.6, then the rate of growth of the tissue would be enormous. For the model under consideration, $q = (1 - \alpha)/\alpha$. It follows that, because α must be only slightly > 0.5, $q \sim 1$. Thus, the stochastic models predict that most stem cells and hence many crypts should eventually regress. Those crypts that did not die would grow and, presumably, would eventually branch to form new crypts (crypt branching is known to occur during growth and repair; Cairnie and Millen, 1975; Maskens and Dujardin-Loits, 1981; Cheng and Bjerknes, 1985). Thus, the stochastic theories of stem cell behavior predict a rather extensive turnover of crypts, but just how extensive?

As a simple approximation, let us determine the probability of crypt extinction with each stem cell cycle (in other words, discretize the model by concentrating on stem cell mitosis). The probability generating function (PGF) of the nth cycle of the discrete branching process may be shown (Karlin and Taylor, 1975; Harris, 1963) to obey the recursive relation

$$\phi_{n+1}(s) = \phi_1(\phi_n(s)),$$

where $\phi_1(s) = 1 - \alpha + \alpha s^2$ is the PGF for the number of offspring of a stem cell mitosis and $\phi_0(s) = s$. It follows by substitution that

$$\phi_{n+1}(s) = 1 - \alpha + \alpha[\phi_n(s)]^2. \quad (1)$$

Since the probability of extinction of a stem cell by the $n+1$th cycle is $\phi_{n+1}(0)$, the probability of extinction by the $n+1$th cell cycle in a crypt initially containing k stem cells is $[\phi_{n+1}(0)]^k$. An average intestinal epithelial stem cell has a cell cycle time of ~16 h (Al-Dewachi et al., 1979). Thus, an average stem cell would have undergone ~31 mitoses in 3 wk. Under the assumptions of the stochastic model (i.e., by Eq. 1 with $\alpha \sim 0.5$[2]), the probability that the offspring of a stem cell present at time 0 would be extinct 3 wk later is ~0.945, that is $\phi_{31}(0) = 0.945$. The probability that a crypt containing k stem cells at time 0, and all of the offspring of the crypt, would die out within 3 wk would then be $\sim 0.945^k = 0.945^{15} = 0.43$, if $k = 15$. Thus, under the assumptions of the stochastic theories we might expect that in 3 wk ~40% of crypts observed at time 0 should die out from loss of their stem cells. I report here that no evidence of crypt loss was found.

[2]Estimates of α based on the stochastic theories may also be derived from the data and the fact that a super-critical branching process grows exponentially (see Harris, p. 142 and p. 150). If we assume that the stem cell cycle time follows a gamma distribution with parameters b and n, then it may be shown that $\alpha = (1 + B/b)^n/2$, where B is the exponential growth rate ($B \sim 2.708 \times 10^{-4}\ h^{-1}$ from the results here). Estimates for b and n may be derived from the literature, $b \sim 0.901$, and $n \sim 14$ (computed from Table I, rows 1 and 2 in Al-Dewachi et al., 1979). From these results, an estimate of α is $\alpha \sim 0.502$, which is near the 0.5 expected from the arguments in the text. I use 0.5 for simplicity, but the reader should remember that $\alpha > 0.5$.

METHODS

In Vivo Recording of Crypts

Three male Swiss albino CD-1 mice were given a 5% glucose solution but no solid food over night. The next morning, the mice were anesthetized (sodium pentobarbitol, 60 mg/kg) and the loop of jejunum immediately distal to the ligament of Trietz exteriorized. The gut was placed on a sterile slide and bathed in saline. Video recordings of crypts seen through the muscularis mucosa were made with transillumination on an ACM microscope (Carl Zeiss Inc., Thornwood, NY). The field diaphragm of the condensor was closed as far as was possible to provide the necessary contrast. When the recording was completed, the animal was closed, and allowed to recover. Then, the same region of gut was observed 1 and 3 wk later. The vascular pattern made possible the ready identification of the same point in the gut. The animals weighed an average of 20.7, 26.4, and 29.6 g at the initial timepoint and 1 and 3 wk later, respectively. The recordings were compared to look for changes in the crypt population. Only small groups of crypts (<25) encircled by a vascular net were used in the comparisons. The recordings allowed a direct crypt by crypt study of crypt survival. A potential flaw in this experiment is that I may have missed a lost crypt because a new one filled its place. I must say that this is a possibility, however, the chances of it are lower than might appear to be the case. When crypts did not branch, I was able to match up crypt by crypt the data from different time points. Crypts or their neighborhood had distinguishing features that allowed a precise matching crypt for crypt. If a branching occurred, the new crypts obviously did not match up with their predecessor, however, even in many of these cases a precise matchup was possible because the week before the predecessor showed signs of getting ready to branch, for example it had a slight constriction or had a large base.

Branching Index

Epithelial sheets from five mice (29 g) were isolated by ventricular EDTA perfusion as described in Bjerknes and Cheng (1981*a*). Proximal jejunum comparable to that studied in the animals described above was used. Counts were made of the percentage of crypts that were in the process of branching (Cheng and Bjerknes, 1985).

RESULTS

I followed the fate of 328 crypts over a period of 3 wk (Fig. 1). While I found that 49 of the crypts divided within that period (20 crypts within the first week, and 29 in the last two weeks), I found no missing crypts. I also estimated the proportion of crypts in the process of branching in isolated epithelium from five other animals. This was ~3.2% ± 0.32% ($\bar{X}$ ± S.E.).

DISCUSSION

The results of the study appear inconsistent with a simple stochastic model of stem cell behavior. It was indicated in the Introduction that under the assumptions of the stochastic theory of stem cell control, ~40% of crypts and their offspring should have been lost within the 3-wk observation period. I observed no evidence of crypt loss. To determine the degree of incompatibility between the stochastic model and the results, we should note that in the stochastic model,

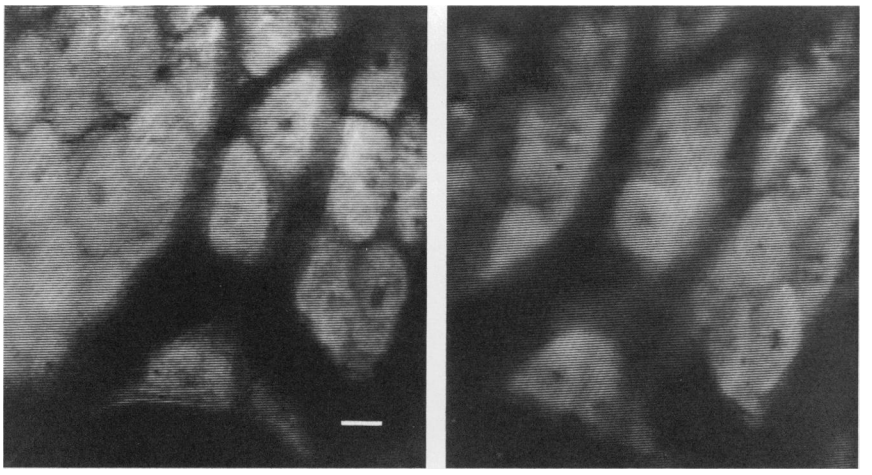

FIGURE 1 Small intestinal crypts in vivo. (*a*) shows a photograph from a video monitor of an initial recording made of crypts. Note the two crypts in the region encircled by the small vascular ring. (*b*) same region three weeks later. Note that where there were two crypts there are now three. The change probably resulted from a crypt branching. Scale bar; 30 μm.

stem cells, and hence crypts are assumed to behave independently. Thus, the number of randomly selected crypts observed at time 0 that survive until time t should approximate a binomial distribution. It follows that the probability that each of 328 crypts observed at time 0 survives until time t is $\sim r^{328}$, where r is the probability that a single randomly selected crypt survives until time t. Since all 328 crypts studied survived, we can be certain at the 95% level that $1 \leq r \leq (1 - 0.975)^{1/328} = 0.99$,[3] yet under the stochastic model we would expect that $r = 1 - [\phi_{31}(0)]^k = 1 - 0.43 = 0.57$ if the number of stem cells in a crypt, $k = 15$. To state this in another way, under the stochastic theory, if we followed 328 crypts for 3 wk an average of $\sim 0.43 \times 328 = 141$ crypts should have gone extinct. I found no evidence of crypt loss indicating that the probability of crypt loss in 3 wk, $1 - r$, is somewhere between 0 and 1%. Thus it would appear that if crypts contain an average of ~15 stem cells, the observations reported here are inconsistent with the stochastic theory.

An important feature of the stochastic theory is that the larger the number of stem cells in a crypt, the lower the probability of extinction of that crypt. Thus, it would be of interest to compute an estimate of the minimum number of stem cells that crypts must contain so that the lack of evidence of crypt loss does not conflict with the stochastic theory at the 95% level. This would be the point at which the 95% confidence bounds for r estimated from the data was equal to the value for r computed from the stochastic theory with k stem cells in a crypt. Recall that from the stochastic theory $r = 1 - [\phi_{31}(0)]^k$, while the results indicate that $1 \leq r \leq (1 - 0.975)^{1/328} = 0.99$. We want to find the value of k for which the former value of r is equal to the lower confidence bound of the latter value of r. After substituting and solving for k, the number of stem cells in a crypt, it is found that unless crypts contain more than

$$\log (1 - 0.025^{1/328})/\log (\phi_{31}(0))$$
$$= \log (1 - 0.025^{1/328})/\log (0.945)$$
$$= 79.4$$

stem cells, the results are inconsistent with the stochastic model at the 95% confidence level.

It is possible that some time would be required for a crypt to disappear after its stem cells were lost. However, even if it took one week for a crypt to disappear after losing its stem cell pool, the results of this study would still be inconsistent with the stochastic model unless crypts contained more than about $\log(1 - 0.025^{1/328})/\log[\phi_{21}(0)] = 56$ stem cells.

A critical issue in the interpretation of my results is the reliability of the estimate of the number of stem cells in a crypt. This can be a confusing point because a distinction between functional stem cells and potential stem cells or clonogenic cells has arisen in the field. The evidence, primarily morphological and behavioral, is that in normal crypts there are ~10–20 cells in the base of the crypts that act as stem cells under normal conditions (Bjerknes and Cheng, 1981; Wright and Alison, 1984). These are the functional stem cells. The other proliferating cells in the crypt are nonstem cells thought normally to have limited proliferative potential. The concept of clonogenic cells comes from studies of crypt regeneration after irradiation. After sufficiently high doses of radiation, it is thought that many regenerating crypts represent clones of a single surviving cell, hence cells with the ability to regenerate crypts are called clonogenic cells. It is not known whether the functional stem cells form a part of the clonogenic cell

[3]This result follows from the confidence interval theory outlined in Kendall and Stuart 114–115 pp., or may be read off of the confidence limit table for the binomial parameter found in the Biometrika Tables (Pearson and Hartley, 1966).

population, but this is usually assumed to be the case. Experiments involving regeneration after irradiation have been interpretated as indicating that crypts contain 2–305 with a mean of ~80 clonogenic cells per crypt (for review see Potten and Hendry, 1983; Wright and Alison, 1984). The latest estimate from C. S. Potten, J. H. Hendry, and J. V. Moore (submitted for publication) is that there are ~32 clonogenic cells per crypt. The wide variation is an indication of the many difficulties inherent in this type of experiment. In addition to the experimental difficulties, the conclusions drawn from the data are very model dependent (see for example Yau and Cairnie, 1979; Hendry, 1979), the computed estimate of the number of clonogens is very sensitive to model parameters, and the assumptions underlying the models are not without serious pitfalls. If the results of these radiation experiments are to be taken seriously, they would suggest that either our understanding of normal renewal in the epithelium is incorrect, that is that the crypt base columnar cells are not the only stem cells, and thus that the number of stem cells in the normal crypt could be >15, or we must conclude that drastic injury results in dedifferentiation of some nonstem cells. The latter possibility conflicts with a major premise of the stochastic model as presented (assumption *c* of Introduction), while the former may or may note be at odds with the stochastic theory depending on the true number of clonogenic cells in the crypt. This discussion points up the urgent necessity for an indisputible and independent estimate of the number of stem cells in a normal crypt, for example, an estimate based on gene function.

If k, the initial number of stem cells in a crypt, is a random variable, and hence may change from crypt to crypt (as would be expected under the stochastic theories or under the crypt cycle model presented below), then my computations using the mean number of stem cells in a crypt, $\bar{k} \sim 15$, are not correct. I have underestimated the extinction probability of some crypts and overestimated that of others. I will show, however, that as long as the distribution of the number of stem cells in a crypt meets certain reasonable criteria, then the probability of extinction I have computed using the mean number of stem cells is a reasonable lower bound to the true crypt extinction probability. To begin, note that the probability of extinction of a crypt containing k stem cells, $[\phi_n(0)]^k$, is a decreasing function of k ($0 \leq \phi_n(0) \leq 1$). The probability of extinction of a randomly chosen crypt is $\Sigma_{k=1}^{\infty} P(k)\ [\phi_n(0)]^k$, where $P(k)$ is the probability that a crypt contains k stem cells. Assume for the moment that the distribution of k is symmetric about its mean $\bar{k}$. Since $[\phi_n(0)]^k$ is a decreasing function, the probability of extinction for a crypt with $<\bar{k}$ stem cells will be higher than that for a crypt with $>\bar{k}$ stem cells. It follows that $\Sigma_{k=1}^{\infty} P(k)\ [\phi_n(0)]^k > [\phi_n(0)]^{\bar{k}}$, since $P(k)$ was assumed to be symmetric about $\bar{k}$. Furthermore, since we have assumed that the number of stem cells will not grow above a maximum value, because crypts that get that large begin to branch dividing the stem cells among smaller daughter crypts, the distribution of the number of stem cells in a crypt will in fact be skewed towards smaller numbers of stem cells. If this is so, then similar arguments show that since the distribution is skewed, more crypts contain $<\bar{k}$ stem cells than contain $>\bar{k}$, and hence that $\Sigma_{k=1}^{\infty} P(k)\ [\phi_n(0)]^k > [\phi_n(0)]_v^{\bar{k}}$. It follows that the conclusions reached here, that the stochastic theories are not consistent with the results at the 95% level unless crypts contain a mean of <80(56) stem cells each, are conservative.[4]

A Crypt Cycle?

The observation of crypt branching but no crypt loss suggests that there may be a crypt cycle (by analogy with cell cycle) at the end of which crypts branch. Under the simplest of assumptions about this process, all crypts would cycle and the crypt population would undergo exponential growth, $N(t) = N_o \exp[(t \log 2)/t_c]$; where $N(t)$ is the number of crypts at time t, N_o is the initial number of crypts, and t_c is the crypt cycle time. A least squares fit to the observed rate of expansion in crypt numbers ($N(0) = 328$, $N(7) = 348$, $N(21) = 377$; t is in days) yielded $N(t) = 330\exp(0.0065t)$, and hence $t_c = (\log 2)/0.0065 = 107$ d. The counts of branching crypts in the isolated sheets of epithelium yielded a fraction of crypts in the process of branching, $I_b = 0.032$. From this result, I may estimate the time for completion of the branching process by the approximate formula $t_b = I_b t_c/\log 2 = 5$ d (see for example Steel, 1968). Exponential growth of the crypt population is consistent with the observation of a steady increase with age in the number of crypts associated with villi (Cheng and Bjerknes, 1985). Thus, it is possible that crypts in the upper jejunum in Swiss albino CD-1, mice of 21–29 g have a crypt cycle time of ~107 d, 5 of which are spent branching.

In conclusion, while I found ample evidence of crypt branching, I found no evidence of the extensive crypt loss predicted by stochastic models of stem cell control. I would conclude that the simple stochastic models of stem cell renewal do not provide an adequate representation of reality, and hence one or all of the assumptions on which

[4]It is worth noting that a subset of the data can be used to obtain an estimate of the lower bound of k consistent with the stochastic theory which might be less sensitive to variation in stem cell numbers per crypt. Twenty of the original 328 crypts divided in the first week producing 40 new crypts. It is likely that new crypts resulting from crypt branching contain similar numbers of stem cells. Thus, we may identify a subpopulation of crypts with a similar initial number of stem cells (which, incidentally, may be $<\bar{k}$). With this assumption, we can use the arguments above to compute a lower estimate of the survival probability of a new crypt over the remaining two weeks of the experiment. The probability of extinction of a single stem cell in two weeks is $\sim\phi_{21}(0) = 0.923$. The result is that unless newly formed crypts contain more than about $\log(1 - 0.025^{1/328})/\log(0.923) = 30$ stem cells, the observed survival of the 40 new crypts for two weeks is not consistent with the stochastic theory.

these models are based are invalid. For example, it is likely that there is at least some degree of dependence of stem cell behavior on local conditions (Bjerknes and Cheng, 1981*b*, 1985). The stochastic model of stem cell behavior have formed the basis of a number of recent theories of cancer (Whittemore and Keller, 1978; Moolgavkar and Venzon, 1979; Knudson el al., 1975; Mackillop et al., 1983). If the results and interpretation presented in this paper is correct, these important theories may need modification.

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