Simulation of cell proliferation in mouse oral epithelium, and the action of epidermal growth factor: evidence for a high degree of synchronization of the stem cells

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Abstract. Computer simulation has been carried out to help to determine the cell-proliferative mechanisms underlying data gathered from a double-labelling experiment on the dorsal tongue of the mouse. Good fits to the data have been obtained by assuming that there is a high degree of synchrony in the stem cells, which have a 24-h cell cycle time, and that daughters of these cells undergo two further divisions, with mean cell cycle times of 48 h, before differentiating. This results in one-seventh of proliferative cells being stem cells, which ties in well with the concept of epidermal proliferative units. There is no need to assume that S-phase duration changes diurnally. The administration of epidermal growth factor seems to increase the degree of synchrony. In such systems, the influx to S-phase and the efflux from it have very sudden short peaks, which it is impossible to observe unless observations are taken very frequently. There are therefore implications for the designs of experiments that attempt to study diurnal rhythms or the effect of factors that disturb the normal proliferative pattern of cells.

Keywords: cell synchronization, circadian rhythm, epidermal growth factor, simulation model, stem cells

INTRODUCTION

The mechanisms governing the formation of hyperplasia and carcinoma in the oral epithelium need to be understood in terms of their effects on cell-proliferative activity (Jordan *et al.* 1998), and our understanding of the response of the oral epithelium to cytotoxic treatment of cancers elsewhere in the body also needs to be improved. This can be achieved through studies of cell proliferation; of particular importance is the explanation of the strong circadian rhythm that the tissue exhibits, particularly in some animals.

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Figure 1. Graphs of: (a) observed labelling index; (b) observed rate of entry to S; and (c) observed rate of exit from S over a 48-h period, for the dorsal tongue of control animals, with fitted sinusoidal curves. (Adapted from Figure 3 of Thomson *et al.* 1999.)

In a previous paper we demonstrated significant distortion of the normal rhythmical proliferation of epithelial basal cells in mouse dorsal tongue following exogenous growth factor administration, and postulated a potential role for growth factors in oral carcinogenesis (Thomson *et al.* 1999).

Normal mouse dorsal tongue epithelium is characterized by a circadian variation in the number of cells undergoing DNA synthesis, with peak activity occurring between 24.00 and 03.00 h and lowest levels from 12.00 to 15.00 h (Hume & Potten 1976; Potten *et al.* 1977). Peaks and troughs in mitotic activity follow these S-phase peaks and troughs a few hours later. Although the precise cause of such rhythms has not been fully established, factors such as physical activity, the feeding regime and the light/dark cycle may all be involved. Similarly, oral keratinocytes possess receptors for various growth factors, such as epidermal growth factor (EGF), which is present in saliva and known to influence epithelial cell replication.

We studied groups of male BDF1 mice over a 48-h period (05.00 h on day 1 to 05.00 h on day 3) using a sequential double-labelling method, comprising tritiated thymidine followed by bromodeoxyuridine, to determine S-phase labelling indices, together with rate of entry (influx) into and rate of exit (efflux) from S, at 4-hourly time points (Thomson *et al.* 1999). As summarized in Fig. 1, cyclical peaks in labelling index occurred in control animals at around 02.00 h, with troughs 12 h later at 14.00 h. Rate of entry into S showed a similar circadian rhythm, being highest at about 01.00 h and lowest between 13.00 and 14.00 h. The peaks and troughs in rate

Figure 2. Graphs of: (a) observed labelling index; (b) observed rate of entry to S; and (c) observed rate of exit from S over a 48-h period, for the dorsal tongue of EGF-treated animals, with fitted sinusoidal curves. (Adapted from Figure 4 of Thomson *et al.* 1999.)

of exit from S appeared to be about 5 h later; S-phase duration was estimated at 4.9 h with a standard error of 0.3 h.

Variations in labelling index may reflect changes in cell-cycle phase transit times. Kirkhus & Clausen (1990, 1992) favoured fluctuation in the durations of S and G₂, but Wright & Alison (1984) believed synchronous waves of cells entering S and then proceeding to mitosis was the principal mechanism determining circadian variation in cell-cycle populations. In spite of the many studies carried out, there appears to be no agreed explanation for the observed circadian rhythms (Warnakulasuriya & MacDonald 1995). In our initial report, we chose to present our results in terms of changing phase durations, but in this paper we shall change our approach and we hope to show that it provides a more comprehensive explanation of our data.

In our study a second group of mice received a subcutaneous injection of recombinant EGF (Ciron Corp., USA) at a dose of 50 µg in 0.2 mL physiological saline, at 05.00 h on day 1, to determine the effects of growth factor stimulation upon cell proliferative activity (Fig. 2). Whilst there were no significant differences between EGF and control animals for overall labelling index, there was a significant reduction in both influx and efflux throughout the 48-h period ($P < 0.001$), suggesting a possible slower transit of cells through S-phase. The duration of S for these animals was calculated at 21.7 h, which was a massive lengthening compared to the control group ($P < 0.001$), so massive that it is unlikely to be true, for it is difficult to see how

an S-phase duration of greater than a day could be consistent with the virtually unchanged rhythm in labelling index.

The reduction in both cell influx and efflux throughout the 48-h period following EGF injection, together with the maintenance of normal labelling indices was an unexpected and interesting finding. EGF was administered at 05.00 h, a time normally associated with high levels of DNA synthesis, in an attempt to produce a maximum effect 8–10 h later when DNA synthesis is known to be low. Previous workers, however, have also observed an EGF-dependent inhibition of cell cycle progression (Kinzel *et al.* 1990; Komiyama *et al.* 1992).

The mechanisms governing stimulation and inhibition of cell-proliferative activity may play key roles in the process of neoplastic transformation. Increased understanding of the mechanisms underlying these changes is of paramount importance if effective interventional therapies are to be developed.

The aim of this study was to utilize computer simulation to characterize in detail whether movement through the cycle phases could recreate the cell proliferation data in our original experiment on control animals, and to try to reproduce the observed changes in rhythm induced by EGF administration.

METHODS

The study used the computer program CELLSIM (Donaghey 1975). A decision, based on preliminary models, was made that stem cells in the model would have a cell cycle time of exactly 24 h, producing daughter cells at the rate of one per day, whilst the daughter cells would cycle more slowly with a mean cell cycle time of 48 h. With an average labelling index of 20%, cell influx and efflux of about 2% and a cell cycle time of 2 days, S phase duration was calculated to be of the order of 10 h. In the computer simulation, all stem cells would spend precisely 9 h in G1, 10 h in S, 4 h in G2 and 1 h in M. Daughter cells would show some variation but would exhibit the same mean phase durations except that G1 would last on average 33 h.

The model produces a number of cohorts of cells, so that 12 h into a stem cell cycle, for example, there are:

- n stem cells due to divide in 12 h time,
- n cells created by stem cells that divided 12 h ago,
- n cells created by stem cells that divided 36 h ago,
- $2n$ cells created by stem cells that divided 60 h ago, whose daughters divided 12 h ago, and
- $2n$ cells created by stem cells that divided 84 h ago, whose daughters divided 36 h ago.

This process could have been extended, but exactly two divisions by daughter cells provided the best fit to the labelling-index data.

One in seven of the proliferative cells is therefore a stem cell, which means that a possible cell organization is that as described for the epidermal proliferative unit presented by Potten (1974), with a stem cell at the centre of the unit in the basal layer and its six more slowly cycling progeny in adjacent positions. Consecutive stem cell divisions displace older cells upward to the suprabasal layer. Cells in this layer may be in S-phase, or indeed mitosis, but the daughter cells produced by such mitoses will be nonproliferative.

Table 1 shows the CELLSIM model, which requires three generations of cells, namely stem cells (STEM), daughters of stem cells (GEN1), and grand-daughters of stem cells (GEN2).

Table 1. CELLSIM model

1	CELL TYPES STEM, GEN1, GEN2;
2	STATES (1) G1,S,G2,M;
3	STATES (2,3) G1A,G1B,G1C,G1D,S,G2M;
4	FLOW G1(1)-S(1) (ALL), S(1)-G2(1) (ALL), G2(1)-M(1) (ALL), M(1)-G1(1) (0.5), M(1)-G1A(2) (0.5), G1A(2)-G1B(2) (ALL), G1B(2)-G1C(2) (ALL), G1C(2)-G1D(2) (ALL), G1D(2)-S(2) (ALL), S(2)-G2(2) (ALL), G2(2)-M(2) (ALL), M(2)-G1A(3) (ALL), G1A(3)-G1B(3) (ALL), G1B(3)-G1C(3) (ALL), G1C(3)-G1D(3) (ALL), G1D(3)-S (3) (ALL), S(3)-G2(3) (ALL), G2(3)-M(3) (ALL), M(3)-G1A(3) (ALL);
5	FLOW (3), WHILE TOTAL < 7000, G1A-G1B(ALL), G1B-G1C(ALL), G1C-G1D(ALL), G1D-S(ALL), S-G2(ALL), G2-M(ALL), M-G1A(ALL);
6	FLOW (3), WHILE TOTAL > 7000, G1A-G1B(0.02), G1B-G1C(0.02), G1C-G1D(0.02), G1D-S(0.02), S-G2(0.02), G2-M(0.02);
7	TIME IN STATES (1) G1 : 9, S:10, G2 : 4, M:1;
8	TIME IN STATES (2,3) G1A:N(9,2), G1B:N(8,2), G1C:N(8,2), G1D:N(8,2), S:N(10,2), G2:N(4,1), M:1;
9	PROLIFERATION (1,2,3) M:2;
10	PROLIFERATION (3), WHILE TOTAL < 7000, M:2;
11	PROLIFERATION (3), WHILE TOTAL > 7000, M:0.2;
12	INOCULUM (1) S: 1000 AT BEGINNING;
13	SUSPEND (1) BETWEEN 100 and 262 G1S,G2M;
14	SUSPEND (2,3) BETWEEN 100 and 262 G1A,G1B,G1C,G1D,S,G2M;
15	\$BLOCK EXIT (2,3) BETWEEN 506 and 512 G1D: 0.9;
16	\$BLOCK EXIT (2,3) BETWEEN 506 and 512 S: 0.9;
17	\$CHANGE TIME IN STATES (2,3), AT TIME = 505, G1D:5;
18	\$CHANGE TIME IN STATES (2,3), AT TIME = 517, G1D:N(8,2);
19	MAXIMUM NUMBER OF GROUPS = 350;
20	REPORTS 499 h;
21	CHANGE REPORTS, AT TIME = 499, 1 H;
22	NO PRINT *;
23	GRAPH 100*(S(1) + S(2) + S(3))/TOTAL;
24	GRAPH 100*(RI(S(1)) + RI(S(2)) + RI(S(3)))/TOTAL;
25	GRAPH 100*(RI(G2(1)) + RI(G2(2)) + RI(G2(3)))/TOTAL;
26	GRAPH 100*(M(1) + M(2) + M(3))/TOTAL;
27	RANDOM NUMBER SEED = 1234567;
28	\$SIMULATE 551 h;
29	SIMULATE 739 h

While stem cells merely pass through the normal phases, other cells have their G_1 phase divided into subphases (A,B,C,D); this is merely to help model the actions of administered agents and cell migration.

Stem cells flow round the cycle and mitosis leads to one stem cell and one daughter in the next generation; the daughter then passes through two further generations, and all cells divide at mitosis. The model is based upon 1000 proliferative units, so that as long as there are fewer than 7000 total cells, all cells remain in cycle, returning to G_1 after mitosis. Once 7000 is exceeded, the cells in the final generation are modelled to migrate (statements 4–11 in Table 1). Because CELLSIM was not designed to deal with spatial modelling, statements 4–6 are of necessity rather clumsy. Gaussian (normal) distributions have been chosen for the times which non-stem cells spend in the various phases of the cell cycle. The standard deviation for their cell cycle times is 4.6 h.

Figure 3. Graphs of: (a) observed mean labelling index; (b) observed mean rate of entry to S; and (c) observed mean rate of exit from S over a 48-h period, for the dorsal tongue of control animals, with simulated curves. Vertical lines show standard errors.

The simulation begins with 1000 cells at the beginning of S-phase and runs for 14 days, the equivalent of seven cell cycles, to reach a stable state before results are recorded. Statements 13 and 14 are merely to ensure that experimental time zero is reached at a convenient model time (here 500 h, by which time the model has run for this length of time less the 162 h for which it has been suspended, that is 14 days). Statements 21 to the end arrange for hourly graphical reports for the simulated experimental period to be produced, summarizing labelling index, influx to S, efflux from S, and mitotic index. Statement 19 is a programming device to ensure that the amount of storage used by the model does not exceed the amount available. Statements 15–18 correspond to the hypothesized action of EGF administration at 05.00 h on the cell system. (The \$ sign indicates that they are not active in the simulation of the unperturbed system but when we wish to include them in the model the \$ sign is removed.) Increased synchrony between cells is postulated so that, over a 12-h period from the time of drug administration, 90% of non-stem cells within the last 8 h of G_1 , or in S, stop progressing through the cell cycle for a 6-h period. In addition, nonstem cells entering the last 8-h phase of G_1 complete it on average 3 h faster than normal. This is merely one way of introducing a greater degree of synchrony as cells enter S. The experimental data and full details of the methods used to obtain them are presented in Thomson *et al.* (1999).

Figure 4. Graphs of simulated 3-h cumulative mitotic index over a 24-h period, for the ventral tongue of control animals, with observed data from Potten *et al.* (2002).

RESULTS

Figure 3(a) illustrates simulated labelling indices (LI) calculated for hourly time points together with mean LIs for the dorsal tongue of control-experiment animals from Fig. 1(a). The simulated results are the means of 20 replicates of 24 h (performed as 10 replicates of 48 h); this curve is then drawn for both 24-h periods. There is a good fit to the observed data. Analysis of the simulated cell influx data in Fig. 3(b) suggests that highly synchronized cell movement into S takes place at 22.00 h and that relatively small numbers of cells enter S at other times. This peak occurs at times between those at which observations were taken in our original 4-hourly experimental time points. Synchronized cell efflux is calculated to occur at 08.00 h, again not exactly a time at which observations were made, although the highest effluxes were observed very close to that time (Fig. 3(c)).

Figure 4 illustrates simulated data for cumulative mitotic index for a 3-h collection period based upon the previously described program and confirms the very similar pattern to data generated from a 3-h vincristine stathmokinetic metaphase arrest technique utilizing murine ventral tongue tissue (Potten *et al.* 2002). The data in this experiment were not taken into account in determining the parameters of the model. Because a 3-h collection period using vincristine effectively means that cells in metaphase at the time of administration and all those entering mitosis in the next 3 h will be counted, and because we are assuming that mitosis lasts for 1 h, the simulated curve is the total of four successive mitotic indices. The fit is good, particularly bearing in mind that the observed data are for ventral, rather than dorsal, tongue.

Proliferative characteristics after administration of EGF are not the same on each of the two following days, so the simulated curves are based on 20 replicates of the whole 48-h period. Inspection of the LIs for animals treated with EGF at 05.00 h and the simulated data in Fig. 5(a) confirms a less regular rhythm. At 08.00 h the normal fall in LI (due to the expected peak in cell efflux at this time) is interrupted by a hiatus during which little cell movement occurs. There is negligible cell influx between 07.00 h and 11.00 h (Fig. 5b), and little efflux seen between 09.00 h and 11.00 h (Fig. 5c). A second peak in efflux, however, then occurs at 12.00 h leading to resumption of the fall in LI. This is followed by a period of increased

Figure 5. Graphs of: (a) observed mean labelling index; (b) observed mean rate of entry to S; and (c) observed mean rate of exit from S over a 48-h period, for the dorsal tongue of EGF-treated animals, with simulated curves. Vertical lines show standard errors.

influx associated with a gentle rise in LI. Peak influx at 22.00 h then facilitates the rapid rise observed in LI.

The following 24-h period then resumes an almost identical pattern to that of the control groups. The fit to the data is good, allowing for the fact that the experimental protocol of taking observations every 4 h is unable to pick up the cohorts of cells entering and leaving S-phase.

DISCUSSION

Cell cycle control depends upon a complex series of interactions between stimulatory and inhibitory proteins. Promotion of oncogenes, inactivation of tumour suppressor genes and alteration of growth factor activity may lead to abnormal regulation of cellular proliferation, which is increasingly recognized as important in carcinogenesis (Kushner *et al.* 1999).

Fluctuations in oral epithelial proliferative activity are well documented, but it was Scheving *et al.* (1979) who postulated that cyclic alterations in EGF salivary content, or alterations in cellular response to EGF, might be involved in generating circadian rhythms in DNA synthesis in mouse tongue.

In our original animal experiment (Thomson *et al.* 1999), 50 µg of EGF was administered to mice at 05.00 h, a time normally associated with high DNA synthesis, in an attempt to produce a maximal stimulation of synthesis between 8 and 10 h later during an expected nadir. While our data originally appeared to suggest reduction in both influx and efflux, alongside a preservation of overall labelling index, and thus an increase in S-phase duration, the CELLSIM model does not support this assumption, as fits were obtained to all datasets with an unchanging S-phase duration of 10 h.

Instead, the model postulates an interruption to normal cell movement followed by a rapid synchronization of cohorts of cells entering S-phase at approximately 12.00 h. This may have been unidentifiable in our original experimental observations due to it occurring between the 4-hourly time points. Indeed, even if we had seen single a histological section that was heavily labelled with one of the markers of DNA synthesis (tritiated thymidine or bromodeoxyuridine) and lightly labelled with the other, it would have been easy to assume that there was a technical problem with the slide, because in all the other slides to be counted the level of labelling was comparable for the two markers.

The precise mechanisms governing the cell cycle and the manner in which EGF influences cell proliferation, migration and differentiation remain unclear. Certainly, there is increasing evidence that EGF receptors may act not only as mediators for diverse signalling systems within epithelial tissues, but also may well play key roles during carcinogenesis and subsequent tumour growth (Hackel *et al.* 1999; Waksal 1999).

Evidence in the literature, however, is conflicting regarding the mode of action of EGF; for example, Kinzel *et al.* (1990) reported upon an EGF-dependent inhibition or delay in the passage of cells from G₂ into mitosis, while Nass & Dickson (1998) observed a shortened G₁ phase following EGF-induced proliferation of mammary epithelial cells, suggesting that the duration of cell cycle phases may indeed vary.

The CELLSIM model, however, suggests that the circadian rhythm in mouse tongue epithelium occurs without change in the duration of cell cycle phases. The accurate fit of the computer model to single and double labelling indices, and to mitotic indices derived from separate animal experimental data, would appear to support the validity of this model. Furthermore, the S-phase duration is now estimated, for both control and EGF-treated animals, to be more than twice the length we previously suggested, showing the magnitude of the errors that can be made in spite of obtaining satisfactory fits to data by sinusoidal curve-fitting.

It is important that we find out whether stem cells really do maintain the high degree of synchrony we have suggested, and, if so, by what mechanism. It is also important to determine what advantage, if any, the tissue gains from such markedly rhythmical proliferation.

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