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Cell kinetic studies in the murine ventral tongue epithelium: mucositis induced by radiation and its protection by pretreatment with keratinocyte growth factor (KGF)

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Abstract. Radiation kills or reduces reproductive capacity of proliferating cells, including stem cells. In the oral mucosae this can result in a decline in the number of cells in the tissue which, if severe enough, will result in the formation of an ulcer when the cellularity essentially reaches zero. We have used histometric measurements of cellularity following exposure to radiation in mouse ventral tongue epithelium as a model for oral mucositis (ulcer development). Here we provide further measurements of cellularity changes in the basal layer and in the epithelium as a whole at various times following 15, 20 or 25 Gy doses. The protective effects of prior treatment with keratinocyte growth factor (KGF) are also investigated. 20 Gy of 300 kV X-rays has become our standard reference dose and the changes in cellularity seen following this dose are highly reproducible, with minimum values being observed 6 days following irradiation. A higher dose results in a greater reduction of cellularity, although the minimum value also occurs at 6 days. A lower dose (15 Gy) results in a much shallower curve, with a minimum value being observed about 1 day earlier. These changes in cellularity can be related to the less sensitive index of mucositis, namely epithelial thickness. There is also a sharp peak in proliferation about 1 day after the minimum in cellularity, i.e. on day 7. The peak following a lower dose of radiation occurs a little earlier and, following the higher dose, the peak tends to be broader. Previous work and data presented in the preceding paper in this series has shown that KGF, given over a period of 3 days, results in a dramatic increase in epithelial thickness in oral mucosa, including the ventral tongue. As a result of the increased cellularity induced by KGF given before radiation, a delay in the fall in cellularity results, which is the consequence of the increased number of cells in the epithelium at the beginning of the study.

Keywords: cell kinetics, histometry, irradiation, keratinocyte growth factor, mouse tongue epithelium, oral mucositis.

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INTRODUCTION

In the preceding papers in this series (Potten *et al.* 2002a, 2002b, 2002c) we have described many of the cell kinetic and histometric characteristics of the keratinizing stratified mucosa on the ventral surface of the mouse tongue. We have also described in these earlier papers how oral mucositis can be a limiting factor in the effectiveness of cancer chemotherapy and radiotherapy treatments. Previously we have presented preliminary data concerning the cellularity changes in the ventral tongue epithelium following a single dose of radiation (Wardley *et al.* 1998). Here we provide further validation of this quantitative objective model of oral mucositis. We have used radiation as a cytotoxic agent (a model cytotoxic where dose and exposure time can be precisely controlled) but the principles of the model can be applied equally to other cytotoxic agents such as chemotherapy drugs. High-energy radiation is a simple and accurate cytotoxic where damage is induced uniformly throughout the body in a precise quantitative fashion only while the animal is exposed to the radiation source.

In the preceding paper in the current series (Potten *et al.* 2002c), we described some of the dramatic histological changes induced in the ventral tongue mucosa by repeated daily exposures to keratinocyte growth factor (KGF). Here we also demonstrate that KGF delivered prior to a dose of radiation reduces the severity of the cellular depopulation of the mucosa induced by a dose of radiation.

MATERIALS AND METHODS

The materials and methods are largely as described in the preceding papers (see specifically Potten *et al.* 2002a). Briefly, male BDF-1 mice 10–12 weeks of age at the time of use were exposed to doses of radiation, with the animals culled at daily intervals up to 10 days postirradiation. All procedures were performed in accordance with the UK Home Office (Scientific Procedures) Act 1986.

Animals were irradiated using a 300-kVp X-ray (H.V.L. 2.3 mm Cu) set at a dose rate of 55–57 cGy/min (H.V.L 2.3 mm Cu) with the thorax and abdomen shielded by 6 mm of lead. The heads of the animals received a single acute dose of 15, 20 or 25 Gy. 20 Gy is a standard reference dose used in many of the individual experiments.

The ventral tongue was excised, cut longitudinally down the centre and 3–5-μm sections, cut parallel to the longitudinal axis, were prepared after Carnoy's fixation and routine histological procedures. The sections were then either processed for autoradiography or for bromodeoxyuridine (BrdUrd) incorporation by immunohistochemistry and were counterstained with either haematoxylin or thionine. The sections were then analysed as described in the earlier papers using the Zeiss Axiohome interactive microscope (Potten *et al.* 2002a). This allows each cell within a defined area of the tissue to be identified by virtue of their nuclei, with the *x*-*y* co-ordinates of each nucleus also being stored. The labelling characteristics of each cell are recorded, as well as its position in the tissue relative to the basal layer (basal, suprabasal, etc.). Labelling characteristics recorded include: mitotic cells, apoptotic cells, the presence or absence of nuclear BrdUrd antibody staining or autoradiographic silver grains. Measurements of area, length and tissue thickness were also obtained.

Prior to culling, animals were injected intraperitoneally (ip) with l0 mg BrdUrd (Sigma Aldrich, Poole, UK) and then culled 40 min later. Following histological preparation, sections

were then processed for the immunological detection of BrdUrd incorporation using an anti-BrdUrd antibody (Mas 250b; Harlan Sera Laboratories, Loughborough, UK) and routine immunohistochemical procedures as described in Potten *et al.* (2002b). Briefly the procedure is as follows: slides were dewaxed overnight and then washed in several changes of absolute alcohol. Endogenous peroxidase activity was blocked using hydrogen peroxide and the slides were then hydrolysed for 8 min in 1 M HCl at 60 °C, followed by neutralization in boric acid buffer for 6 min. After washing in phosphate-buffered saline (PBS) the samples were then incubated for 30 min in normal rabbit serum (Sigma) to block nonspecific binding and then incubated in a 1 : 5 dilution of the anti-BrdUrd antibody for 1 h at room temperature. The slides were washed in PBS and incubated for 1 h in a 1 : 100 dilution of rabbit antirat peroxidase (Dako, Cambridge, UK) which had been diluted to this working concentration using 10% normal mouse serum. Finally slides were then washed three times in PBS, developed using diaminobenzidine (DAB) and counterstained with thionine (Wardley *et al.* 1998).

In one experiment animals received a daily dose of 125 μg human recombinant keratinocyte growth factor (KGF) delivered subcutaneously in 0.2 mL of saline at 15.00 h on each of the 3 days preceding radiation exposure. The mice were irradiated 24 h after the last KGF injection, i.e. at 15.00 h on day 4. Groups of animals were culled 2 days and 4 days post-irradiation and at daily intervals from days 6–11.

At 40 min prior to the time of culling, some animals were injected with methyl tritiated thymidine (^{3}HTdR) of specific activity 222 GBq/mM (NEN Life Sciences, Hounslow, UK). This was administered intraperitoneally as 25 μCi (0.925 MBq) in 0.1 mL saline. After the animals were culled, the tissue was excised, cut longitudinally and the two halves of the tongue fixed in Carnoy's fixative for a period of 30 min Following routine histological processing, sections were cut through the long axis of the tongue to provide samples for autoradiography. These sections were dewaxed, hydrated and then dipped in autoradiographic emulsion (Ilford K5 emulsion; Ilford, Mobberley, UK). After 14 days' autoradiographic exposure slides were then developed, fixed and counterstained with haematoxylin.

In one experiment animals were irradiated at 15.00 h with 20 Gy X-ray and then pulsed either immediately following irradiation or at time points ranging from 2 to 10 days post-irradiation with 50 μCi of ^{3}HTdR. Animals were culled over a period of 1–4 days after ^{3}HTdR labelling.

CELL PRODUCTION RATE ESTIMATION

The methods here are adopted from Potten *et al.* (2000) and are described in Barthel *et al.* (2002) and are based on a two-compartment model, the basal and suprabasal compartments with their cells. Provided that cell division occurs only in the basal layer and that cells are only labelled when in S, the increase in the number of labelled cells in the combined basal/suprabasal compartment, L_{tot} is a measure of the cell production, whereas the increase in the suprabasal layer, L_s is a measure of the cell migration from the basal to the suprabasal layer. When relating these changes to the number of basal cells, L_{bas} one obtains the cell production rate

$$r = \mathrm{d}L_{tot}/\mathrm{d}t/L_{bas}$$

and the cell migration rate

$$q = \mathrm{d}L_s/\mathrm{d}t - L_{bas}$$

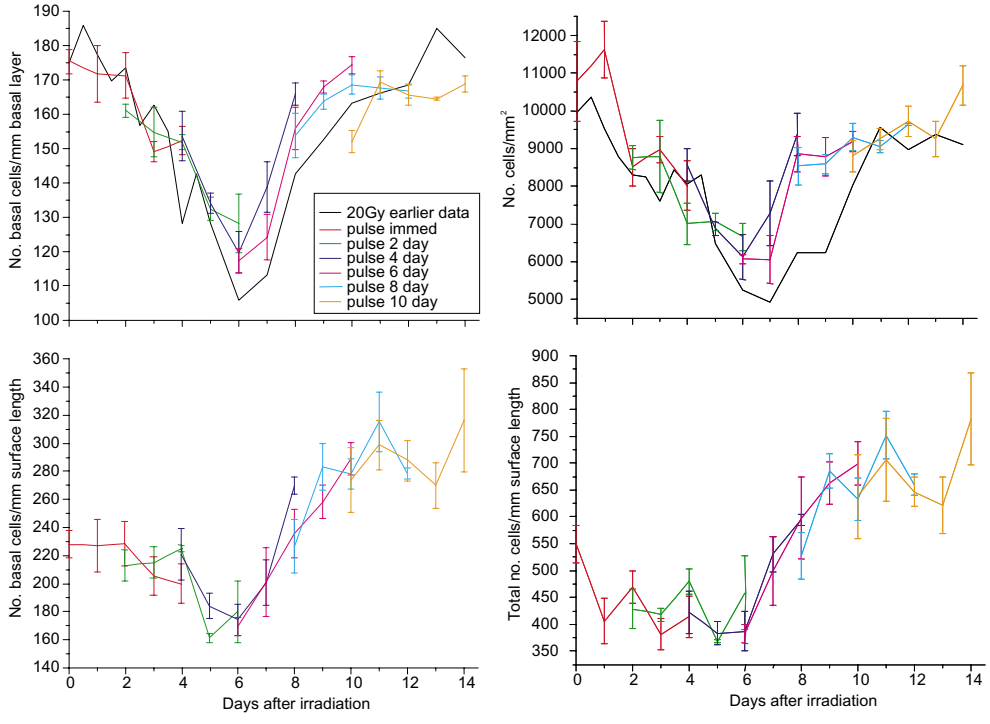

Figure 1. Cellularity measurements for mouse ventral tongue at various times after 20 Gy of X-rays. Left-hand panels, data for basal cells; right-hand panels, total cells. The solid line in the upper two panels shows pooled data including the data of Wardley *et al.* (1998). The animals received an injection of tritiated thymidine (a pulse labelling protocol) on the days indicated. Labelling data were not used in the generation of these graphs, but were used in Figs 6 and 7. Each group of pulsed animals gives a fragment of the total picture of the cellularity changes and each fragment of the total picture provide information on the reproducibility. Mean and standard error limits are shown.

The time derivatives can be estimated by linear regression. In order to avoid errors because of geometrical differences between individual sections, the cell numbers should be normalized by relating to a characteristic length, e.g. the top layer width.

RESULTS AND DISCUSSION

The standard dose that we have generally used for our murine oral mucositis model is an acute dose of 20 Gy delivered to the head only. From the data acquired, the average thickness of the stratified epithelium was determined, together with the number of basal or total cells per unit length of basal layer or cells per unit area. It is difficult to know which of these is the most informative. However, the total number of cells beneath 1 mm length of the surface of the epithelium (and the corresponding number of basal cells beneath the same 1 mm length) were considered useful because they should provide information on both the proliferative compartment and the total cells, but also take into account any increases in basal layer undulation and cell size. When the immunohistochemical detection of bromodeoxyuridine was used, the number of basal and suprabasal was also recorded. The fraction of S-phase cells (labelled cells) was expressed

Table 1. Control unirradiated data pooled from several experiments. The saline treatment itself results in some changes. In some of the figures the pooled controls have been used, whereas in other data presentations the saline-treated controls have been used (see individual figures)

	No. of mice	Cells/unit length (mm)	Cells/unit area (mm^2)	Basal cells/top layer (mm)	Total cells/top layer (mm)	Thickness (μm)
Untreated	42	175.5	10574.4	234.6	604.4	54.9
Saline	81	145.5	11627.4	167.6	507.2	46.8
Pooled	123	155.9	11262.4	190.5	540.4	49.6

Table 2. Cellularity measurements for mouse ventral tongue on days 5–7 following 20 Gy of X-rays. Data have been pooled from several experiments

No. of days after irradiation	No. of basal cells/mm basal layer	No. of basal cells/mm surface length	Total no. of cells/mm^2	Total no. of cells/mm surface length	Thickness (μm)
5	131.7	172.6	6802.5	375.4	46.8
6	117.9	174.6	6022.7	409.0	60.4
7	125.6	200.8	6082.8	514.9	68.0
Control (saline)	145.5	167.7	11627.4	507.2	46.8

as a labelling index for the basal layer and for the suprabasal layer but could also be expressed as the total number of labelled cells per unit length of the upper surface.

Groups of animals were culled at various times after a dose of 20 Gy and parts of this response curve have been repeated several times. The data shown in Fig. 1 illustrate the reproducibility of the quantitative measurement of change in cellularity and illustrates the various ways of expressing the data (several panels) (see also Tables 1 and 2). There was a steady decline in cellularity with minimum values of about 50–60% of control obtained on days 5–7. The levels of decline in cellularity do depend on the control data used (Table 1) because saline treatment itself causes some changes. It would be expected that cellularity in the basal layer would fall initially and that this would be followed by a fall in total cellularity as the reduction in cell output from the basal layer takes effect. Generally, the basal layer depletion reached a minimum on days 5–6 and the total cellularity on days 6–7 (see Fig. 1). The data in Fig. 1 can be pooled to give an average response curve (Fig. 2). Figure 3 shows the histological changes seen at day 6 following a dose of 20 Gy.

Figure 4 shows the results obtained when doses of 15 and 25 Gy were delivered in comparison with the standard 20 Gy response curve. Subtle differences were observed, depending on which measure of cellularity was considered. However, the general response was a progressive decline in cellularity reaching minimum values between 5 and 7 days postirradiation. The minimum value was lower following 25 Gy (50% of control on day 6) and higher following 15 Gy. The minima was earlier after 15 Gy at day 5. (Table 3)

Figure 5 shows the changes in epithelial thickness following the standard dose of 20 Gy. The changes in epithelial thickness roughly mimicked those seen as changes in cellularity but did not show dramatic changes. The minimum values were seen on day 5, which is followed by an expansion presumably due to a combination of regeneration and possible oedema or cellular swelling. Thickness measurements are not as sensitive an indicator of the postirradiation changes as are the changes in cellularity.

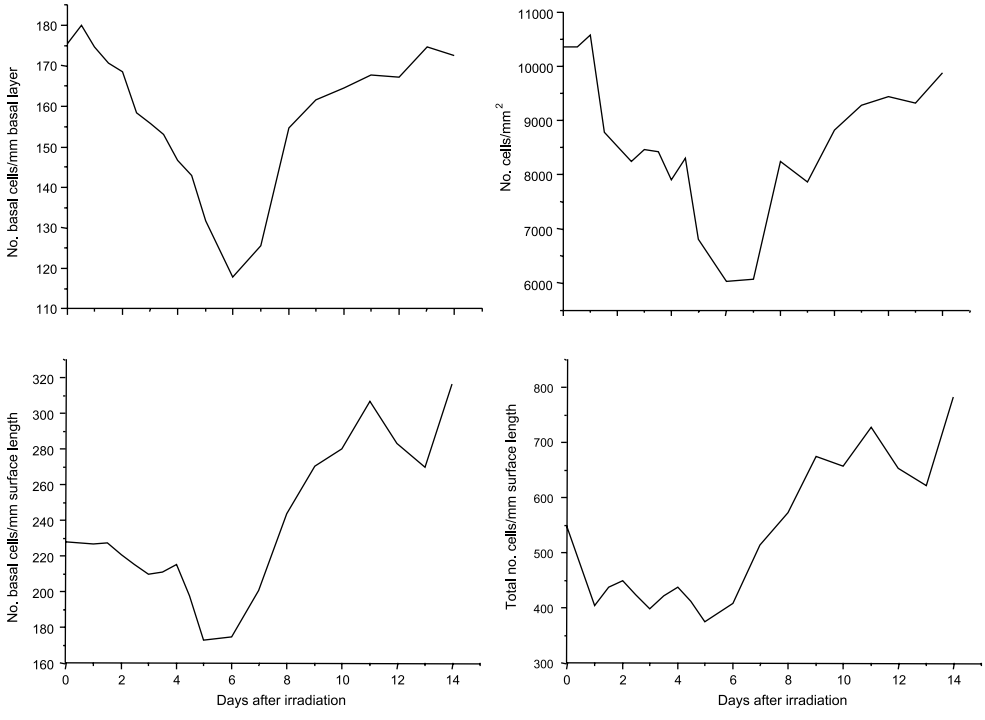

Figure 2. Shows overall pooled data taken from Figure 1 to provide a series of reference curves for subsequent analysis.

Figure 6 shows the regenerative response as demonstrated by the changing pattern in BrdUrd labelling index (LI) on different days post-irradiation. A peak value of 47.3 ± 6.67% labelling was seen on day 7 post-20 Gy, with a rapidly ascending series of values between days 5 and 7. The occurrence of this sharp rise in labelling (proliferation in the basal layer) precedes the changes in cellularity by about 24 h. These observations are similar to those reported by Wardley *et al.* (1998). The response after 15 Gy was less dramatic and the peak was slightly earlier, broader and somewhat lower than that seen after 20 Gy. After 25 Gy the peak was slightly later (days 7–9) and less accentuated. The response seen in the suprabasal layers was about a day later than for the basal layer and the size of the peak was in proportion to the dose.

Thus, it is clear that a single acute dose of radiation induces a fall in the cellularity in the ventral tongue between days 5 and 7, with the levels of cellular depopulation and timing depending on dose. A regenerative response is triggered before the cellularity is at its minimum and the peak time and magnitude of the response also varies depending on dose, with the earliest peak times occurring following the lowest dose. The highest peak values were seen following a dose of 20 Gy where the labelling index may reach a value of nearly 50%, i.e. almost half the basal cells were in S phase at any one instant in time, suggesting very short cell-cycle times at this point in the regenerative response.

Figure 7 shows the result of an experiment where ^{3}HTdR was injected into groups of animals, either immediately after 20 Gy irradiation or 2, 4, 6, 8 or 10 days after irradiation and groups of animals were culled 1–4 days after the ^{3}HTdR administration. The initial group of animals in each case here provide a picture of the changes in labelling index (LI) at each of the times after irradiation, i.e. data comparable to those shown in Fig. 6. The samples taken 1–4 days

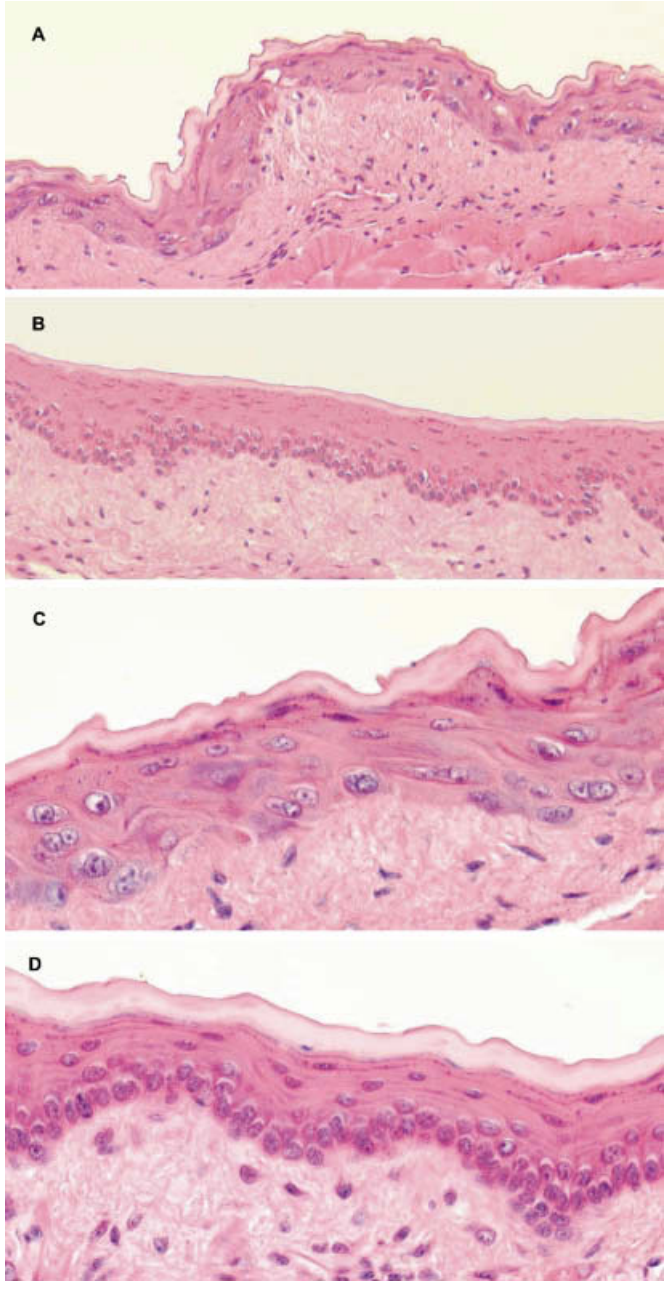

Figure 3. Shows the histological appearance of mouse ventral tongue 6 days after a dose of 20 Gy. (a) Ventral tongue 6 days after irradiation with 20 Gy (× 109); (b) Unirradiated ventral tongue (× 109); (c) Ventral tongue 6 days after irradiation with 20 Gy (× 211); (d) Unirradiated ventral tongue (× 211). The reduction in cellularity in the epithelium as whole and in the basal layer can be clearly seen, while the thickness shows less of a change in c and d. Haematoxylin and eosin staining.

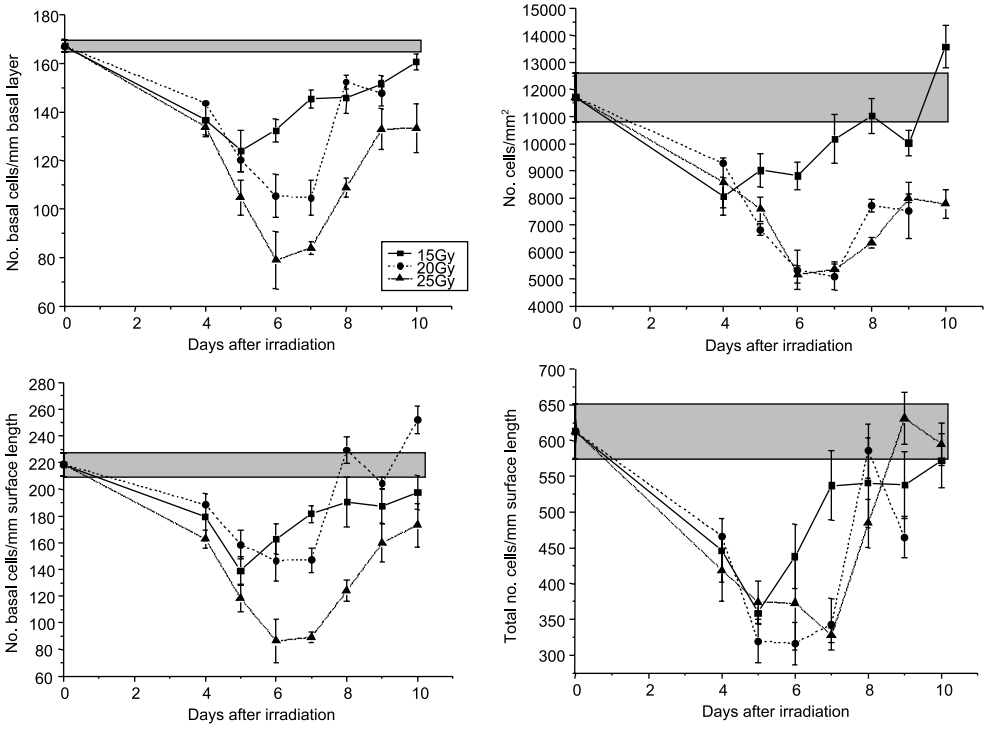

Figure 4. Cellularity changes in mouse ventral tongue following doses of 15, 20 and 25 Gy of X-rays. Left hand panels: data for cellularity changes in the basal layer. Right hand panels: data for total epithelium. The horizontal bar shows the control values with standard errors.

Table 3. Cellularity measurements for mouse ventral tongue on days 5 and 6 following 15, 20 and 25 Gy of X-rays

No. of days after irradiation	Dose of irradiation (Gy)	No. of basal cells/mm basal layer	No. of basal cells/mm surface length	Total no. of cells/mm^2	Total no. of cells/mm surface length	Thickness (μm)
5	15	123.9	139.2	9014.4	358.7	41.2
	20	120.1	158.5	6829.5	319.8	46.8
	25	104.8	118.3	7589.4	373.5	50.4
6	15	132.4	162.9	8819.3	437.9	50.8
	20	105.5	146.7	5341.2	315.9	60.4
	25	78.9	86.4	5175.2	372.7	71.6
Control (saline)	0	145.5	167.7	11627.4	507.2	46.8

after each ^{3}HTdR dose provide information on cell emigration from the basal layer and on the rate of cell proliferation.

Figure 8 shows the calculated cell production rate (r) and the cell emigration rate from the basal to suprabasal layer (q) for various times between 2 and 10 days post-irradiation (20 Gy). Estimates for the control values derived from the data over the first 24 h using the continuum model and from the vincristine accumulation studies, are shown on the vertical axis. The actual values are shown in Table 4 with their standard errors and calculated basal layer turnover times.

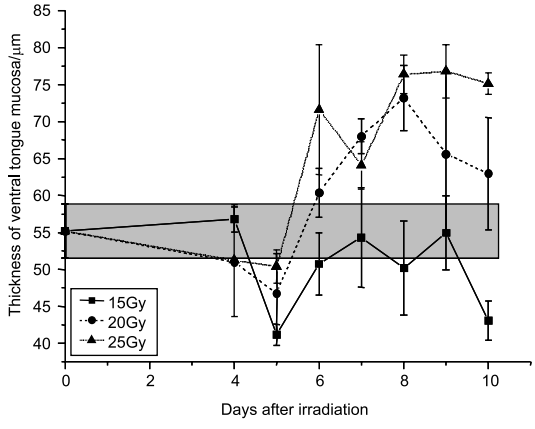

Figure 5. Changes in the thickness of the ventral tongue epithelium at various times after doses of 15, 20 and 25 Gy X-rays. The horizontal bar indicates the normal control level.

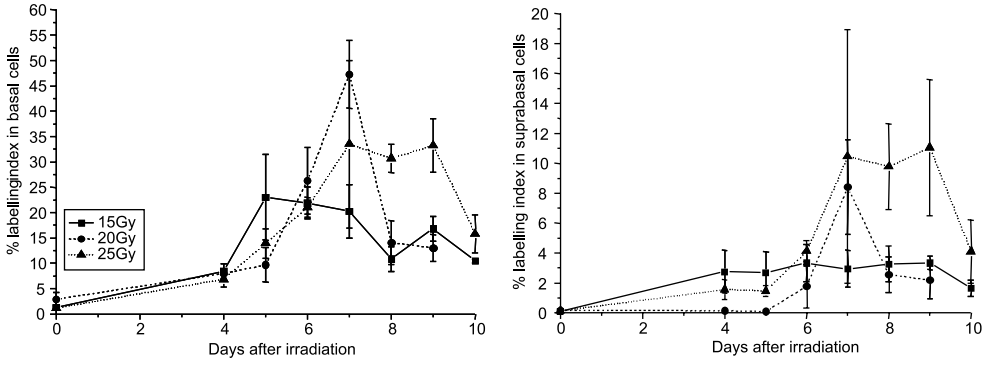

Figure 6. Changes in the labelling index in the basal layer (left panel) and suprabasal layers (right panel) of mouse ventral tongue epithelium at various times after 15, 20 and 25 Gy of X-rays.

Table 4. Cell production, cell migration and basal layer turnover times at various times post-20 Gy

Time (h)	Cell production rate [r] (h)	Cell migration rate [q] (%/h)	Turnover time (h)
48	9.5 ± 2.1	0.3 ± 0.5*	10.5 ± 2.4
96	18.0 ± 2.3	2.4 ± 0.6	5.6 ± 0.7
144	15.6 ± 2.5	10.6 ± 2.4	6.4 ± 1.0
192	16.0 ± 3.2	7.3 ± 1.8	6.5 ± 1.3
240	7.4 ± 2.3	3.4 ± 1.0	13.4 ± 4.2

*Not significantly different from zero.

The fastest rate of increase in LI after ^{3}HTdR injection was seen in the basal layer of groups injected with ^{3}HTdR 4 days postirradiation, i.e. just prior to the surge in proliferation seen with the pulse labelling index in the basal layer. For the 4-day ^{3}HTdR group, the labelling index rose from 10.8 ± 1.4% in the basal layer to 73.1 ± 3.5% within a period of 48 h (i.e. 3 doublings).

Labelling appeared in the suprabasal layers about 24 h later for all the groups. The fastest rise in suprabasal labelling in this experiment was observed in animals pulsed at 4 and 6 days post-irradiation where the suprabasal labelling increased rapidly on day 1, 2 and 3, reaching a plateau value of close to 80% labelling in the suprabasal layers 3 days following ^{3}HTdR administration for the 6-day labelling group. Animals pulsed immediately after irradiation showed a fall in labelling index in the basal layer from 6.8 ± 1.1% to 4.5 ± 1.2% at 24 h and then a gradual rise over the subsequent 3 days to give values of 12.2 ± 2.9% 4 days after the ^{3}HTdR labelling.

The data presented in this paper together with observations presented in Potten *et al.* (2002a, 2002b, 2002c) and the analysis performed by Barthel *et al.* (2002) enable one to estimate the turnover time of cells in the basal layer of mouse ventral tongue epithelium and the transit time through the suprabasal layers. The turnover time in the basal layer can be estimated from three different approaches. The first of these is from the vincristine arrest experiment (Potten *et al.* 2002b). This indicates that 54% of the basal cells enter mitosis within a 24-h period. This will give a cell production rate of 2.25% per hour and the reciprocal of this gives a turnover time of 44 h. This provides an average turnover time for the entire basal layer and excludes any considerations about the growth fraction in the basal layer. The uncertainties associated with this estimate are related to any underestimation of the total mitotic accumulation due to any delayed action of the vincristine in blocking mitotic activity, which is counteracted in an unknown fashion by a tendency to overestimate the number of accumulated mitotic figures due to the initial number of mitotic figures present (i.e. the initial intercept on any vincristine accumulation curves).

The second method of estimating the turnover time comes from an analysis performed using the continuum model (Barthel *et al.* 2002), which provides an estimate of the basal to surface transit time of about 50 h. If the basal layer compartment and the suprabasal compartments were the same in size and assuming steady-state conditions, the basal layer would need to turnover every 50 h to replace the suprabasal compartment. However, there are 1.58 times more cells in the suprabasal layers than in the basal layer (102.7 cells per area, compared with 65 cells per area in the basal layer). Thus, the basal layer would need to turnover faster than 50 h to account for this and this correction gives a turnover time of 31.6 h. This is likely to be an overestimate, as the suprabasal compartment may be underestimated due to difficulties in defining the uppermost cells and outermost layers, and the fact that the nuclei used to identify cells are degraded and shrink in size and are hence more difficult to identify in the upper layers.

The third method of estimating the turnover time is also described and presented in the continuum model paper (Barthel *et al.* 2002) and is based on estimations of the cell emigration rate from the basal layer over the initial 24 h after pulse labelling (data presented in Potten *et al.* 2002a). These observations indicate a dramatic circadian dependence on the cell emigration rate with two peaks occurring within the 24 h, but the average cell emigration rate (cell production rate) is 3.8% per hour, the reciprocal of which gives a turnover time for the basal layer of 26 h. Although it is thought that the delayed uptake phenomenon does not contribute significantly to this estimate, any influence it would have would tend to overestimate the production rate and therefore, underestimate the turnover time.

We thus have three independent, indirect estimations using models, assumptions, and data presented within this paper and Potten *et al.* (2002a, 2002b) of the turnover time of the basal layer in BDF1 ventral tongue epithelium of 26, 32 and 44 h. The data presented in the present paper indicate that the turnover time between 4 and 8 days postirradiation (20 Gy) is reduced from 26–44 h down to 5.6–10.5 h (Figs 7 and 8), i.e. a reduction of between two- and eight-fold, depending on which initial turnover time is assumed.

Figure 9 shows the effects of 3 days of pretreatment with KGF prior to a dose of 20 Gy irradiation on the changes in cellularity and thickness in the ventral tongue mucosa. KGF resulted

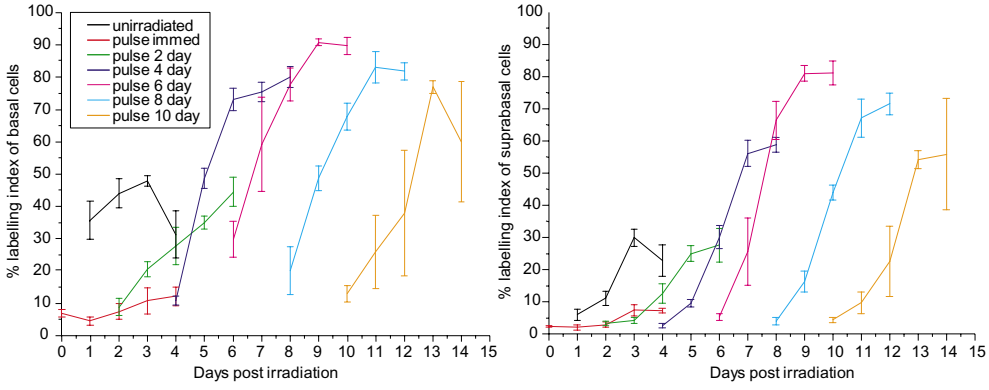

Figure 7. The changes in the labelling index of mouse ventral tongue epithelium on days 1–4 following a tritiated thymidine pulse injection on the days specified. Data for basal cells is shown in the left panel, and suprabasal cells in the right panel. If tritiated thymidine is given immediately after 20 Gy, the labelling index over the following 4 days shows an initial fall and a slight increase thereafter. Labelling given at day 2, results in a progressive increase in the labelling index over the subsequent 4 days. The rate of increase is dramatically raised when the ^{3}HTdR pulse is given on day 4, 6 or 8. The labelling index rises in the suprabasal layers following the rise in the basal layer by about 24 h. The control unirradiated data points are considerably higher than those for labelling immediately after irradiation.

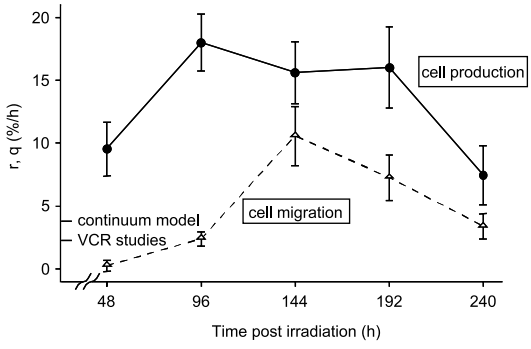

Figure 8. Cell production rate and migration rate at different times post irradiation as determined using the continuum model (Potten *et al.* 2000; Barthel *et al.* 2002).

in a less severe response (66% of control cellularity compared with 56% in the saline-treated animals), i.e. protection against the cellular depopulation associated with ulceration of the ventral tongue epithelium. The minimum levels tended to occur a day later in the KGF treated group, which is consistent with the fact that the cellularity was initially higher in the KGF group (see data on total cells/mm surface and the data in Potten *et al.* 2002c). The return to normal values also seemed to be slightly delayed in the KGF-treated animals, which is consistent with the labelling data (see below). Daily injections of vehicle for the KGF (saline) can result in a significant drop in cellularity measurements such that the initial control time point on some of the graphs in Fig. 9 is lower than the control untreated values (the horizontal bars in the figures). The reasons for this are unclear.

The thickness of the epithelium is greater in the KGF-treated animals and hence takes longer to fall to a minimum on day 6, which is less than the minimum seen in the saline-treated animals

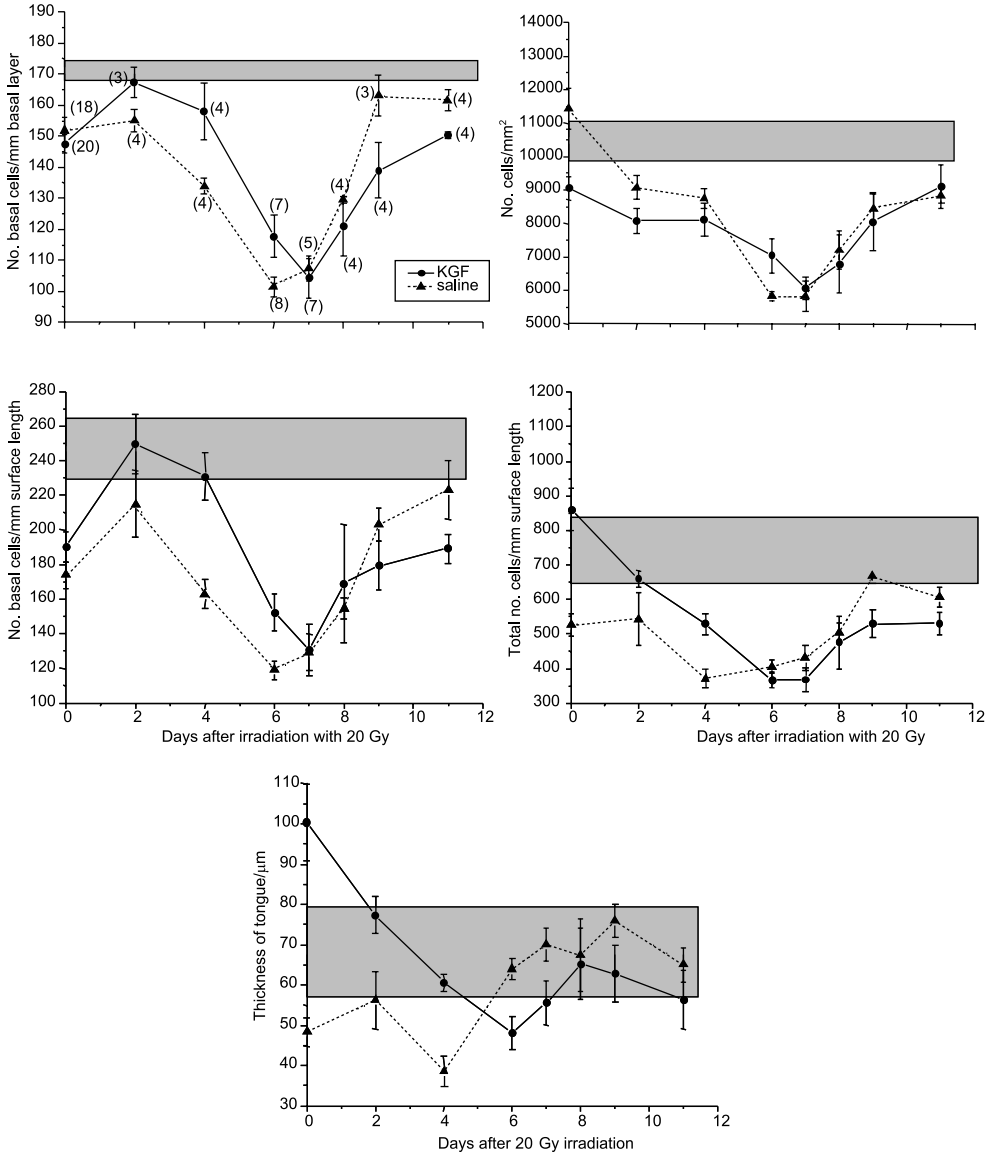

Figure 9. Changes in the cellularity of mouse tongue epithelium at various times following doses of 20 Gy X-rays. Left-hand panels, data for basal cells; right-hand panels, data for total cells. In each case, normal control values for untreated animals are indicated by the horizontal bar. Vehicle (saline) treatment can result in a lowering of the initial values. The animals in this experiment received three daily subcutaneous injections of keratinocyte growth factor (KGF) or saline vehicle at 15.00 h each day and were irradiated 24 h after the last injection. KGF resulted in a significant delay in the onset of cellular depopulation, with the minimum values being seen about 24 h later than in the saline treated groups. The minimal values were slightly greater in the vehicle controls. KGF also resulted in a delay in the return to normality (in the regenerative response). Changes in the thickness of the epithelium of the ventral tongue of the mouse at various times following a dose of 20 Gy X-rays in KGF treated and saline treated animals are shown in the lowest panel.

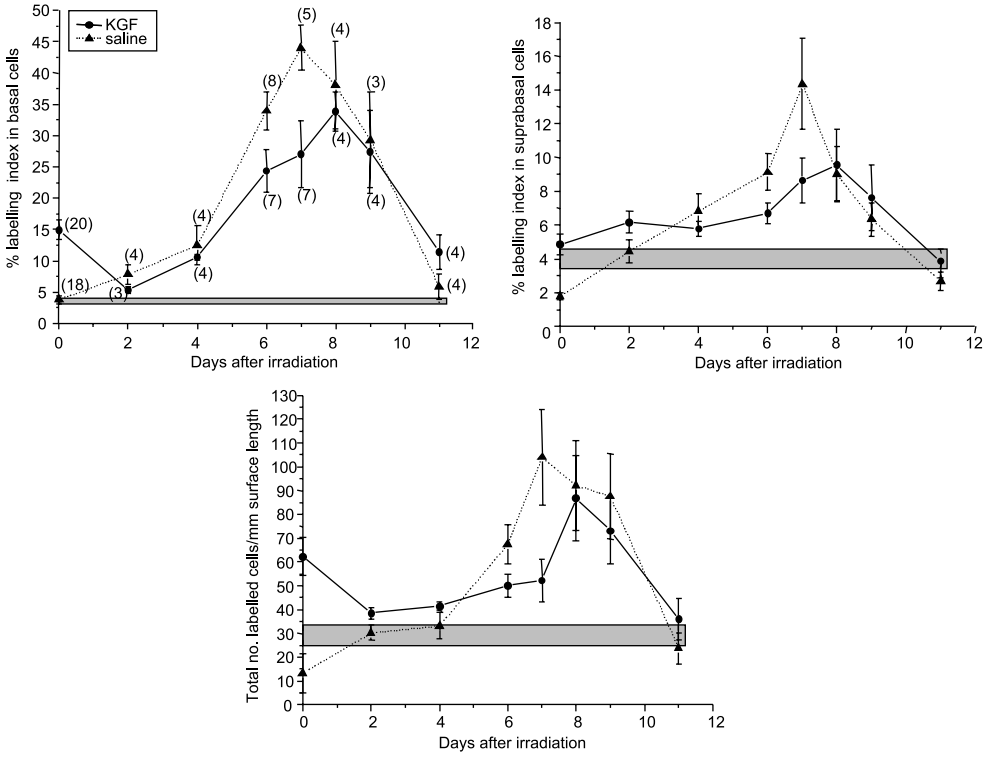

Figure 10. Changes in the labelling index in the ventral tongue epithelium of mice treated with keratinocyte growth factor (KGF) KGF as vehicle followed by a dose of 20 Gy X-rays. Data are shown for the labelling index of the basal cells, suprabasal cells and the total number of cells beneath 1 mm surface length. KGF results in a suppression of the peak in labelling seen around day 7.

on day 4 (Fig. 9). It is this change in thickness of the epithelium that seems to correlate best with the ability of the epithelium to withstand injury and not succumb to ulceration.

Figure 10 shows the effects of KGF pretreatment on the regenerative response as shown by BrdUrd labelling index. KGF pretreatment resulted in a lowering of the peak value and a slight shift in the peak to the right (later response) following a dose of 20 Gy, consistent with the fact that the KGF induced less of a cellular depopulation, which therefore required a less energetic regenerative response (see the 15 Gy response in Figs 4 and 6). The data are consistent with the concept that the size of the regenerative response is determined by the level of cellular depletion while its timing is more determined by the time of the minimum in cellularity. In the KGF-treated groups the initial thickness and cellularity was increased, hence it took longer to reach the cellular nadir. The regenerative response was also triggered slightly later. The initial increased cellularity and epithelial thickness may also provide a protective environment for stem cell repopulation, which may start earlier than the regenerative response shown in Fig. 10.

There have been other studies on ulcer induction in mouse tongue and some other studies, using hamsters, on the effects of TGFβ (Sonis *et al.* 1994), EGF (Sonis *et al.* 1992), or KGF (Dörr *et al.* 1994, 2000, 2001, 2002a) in protecting against radiation- or drug-induced damage.

In a study on ulcer induction in the ventral tongue of C3H mice, using 25 kV soft X-rays, Dörr & Kummermehr (1991) observed maximum ulcerative lesions at about 10–12 days post-irradiation. They noted erythema and oedema appearing after a latent period of about 5 days.

Ulcers were induced by doses within the range 14–20 Gy. At 20 Gy, cell counts per mm length of epithelium showed a progressive fall from an initial figure of about 440 cells per mm, to a minimum on day 8 of about 75 cells per mm (17% of control values), a much more severe response than seen here. Mitotic counts fell initially with a transient peak at 4–5 days and a subsequent major regenerative peak between days 10 and 12. In a subsequent study, Dörr *et al.* (1994) used harder X-rays (300 kV) at 13 Gy and showed a reduction in the cellularity from a value of about 425 to about 300 nuclei/mm epithelium at day 4 postirradiation (70% of control). There was a subsequent recovery reaching normal levels at about day 7. Although the time scale for this response and its recovery was more rapid than seen after the larger doses of soft X-rays, the mitotic response was somewhat similar to that seen using the 25 kV X-rays, where there was a transient peak at around 3 days, with a subsequent peak or levelling off between 7 and 10 days.

It is clear that some major differences exist between the C3H mice used by Dörr and colleagues and the BDF1 mice used here (see Dörr *et al.* 2002). The C3H mice have cell proliferation parameters about two times longer (slower) than the BDF1 mice. There are also differences in the levels of cellularity: the C3H mice appear to have about half the number of cells beneath a 1-mm surface length. It is possible that variations in technical approaches and histometric methods, contribute, in part, to the differences, but the biological reasons remain obscure.

In a recent paper, Dörr *et al.* (2001), using C3H mice and soft X-rays and various different protocols for the delivery of KGF prior to, post, or prior and postirradiation, noted that KGF in all the protocols resulted in a significant reduction in oral mucositis. The effects were greatest in the protocols where KGF was administered after irradiation. This caused a significant shortening in the latent time to ulceration from about 11 to 7 days. The KGF treatment generally increased the iso-effect dose from about 10.9 to 24.9 Gy, which corresponds to dose modification factors ranging between 1.7 and 2.3. This dose modification factor is roughly equivalent to the protection factors observed in our studies involving KGF protection of clonogenic stem cells in the small intestine (Farrell *et al.* 1998).

In a further study using 18 Gy of soft X-rays and KGF at a dose of 5 mg/kg (Dörr *et al.* 2002) found that KGF, given for 5 days from the time that ulcers developed, was ineffective in reducing the mucositis symptoms. In contrast, KGF given before or after a single or fractionated dose, or given during fractionation, had a pronounced mucoprotective effect.

Sonis *et al.* (1994) used a slightly different model for oral mucositis to show that factors such as TGFβ3 can significantly reduce the incidence, severity and duration of oral mucositis in the Syrian golden hamster cheek pouch. The mode of action here is probably a cell-cycle block induced by the TGFβ3, as the growth factor reduced proliferation of oral tissue both *in vitro* and *in vivo.* TGFβ3 also protected stem cells in the gastrointestinal tract probably by inducing a similar reduction in the number of S-phase stem cells (Potten *et al.* 1997; Booth *et al.* 2000). The golden Syrian hamster model involves exposure of the animals to a cytotoxic agent (5-fluorouracil over 2–5 days in Sonis *et al.* 1990, 1992, 1994), but radiation could in principle also be used. The epithelium was then mechanically irritated on day 4 to induce an erythema using the tip of an 18 G needle to scratch the epithelium. The cheek pouch epithelium was everted, after anaesthetization of the animals, and photographed. The severity of mucositis was then recorded using a subjective visual scoring procedure.

CONCLUSIONS

The data presented here provide evidence indicating that the murine ventral tongue epithelium is a useful model system for oral mucositis studies. Histometric changes can be measured in an

objective and highly quantitative fashion and complex cell kinetic studies have been performed. The system has become well defined. The data presented here and in the preceding papers suggest that the turnover time of the basal layer is between 26 and 44 h, with a transit time from the basal layer to the uppermost layers of about 50 h. Radiation induces a well-defined cellular depopulation with minimal values on days 5–6. Similar cellular depopulation changes have been observed following a variety of cytotoxic drugs (data not presented). Using this model we can demonstrate the efficacy of using modulating factors, such as KGF, delivered prior to exposure in reducing the severity and timing of oral mucositis as measured by changes in epithelial cellularity. This model complements the established small intestinal mucositis model that has been used to demonstrate the efficacy of interleukin 11 (Potten 1995, 1996), TGFβ3 (Potten *et al.* 1997; Booth *et al.* 2000), and KGF (Farrell *et al.* 1998; Potten *et al.* 2001) in reducing the severity of gut damage.

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