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Cell kinetic studies in the murine ventral tongue epithelium: the effects of repeated exposure to keratinocyte growth factor

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Abstract. Keratinocyte growth factor (KGF) stimulates proliferation and differentiation in various epithelial systems. Three daily subcutaneous injections of 125 μg of this protein into mice induce dramatic changes in the histology and histometric measurements of the ventral tongue epithelium. The thickness of the epithelium is increased two-fold and the number of cells beneath a 1-mm length of the surface is increased 1.6-fold. KGF also induces a four-fold increase in the number of S phase cells labelled with tritiated thymidine in the basal layer on the third day after KGF administration. The increase in thickness and cellularity persist for at least 4 days after the end of the KGF injections. However, there is a dramatic fall in the number of S phase cells detected by ^{3}HTdR pulse labelling 2 days after the end of the KGF treatment. There are indications that by 7 days after the 3-day regimen of KGF treatment, both thickness and cellularity have fallen back to near control levels. Continued exposure to KGF over a period of 7 days does not result in any further increases in thickness, cellularity or proliferation. In fact, the proliferation decreases on the fifth, sixth and seventh days of KGF injection to control values on day 7. These changes in the epithelium following KGF treatment suggest that the thicker and more cellular epithelium may be more able to cope with an exposure to a cytotoxic agent and hence be protected in comparison with normal or vehicle-treated epithelium.

Keywords: cell kinetics, histometry, keratinocyte growth factor, mouse tongue.

INTRODUCTION

Oral mucositis is one of the more distressing side-effects of cancer chemotherapy and radiotherapy (head and neck). The severity of oral mucositis and similar gastrointestinal damage often limits treatment regimes and dose levels and hence limits treatment efficacy and cure rates. There is considerable interest in developing strategies to reduce the adverse side-effects of these cancer treatment regimens and thereby improve the quality of life for cancer patients, and possibly allow for dose escalation and hence improvement in cure rates. The use of various

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growth factors delivered either before or after cytotoxic exposure has been studied in terms of their ability to reduce the sensitivity of normal tissue stem cells to cytotoxic exposure and/or to speed up the regenerative response from surviving normal tissue stem cells. These studies have included work using interleukin-11 (IL-11) (Potten 1995, 1996), transforming growth factor β-3 (TGFβ-3) (Potten *et al.* 1997; Booth *et al.* 2000), keratinocyte growth factor (KGF) (Farrell *et al.* 1998) and fibroblast growth factor (FGF) (Houchen *et al.* 1999; Paris *et al.* 2001). All these growth factors have been shown to alter the sensitivity of intestinal clonogenic stem cells using the crypt microcolony assay for stem cell survival and functional competence and have also been shown to significantly protect whole animal survival. There remains an issue as to whether these treatments also afford protection to tumour stem cells. This has often not been thoroughly tested. However, tumour cells are less responsive to certain growth factors as suggested by some *in vitro* (Robson *et al.* 1997) and *in vivo* (Zaghloul *et al.* 1994) studies.

In the first two papers of this series (Potten *et al.* 2002a, 2002b) we presented an extensive cell kinetic description of our murine oral mucositis model (Wardley *et al.* 1998) and we present data in the following paper (Potten *et al.* 2002c) showing how pretreatment with KGF reduces the severity of the oral mucositis induced by irradiation. In this paper we present cell kinetic data showing the effects of repeated KGF treatment on murine ventral tongue mucosa. The changes, including mucosal hyperplasia induced by repeated KGF treatment, form the basis of the rationale that KGF may be an effective protector against oral mucositis.

KGF was initially identified from its stimulatory activity on epithelial cells *in vitro* (Finch *et al.* 1989; Rubin *et al.* 1989). It was subsequently found also to stimulate differentiation in various epithelial tissues *in vivo*, including lung (Ulich *et al.* 1994a), breast (Ulich *et al.* 1994b), pancreatic ducts (Yi *et al.* 1994) and uroepithelium (Yi *et al.* 1995). KGF-1 is a member of the fibroblast growth factor family (FGF-7) and has been shown to dramatically increase proliferation, thickness and cellularity (hyperplasia) of various surface epithelia *in vivo* (Farrell *et al.* 1999, 2002). A detailed study of the effects of repeated daily doses of 125 μg of KGF, given subcutaneously (sc) to 25-g mice, showed general changes in proliferation in the intestinal epithelium, with significant increases in proliferation in the stem cell region of small intestinal crypts (Potten *et al.* 2001). In the present experiment the effects of repeated daily 125 μg sc injections of KGF have been studied in relation to tissue thickness, cellularity and proliferation in the mouse ventral tongue. Studies are presented both during repeated exposure to KGF and on the return to normality once exposure has ceased.

MATERIALS AND METHODS

The materials and methods are essentially the same as reported in the first paper in this series (Potten *et al.* 2002a). Briefly, male BDF-1 mice 10–12 weeks of age at the time of experimentation (~25 g) received subcutaneous injections of recombinant human KGF (Amgen, Thousand Oaks, CA, USA). The animals were injected with 125 μg per 0.2 mL injection, equivalent to about 5 mg/kg. The injections were given sc on a daily basis at 15.00 h. For each group treated with KGF, a corresponding group of animals received an identical volume of saline containing 0.1% bovine serum albumen (BSA), which was the dilution vehicle for the KGF. When the animals were culled, the tongues were removed and fixed in Carnoy's fixative and then cut longitudinally. After embedding, 3–5-μm sections were prepared parallel to the cut longitudinal axis. The sections were dipped in autoradiographic emulsion (Ilford K5 emulsion; Ilford, Mobberley, UK) and exposed for 2 weeks, developed and the sections counterstained with haematoxylin and eosin.

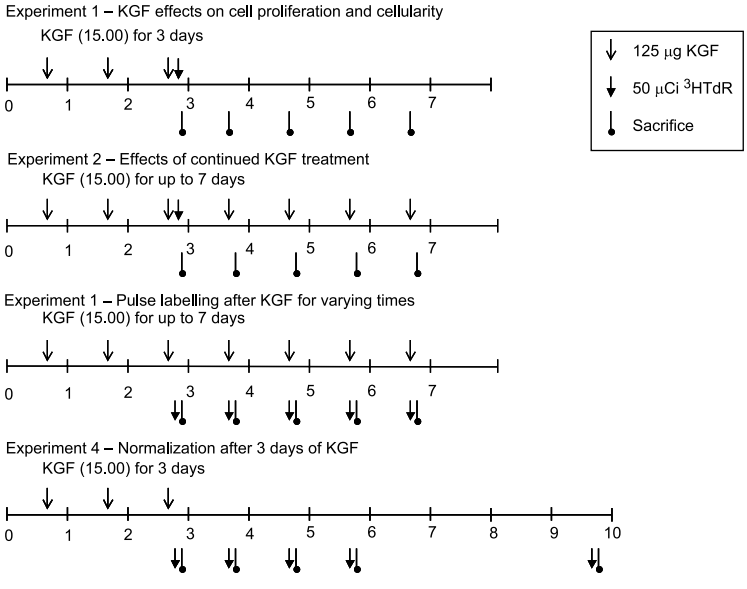

Figure 1. Schematic plan of the four experiments.

Animals were treated in groups of four and various experimental protocols were tested. All experiments were performed within the regulations of the United Kingdom Animal Scientific Procedures Act 1986. The following experiments were performed. (see also Fig. 1).

KGF effects on cell proliferation and cellularity

Mice received a daily sc dose of KGF delivered at 15.00 h for 3 consecutive days. The animals were pulsed with tritiated thymidine (^{3}HTdR) 40 min after the last KGF injection and groups of animals were then culled at 40 min, 1, 2, 3 and 4 days post-^{3}HTdR. Sections were analysed for the number of labelled cells in the basal layer and suprabasal strata. KGF-treated animals were compared with vehicle-treated controls.

The effects of continued KGF treatment

Animals were treated with KGF and pulsed with ^{3}HTdR as above, i.e. on day 3. Groups of animals were culled at the same times as above, i.e. at varying times after the first 3 days of KGF and the ^{3}HTdR pulsing. The difference in this experiment was that daily KGF injections continued during all of the post ^{3}HTdR period (days 1–4 post ^{3}HTdR). Proliferation and cellularity were analysed as above.

Pulse labelling following KGF treatment for varying times

Animals received daily sc injections of KGF for 3, 4, 5, 6 or 7 days. A ^{3}HTdR pulse was administered 40 min after the last injection in each time group and animals were culled 40 min later. The effects on proliferation, mucosal thickness and cellularity were determined.

Time of normalization after KGF treatment

Animals were injected daily sc with KGF for 3 days at 15.00 h as above and groups of animals were labelled with ^{3}HTdR at 40 min 1, 2, 3 and 7 days after the last KGF treatment. The animals were culled 40 min after the ^{3}HTdR administration. Labelling, mucosal thickness and cellularity measurements were made.

Scoring of histological parameters was as described in the earlier papers (Potten *et al.* 2002a). Briefly, the sections were analysed using the Zeiss Axiohome interactive microscope, with measurements commencing at a 2-mm distance from the tip of the tongue. Areas of epithelium were defined with the use of the computer mouse and microscope and the basal cells located in this area were identified by their nuclei and marked with specific icons according to their labelling characteristics. The *x* and *y* co-ordinates of each of these cells were then recorded together with measurements of the area of the defined field and a measurement of the length of the basal layer. All suprabasal nuclei were also defined in the circumscribed area of mucosa. Each area measured covers all the mucosa from the basal layer to the superficial corneocytes contained within the defined field. Data acquisition and analysis were as described in the earlier paper (Potten *et al.* 2002a).

RESULTS AND DISCUSSION

From the four experiments described here, various groups of animals were identical, representing 40-min samples taken after the end of 3 days of KGF treatment. These data have been pooled to give groups of up to 20 animals for KGF- and control-treated groups, respectively.

Three days of KGF treatment increased the epithelial thickness from 48.3 ± 3.5 μm to 100.3 ± 9.6 μm and the total cellularity from 526.7 ± 33.1 cells/mm surface length to 859.5 ± 61.2 cells/mm surface length (see Table 1), i.e. approximately two-fold increases. However, the size of the cells was also increased as there were fewer cells per unit area in the KGF-treated group. The labelling index (LI) in the basal layer showed a dramatic rise over the first 24 h as was seen in the control data and in the data presented in Potten *et al*. (2002a) (Fig. 1, upper panel). The LI rose from $3.8 \pm 0.6\%$ to $38.2 \pm 3.0\%$ in the saline controls, and from $14.9 \pm 1.6\%$ to $38.5 \pm 5.2\%$ in the KGF-treated animals over the first 24 h. At 24 h the KGF- and saline-treated groups had the same LI. During the rest of the experiment time course, the LI values remained essentially the same at about 39–50% for KGF- and control-treated groups (Fig. 2). The LI in the basal layer immediately after 3 days of KGF treatment was about

Table 1. Histometric parameters for KGF-treated and vehicle-treated groups of animals. Treatment consisted of three daily injections of KGF or vehicle

Treatment	No. of mice	% LI basal cells	% LI suprabasal cells	No. basal cells/mm basal layer	No. basal cells/mm surface length	Total no. cells/mm surface length	Cells/mm^2	Thickness (μm)
125 μg KGF once daily for 3 days. Wait 40 min. Pulse with 50 μCi ^{3}HTdR. Wait 40 min and cull.	20	14.9 ± 1.6	4.9 ± 0.6	147.6 ± 3.1	190.2 ± 8.8	859.5 ± 61.2	9055.3 ± 349.0	100.3 ± 9.6
0.2 mL saline once daily for 3 days. Wait 40 min. Pulse with 50 μCi ^{3}HTdR. Wait 40 min and cull.	20	3.8 ± 0.6	1.8 ± 0.2	151.8 ± 4.3	173.8 ± 7.7	526.7 ± 33.1	11441.3 ± 613.0	48.3 ± 3.5
Ratio of KGF-treated to saline-treated.	–	3.92	2.72	0.97	1.09	1.63	0.79	2.08

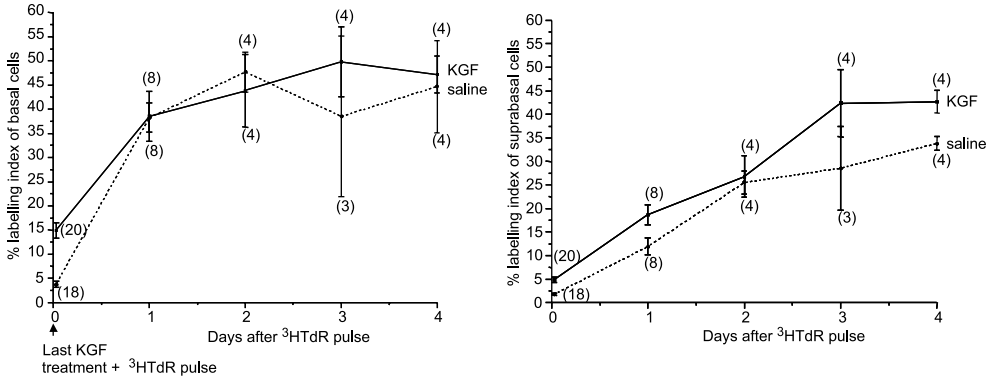

Figure 2. The labelling index in the basal layer (left panel) and suprabasal layer (right panel) when animals received three daily injections of KGF or vehicle (saline). All animals were pulsed with tritiated thymidine following the third of the daily injections and groups of animals were culled at the times shown following ^{3}HTdR injection. Means and standard errors are shown. The number of animals is shown in parenthesis.

four-fold higher in the KGF-treated group (Tables 1 and 2) and about 2.7-fold higher in the suprabasal layer. Thus, KGF increased the rate of proliferation. The labelling in the suprabasal layers continued to rise at approximately the same rate in the KGF and control groups up to day 3 at a rate of about 0.54%/h or 13%/day (Fig. 2, lower panel). The suprabasal LI was consistently 5–10% higher in the suprabasal layers of the KGF-treated groups (Fig. 2, lower panel). Although the LI in the basal layer was about four-fold higher in the KGF-treated group immediately after ^{3}HTdR administration, this difference is lost at 1 day (Fig. 2, upper panel) presumably due to the delayed uptake phenomenon reported in Potten *et al*. (2002a).

As can be seen from Table 1, KGF could induce changes in histometric parameters by factors of 2–4. It is also clear that in some cases little change could be detected (number of basal cells) or even a reduction after KGF could be seen (total cells/mm^2). This is due to the fact that even though the epithelium is considerably thicker with more undulations in the basal layer and extensions of the rete ridges, the individual cells are larger (see Fig. 3). Thus, one effect of KGF is to increase cell size by inducing more differentiation products, delaying emigration and loss, or some oedematous effects.

Although the initial LI of the basal cells in the KGF-treated group was nearly four times higher than in the vehicle controls, this difference was not maintained at 1 day post-KGF treatment (Fig. 2). For all of the 4 days post-KGF treatment, the labelling in the suprabasal layers was above the controls, indicating that more labelled cells migrate from the basal layer in the KGF-treated groups.

Figure 4 shows the pooled data for the mean epithelial thickness and the cellularity as seen beneath a 1-mm length of epithelial surface when KGF treatment continued for up to 7 days. For both parameters, the KGF-treated groups were significantly higher than the vehicle-treated controls for the 7 days of KGF treatment. However, continued exposure to KGF did not increase the thickness or cellularity further than seen on day 3. If KGF treatment was prolonged beyond the original 3-day period (Fig. 2) there was a further increase in the LI in the basal layer on day 4 and then no further increase (note that ^{3}HTdR was given to all the animals in this experiment on day 3 so the increase seen on day 4 is a doubling in LI due to cell division or delayed thymidine incorporation (see Potten *et al.* 2002a). In fact, if an additional KGF injection was given on the fourth day, 1 day after the tritiated thymidine pulsing, the vehicle-treated LI in the basal layer was higher than that for the KGF-treated groups (Fig. 5).

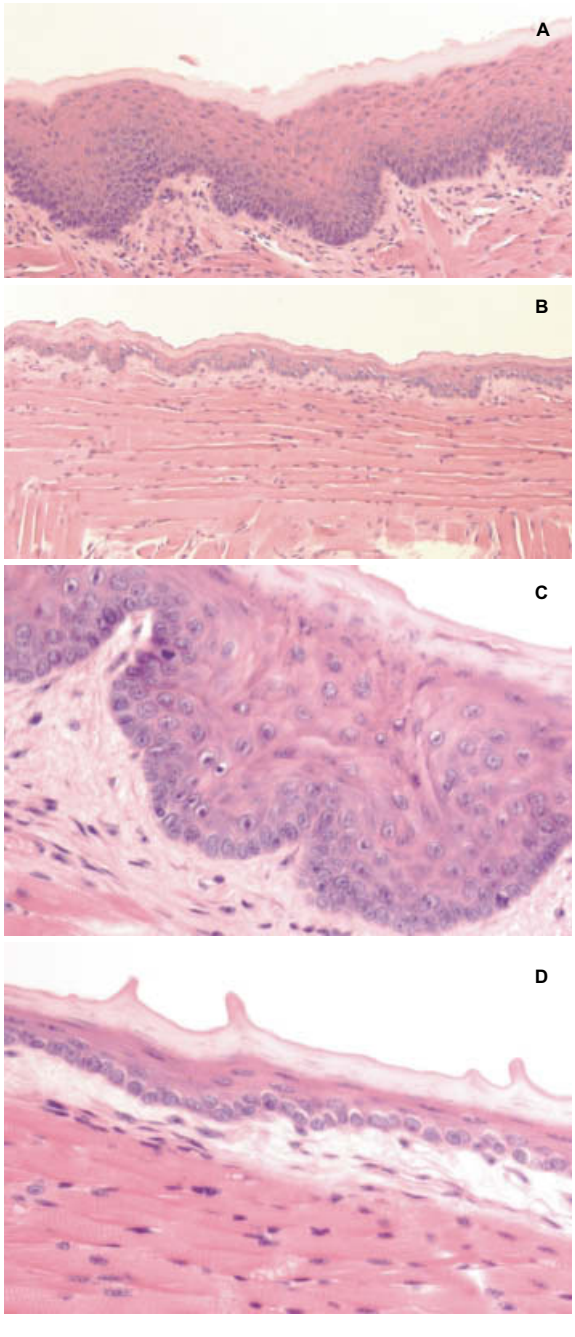

Figure 3. Photomicrographs showing typical areas of ventral tongue epithelium from animals that had received three daily injections of KGF or saline. (a) Ventral tongue treated with 125 μg of KGF for 3 days (× 109); (b) Ventral tongue treated with saline for 3 days (× 109); (c) Ventral tongue treated with 125 μg of KGF for 3 days (× 211); (d) Ventral tongue treated with saline for 3 days (× 211). These photomicrographs relate to the information shown in Table 1.

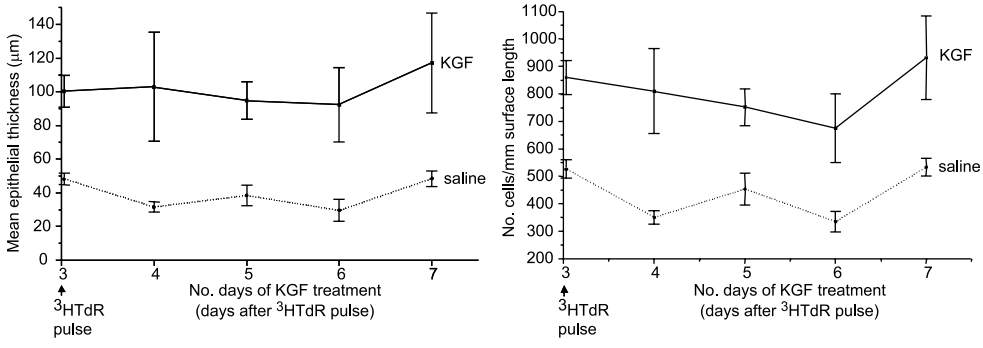

Figure 4. Epithelial thickness (left panel) and cellularity (right panel) in groups of animals that received three daily injections of KGF or vehicle (saline) with sampling performed on the days shown. The animals received tritiated thymidine on day 3 and the changes in labelling index are shown in Figure 5.

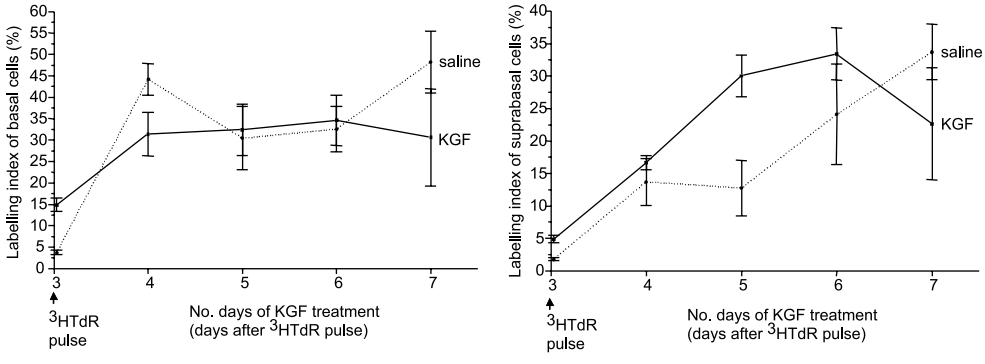

Figure 5. Labelling index in the basal layer (left panel) and suprabasal layers (right panel) for ventral tongue epithelium from animals that we received three daily injections of KGF or vehicle (saline), with sampling at the times shown. Tritiated thymidine was given following the third of the KGF or saline injections. Mean ± SE are shown.

When animals were pulse-labelled and sampled on days 3–7 in an experiment where KGF was continued on a daily basis up to day 7, the basal layer LI remained high on days 3 and 4, but then declined to reach almost the saline control values on day 7 (Fig. 6). The saline-treated groups remained roughly constant for the 7 days as did the levels of suprabasal labelling (Fig. 6, right panel). The thickness and cellularity measures in this experiment were very similar to those shown in Fig. 5. Thus, in spite of continued daily injections of KGF, the proliferative activity seen at 3 days (see Table 1) was maintained for another 24 h and then declined rapidly on days 5, 6 and 7. The epithelium therefore appears to correct the overproduction of cells (endogenous homeostatic processes) or the KGF switches from stimulating proliferation to stimulating differentiation.

When the KGF treatment was stopped at day 3 but groups of animals were pulsed with ^{3}HTdR and culled 40 min later at various times after the last KGF treatment up to day 7 (washout experiment), the LI was as shown in Fig. 7. The LI fell rapidly in the KGF-treated groups on days 1 and 2 after the 3 days of KGF (Fig. 7). On days 2 and 3, the KGF-treated groups were dramatically lower than the vehicle-treated groups. However, by day 7 the KGF-treated animals again had a slightly higher LI. There were some unexplained fluctuations in the LI of the vehicle-treated groups (Fig. 7). Both the thickness and the cellularity in the KGF-treated groups

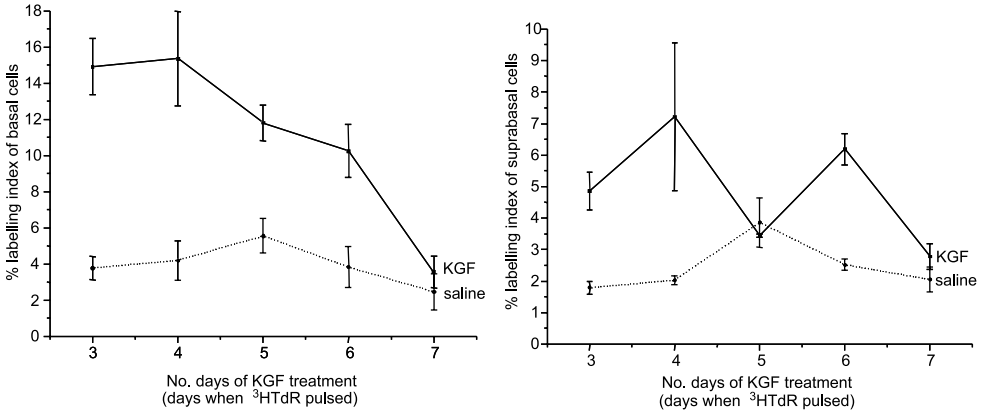

Figure 6. Labelling index in the basal layer (left panel) and suprabasal layer (right panel) in mouse ventral tongue when daily injections of KGF are continued to be given beyond the initial first 3 days. Animals were pulsed with ^{3}HTdR 40 min before sacrifice. Data are presented for KGF-treated and vehicle (saline)-treated animals. Mean ± SE are shown.

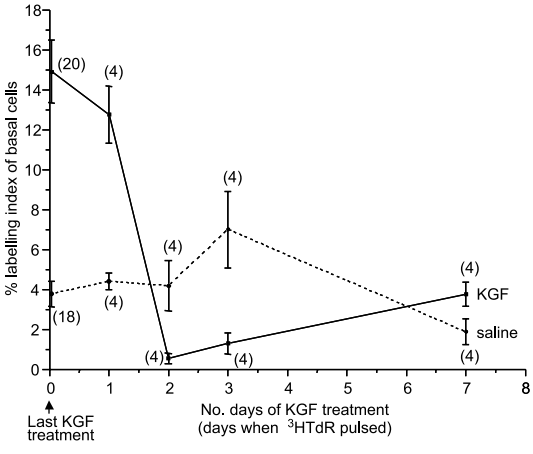

Figure 7. Labelling index in the basal layer of the ventral tongue epithelium from animals that received three daily injections of KGF or vehicle (saline) and were pulsed with tritiated thymidine on the days shown. The changes in epithelial thickness and cellularity in the experiment are shown in Figure 8. Mean ± SE are shown.

remained roughly constant for the first 3 days after the end of the 3 day KGF treatment and then declined to the values at the 7 day point where the KGF-treated thickness values were still above the vehicle-treated controls, while the cellularity values at day 7 were similar. These data suggest a progressive return towards control values over the period 3–7 days post-KGF treatment (Fig. 8). The falls in thickness and cellularity (Fig. 8) follow the downregulation of proliferation (Fig. 7) by about 1 day but appear less precipitous.

These observations are consistent with those reported elsewhere (Farrell *et al.* 1999, 2002; Dörr *et al.* 2001, 2002), which state that KGF delivered on a daily basis for 3 days results in some dramatic changes in the cell kinetics, thickness and cellularity of mouse ventral tongue. There is an increase in the fraction of basal cells in the S phase of the cell cycle, consistent with an increased cell production rate that is also observed as an increase in the migration of cells

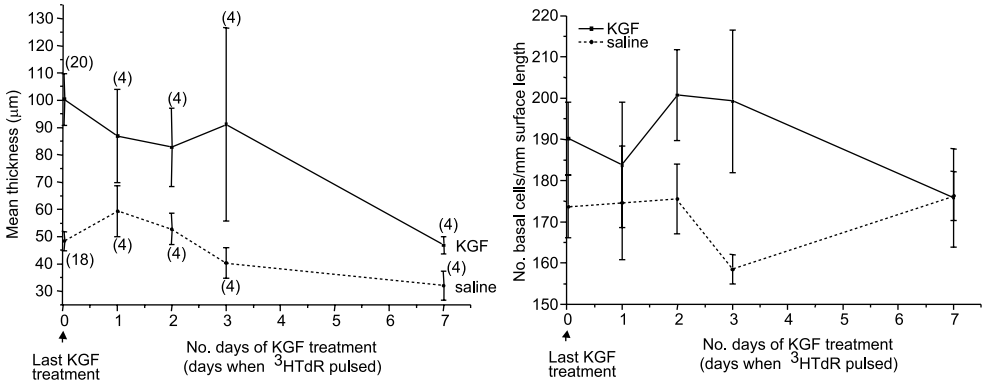

Figure 8. The changes in epithelial thickness (left panel) and cellularity (right panel) in the ventral tongue epithelium of animals that received three daily injections of KGF or vehicle (saline), with groups of animals sampled on the days shown. Mean ± SE are shown.

from the basal layer into the suprabasal layers. This increased production and migration results in an increase in the total cells and thickness of the epithelium. There is also an increase in cell size, possibly indicating a change in some aspect of differentiation. This process is not increased by further exposure to KGF and, indeed, once the 3-day peak is obtained, there is a progressive trend towards normalization, possibly indicating a switch from a proliferative stimulation to a differentiation stimulation by KGF as an attempt is made by the tissue to compensate for the overproduction of cells, i.e. evidence of the natural homeostatic processes being switched on. Once KGF treatment is stopped, the epithelium regresses progressively back towards control values but not all values are reached within the time frame of the present experiments (i.e. 4 days post KGF, 7 days in total). There is no clear evidence of apoptosis being involved in this tissue-compensation process. However, apoptotic studies in tongue epithelium are difficult (Potten 2001) and the timing of the samples in the current experiments may not have been optimal for apoptosis observation.

The increased cellularity and thickness would suggest that if radiation is delivered at the time of this increased thickness the ability of the epithelium to maintain an effective barrier is likely to be increased simply as a consequence of the greater number of cells present. It remains unclear as to whether the increases in proliferation and cellularity are associated with an increased number of epithelial stem cells in the basal layer, which would be required if a long-term protection was to be afforded. The data obtained following KGF treatment in the crypts of the small intestine suggest that KGF does indeed stimulate an increase in stem cell proliferation (Potten *et al.* 2001) and hence possible increases in stem cell numbers. Whether this is the case or not, the increase in total cellularity seen in the present data should provide an epithelium with more cells present post-treatment, which can help maintain the barrier for a longer period of time and hence provide a better environment for stem cell regeneration.

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