The effects of keratinocyte growth factor in preclinical models of mucositis

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Abstract. The epithelium of the oral cavity and small intestine of the gastrointestinal tract have a high rate of cell renewal and as such, are sensitive to cytotoxic therapies that kill rapidly dividing cells. Mucositis is a complication of cancer therapy where impairment of the regenerative capacity of the epithelium leads to atrophy, ulceration and a loss of barrier function. Keratinocyte growth factor (KGF) is an epithelial cell-specific growth and differentiation factor that is trophic for the mucosal epithelium of the gastrointestinal tract. In this study, KGF in normal animals caused epithelial thickening in the squamous epithelium of the oral cavity and increased crypt depth and villus height of the small intestine. It also appeared to regulate gene expression in these tissues including that of some antioxidant enzymes and intestinal trefoil protein. KGF has been shown to be efficacious in several preclinical models of mucositis where KGF pretreatment reduced weight loss typically seen during and after the course of therapy and significantly improved survival. At a tissue level KGF reduced atrophy, accelerated regrowth, and decreased ulcer formation of the oral epithelium after irradiation, and improved crypt survival and prevented villus atrophy in the small intestine of irradiated or chemotherapy-treated mice. Preliminary studies suggest that its efficacy may be partly a consequence of the growth and differentiation effect, and also partly due to regulation of the expression of genes that play a role in mucosal protection. These data suggest that KGF may be useful for the prevention or treatment of mucositis in patients treated with regimens of cancer therapy that have gastrointestinal toxicity.

Keywords: KGF, mucositis, epithelium, radiation, gastrointestinal tract.

KERATINOCYTE GROWTH FACTOR: *IN VIVO* ACTIVITIES

Effects in the gastrointestinal tract in normal animals

KGF is a growth factor for which epithelial cell specificity was initially identified in *in vitro* assays (Rubin *et al.* 1989; Finch *et al.* 1989). Subsequent studies in normal animals showed that it had growth and differentiating activities in a variety of epithelial tissues *in vivo* including the gastrointestinal tract. These activities included a trophic effect on the glandular epithelium

of the gastrointestinal tract including stomach and large and small bowel, a trophic effect on the squamous forestomach (Housley *et al.* 1994), stimulation of hepatic growth (Housley *et al.* 1994) and lipid metabolism (Nonogaki *et al.* 1995), and ductal proliferation in the pancreas (Yi *et al.* 1994). These activities are probably direct effects of KGF in these tissues because transcript for the receptor, a splice variant of the fibroblast growth receptor II, is expressed in the epithelium along the length of the gastrointestinal tract (Housley *et al.* 1994). KGF is also expressed in the same tissues (Housley *et al.* 1994) and *in situ* hybridization studies have shown that the transcript is found in cells adjacent to the epithelium during development and in adults, suggesting that this growth factor–receptor ligand system functions in a paracrine manner to stimulate tissue growth during development and to maintain function in adults (Werner 1998).

More detailed analysis of the effects of administration of pharmacological doses of KGF to animals shows striking activities in the upper aerodigestive tract and the small intestine. These two regions of the gastrointestinal tract are lined by a continuously renewing epithelium where rapid proliferation of stem or germinal cells is followed by differentiation, migration and subsequent loss of postmitotic cells. The upper aerodigestive tract has a squamous epithelium composed of keratinocytes organized into layers; a basal germinal layer, the spinous layer, and the upper granular layer, all of which in rodents are protected by a keratinized surface layer. The epithelium of the upper aerodigestive tract responds robustly to KGF; this was shown in studies where 5 mg/kg KGF was administered daily to mice for 3 days, the tongues resected 24 h after the last dose and processed for light and electron microscopic morphometry. Examination of the toluidine blue-stained semi-thin sections showed a marked increase in the overall epithelial thickness. In addition to this increased cellularity, there was a significant augmentation of the keratohyalin granule component of the granular layer, with a commensurate increase in the density of the keratinized layer (Fig. 1).

In these tongues, measurement showed a two-fold increase in thickness of the nucleated layers of the ventral epithelium as well as a four-fold increase in the keratohyalin granule area (Fig. 2). This increase in area was due to increased numbers of individual granules in combination with an increase in the average size of the granules. In similar experiments, animals were injected with bromodeoxyuridine (BrdU), a marker of proliferation, 1 h before necropsy, and processed for BrdU immunostaining (Housley *et al.* 1994) and haematoxylin and eosin staining of paraffin sections. It was seen that the KGF-treated mice also had increased numbers of BrdU positive cells and a correlated increase in mitotic figures (Fig. 2), both of which were confined to the basal layers of the epithelium.

Studies by Frank *et al.* (1997) showed that KGF specifically upregulated the expression of a non-selenium-dependent glutathione peroxidase (nsGPX) in human keratinocytes in culture. To

Figure 1. Semi-thin sections of the ventral surface of the mouse tongue in normal, control mice and mice that were treated with 5 mg/kg KGF for 3 consecutive days. Increased cellularity as well as increased granule size and number are evident.

Figure 2. The effect of KGF on several measures of epithelial growth as assessed in cross-sections of the ventral tongues of mice. Each bar represents the mean $\pm$ standard error of the mean (SEM) where $n = 5$ mice/group. $*P < 0.05$ compared to control. The granule counts were performed on photographs of cross sections enlarged to the same final magnification so that data are expressed per an identical unit area. The BrdU and mitotic figure counts were also done in cross-sections of tongue where the numbers of BrdU-positive cells or mitotic figures were counted in an identical unit length of tongue for each mouse in both treatment groups.

Figure 3. *In situ* hybridization for mRNA of GST and nsGPX in the epithelium of the ventral tongues of mice. These photomicrographs show that the mRNA transcript for these genes is upregulated in the oral squamous epithelium by treatment of the mice with 5 mg/kg/day for 3 consecutive days.

determine whether KGF had effects on antioxidant enzymes *in vivo*, *in situ* hybridization (Wilcox 1993) was performed with probes for the mRNA of nsGPX and glutathione-S-transferase (GST). In mice treated with 5 mg/kg/day of KGF for 3 days, the hybridization studies showed increased signal for the transcript of both genes (Fig. 3). Gene regulation of other antioxidant

Table 1. Microvillus measurements were made on electron micrographs. The total absorptive area of the jejunum was calculated as the product of microvillus surface area and density on enterocytes, and of the villus surface area and density in a mm² of *en face* jejunum. The final product is expressed as mm² absorptive area/mm² jejunum wall

	Control (mean $\pm$ SEM)	KGF (mean $\pm$ SEM)
Tissue wet weight		
Duodenum (g)	0.31 $\pm$ 0.01	0.35 $\pm$ 0.02
Jejunum (g)	0.52 $\pm$ 0.03	0.62 $\pm$ 0.03
Ileum (g)	0.18 $\pm$ 0.01	0.21 $\pm$ 0.01
Jejunal villi		
Length (μ m)	473 $\pm$ 38	538 $\pm$ 27
Maximum diameter (μ m)	160 $\pm$ 15	194 $\pm$ 32
Minimum diameter (μ m)	99 $\pm$ 10	98 $\pm$ 11
Density (villi/mm ²)	35 $\pm$ 5	31 $\pm$ 4
Enterocytes (/mm villus)	167 $\pm$ 23	169 $\pm$ 16
Enterocyte Microvilli		
Microvilli density (/um ² enterocyte)	48 $\pm$ 7	49 $\pm$ 3
Diameter (nm)	122 $\pm$ 7	122 $\pm$ 6
Length (nm)	1180 $\pm$ 251	1489 $\pm$ 319
Total absorptive area (mm ²)	223	320
Goblet cells (/mm villus)	9 $\pm$ 2	22 $\pm$ 2

enzymes is currently being explored, but the data thus far suggest that KGF may influence the antioxidant capacity of the tissues of the oral cavity.

The above observations are interpreted to mean that KGF has proliferative effects on oral epithelium as well as effects on postmitotic cells, and suggests that there are multiple mechanisms that account for the increase in thickness measured in this epithelium in response to KGF treatment. The rate of tissue generation appears to be increased as more cells are proliferating, but also the rate of cell loss due to desquamation at the surface may be decreased by the KGF treatment due to enhanced desmosomal attachment (Farrell *et al.* 1999) and keratohyalin protection. Additionally, KGF appears to regulate antioxidant genes *in vivo*, suggesting that oxidative tissue stress induced by irradiation may be abrogated by KGF treatment.

The small intestine is also sensitive to KGF and, like the squamous mucosa, KGF has growth and differentiating effects in this segment of the gastrointestinal tract. Housley *et al.* (1994) showed that administration of KGF to rats causes increases in duodenal crypt depth and increased numbers of goblet cells. To characterize the tissue response further, mice were treated with 5 mg/kg of KGF for 3 days, and injected with BrdU 1 h prior to necropsy, when the small intestine was removed and divided into segments that were flushed with saline, blotted and weighed. Tissues were processed for light and electron microscopy, as well as for immunostaining and morphometry; the data are presented in Table 1. Wet weights of the intestinal segments were increased by the KGF treatment and morphometric measurement showed that both crypt depth and villus height were also increased (Farrell *et al.* 1998). Further morphometric analysis of villus features such as microvillus length showed that simultaneous changes in these parameters in response to the KGF treatment lead to a 45% increase in total absorptive surface area. These morphometric measures are consistent with the reported increases in brush border enzyme activities in KGF-treated mice (Russell *et al.* 1997).

Figure 4. *In situ* hybridization of rat small intestine showing mRNA expression for ITF. These photomicrographs show ITF that is normally expressed in the goblet cells of the small intestinal epithelium is apparently up-regulated in response to systemic administration of 5 mg/kg for 6 days.

As in the rat, there were also more goblet cells in KGF-treated mice than in saline-treated controls. The increase was apparent at all levels of the intestine and counts made in cross-sections at the level of the jejunum showed a threefold increase (Table 1). Goblet cells produce mucins that are thought to function as a luminal barrier in the small and large intestines. They also produce intestinal trefoil protein (ITF), which is a member of a family of small protease-resistance proteins shown to contribute to gastrointestinal defence and repair (Playford *et al.* 1996). In studies where rats were treated with 5 mg/kg/day of KGF for 6 days, *in situ* hybridization (Wilcox 1993) showed that the signal for ITF was considerably higher than in control, saline-treated rats (Fig. 4), presumably due to the increased numbers of goblet cells, although a concomitant increase in individual cell production of ITF can not be ruled out. These growth and differentiating effects of KGF, including surface area increase and elevated expression of mucin and related proteins, suggest that KGF may enhance the absorptive and barrier functions of the small intestine.

Effects in the upper aerodigestive tract after radiation injury

Under normal physiological conditions, steady state is maintained in squamous epithelium by continuous production of cells in the germinal layers as the surface layers are eroded by desquamation. Radiation disrupts this steady state by impairment of the reproductive capacity of the tissue, leading to atrophy and ulceration. This has been shown also in mouse tongue (Dörr & Kummermehr 1992) where there was a dose-dependent effect of radiation on frequency and confluency of ulceration on the ventral surface. In this model, it was shown that treatment of the mice with KGF resulted in a significant reduction in oral mucosal ulceration (Dörr *et al.* 2000). In a different mouse model of radiation-induced epithelial atrophy, KGF was able to reverse the trend towards atrophy following single or multiple dose radiation (Farrell *et al.* 1999). Pre- and post-treatment schedules were evaluated and found to have activity in this model, with the most robust effect observed in the mice that received KGF after irradiation. This was attributed to multiple mechanisms including enhanced proliferation and effects on desmosomes and keratohyalin in postmitotic cells. Importantly, it was demonstrated that injured epithelium retained the ability to respond to KGF. Further studies are underway to evaluate the role of antioxidant enzymes as preliminary studies have shown that KGF does regulate the expression of some of

Figure 5. The effect of KGF pretreatment on the small intestines of mice irradiated with 12 Gy. While these measures of tissue wet weight, DNA and protein are reduced to less than half of that in normal, unirradiated mice (data not shown), the mice pretreated with KGF show that these measures of growth are improved relative to the irradiated group.

the same genes in oral cavity tissues from mice that received radiotherapy in combination with chemotherapy (data not shown).

Effects in the small intestine after radiation injury

Radiation also kills rapidly dividing cells in the crypts of the small intestine, impairing reproductive capacity and leading to depopulation of the villus. This results in a wide range of symptoms from mild diarrhoea to sepsis. KGF given to mice as a pretreatment before abdominal or total body irradiation has been shown to improve crypt survival and animal mortality (Kahn *et al.* 1997). In experiments examining tissue effects in the small intestines of irradiated mice, animals were treated with KGF at 5 mg/kg/day for 3 days prior to receiving a single dose of 12 Gy. The tissue wet weight, DNA, and protein were all increased in the KGF-treated animals relative to irradiated controls (Fig. 5), demonstrating that KGF pretreatment spared the small intestine from damage. This correlates with crypt count data showing that crypt survival, and hence growth capacity, was increased in KGF-treated mice compared to control, irradiated mice (Farrell *et al.* 1998), and suggests that KGF may also impact positively on mucosal functions such as nutrient absorption.

The mechanisms of action of these effects are as yet unknown. It may be a result of the pretreatment trophic effect where increased numbers of crypts and larger villus surface area could enhance reproductive and functional capacity, thereby sustaining the mice through cytotoxic injury. Alternatively, as radiation and chemotherapy kill stem cells via apoptosis, it may be that there is an antiapoptotic effect of KGF. Studies are underway to determine whether KGF affects radiation-induced apoptosis in crypts, and whether KGF influences gene regulation of the apoptosis pathway. An alternative explanation is that KGF given prior to irradiation stimulates stem cells to divide, thus increasing the number of radiation target cells (Potten *et al.* 2001). With the same level of fractional stem cell killing, a greater number of stem cells will survive in the KGF-treated groups, increasing the efficiency of the regeneration process. KGF has been shown to enhance the glutathione redox state in rat intestinal mucosa during malnutrition (Jonas *et al.* 1998), and it may be that KGF will reduce radiation-induced tissue oxidative stress also.

CONCLUSION

The rapidly proliferating epithelium of the oral and intestinal mucosae are sensitive to radiation and other cyto-ablative agents that compromise regenerative capacity, leading to nutrition and barrier impairment. The potent growth, differentiation and protective effects of KGF on the gastrointestinal tissues indicate that KGF may be useful for treatment of radiation injury of the gastrointestinal tract. Its effects are directed against epithelial cells where the receptor is expressed, and this specificity for epithelial cells may be an advantage that therapies such as aminothiols, interleukins or TGF β s lack. Data so far suggest that KGF could potentially be used either prior to therapy in order to prevent oral and intestinal toxicity, or after radiation exposure where it has been shown that KGF appears to facilitate the regrowth of the oral epithelium. Further work will be important to understand the mechanisms of the effects of KGF in normal tissues, as well as the interaction of KGF with cyto ablative therapy, so that the clinical application of this growth factor leads to successful treatment of cyto-ablative injury.

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