
Keratinocyte Growth Factor Increases Hair Follicle Survival Following Cytotoxic Insult

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Hair loss is a distressing side-effect of cancer therapy. Factors that might reduce this loss are therefore likely to improve patient well-being and reduce treatment refusal. Keratinocyte growth factor has been shown to regulate proliferation and differentiation in epithelial tissues and may regulate the clonogenic cells (stem cells) of the hair follicle. Using X-irradiation as a model cytotoxic agent we investigated whether keratinocyte growth factor pretreatment could increase hair follicle survival (by implication clonogen survival) and regeneration of differentiated progeny (a hair). Irradiated telogen follicle survival data were consistent with that published previously. Daily keratinocyte growth factor pretreatment increased hair survival during the first hair growth cycle, the level of protection having a slight radiation dose dependence. Protection was maintained after a second hair cycle, but at a lower level (hairs and follicles). Hairs irradiated in anagen and analyzed during the second cycle exhibited a similar level of protection. No difference

in protection levels could be observed between mice treated either once or twice daily with keratinocyte growth factor. Results indicated approximately 10 extra hairs per mm² (14.5% of unirradiated control) could survive the cytotoxic insult if pretreated with 12.5 µg keratinocyte growth factor. This could be doubled by a 10 × higher keratinocyte growth factor dose. The fact that protection was maintained during two cycles of hair growth indicated that the clonogenic cells had been protected. Keratinocyte growth factor pretreatment had no significant effect on the level of mitosis but increased the number of p21^{waf1} expressing cells. Intriguingly, the number of apoptotic fragments per follicle were transiently increased in the keratinocyte growth factor pretreated mice, although this effect was most pronounced in the upper half of the follicle bulb, i.e., above (rather than within) the germinal matrix. *Key words: alopecia/cancer/follicle/hair/KGF/radiation. J Invest Dermatol 114:667–673, 2000*

Hair loss (alopecia) is one of the distressing side-effects of cancer therapy, frequently causing patients to refuse treatment. Factors that might reduce radiation or chemotherapy induced alopecia are therefore likely to reduce treatment refusal and, ultimately, increase patient survival. A wide range of growth factors, including several members of the FGF family, have been implicated in the regulation of hair follicle growth (DuCros, 1993a, b; Hebert *et al*, 1994). One of the most interesting clinical prospects is keratinocyte growth factor, KGF or FGF-7. KGF has been shown to be expressed in the dermis and to regulate epidermal proliferation and differentiation via a paracrine mechanism, stimulating wound healing and hair growth (Finch *et al*, 1989; Pierce *et al*, 1994; Werner *et al*, 1994; Danilenko *et al*, 1995; Guo *et al*, 1996). Indeed, injection of KGF into nude mice directly stimulated hair growth, particularly at the injection site. Targeted overexpression of this factor in the epidermis, however, reduced hair follicle morphogenesis (Guo *et al*, 1993). The receptor has also been identified within the hair follicle (LaRoche *et al*, 1995).

Hence, there is convincing evidence that this factor is a potent regulator of hair follicle growth.

Parallel studies have recently shown that KGF also regulates proliferation and differentiation in another constantly renewing and therapy sensitive epithelial tissue, the small intestinal mucosa (Housley *et al*, 1994). Interestingly, in this tissue short-term pretreatment with the factor also increased stem cell survival and improved epithelial regeneration following cytotoxic insult (Kahn *et al*, 1997; Farrell *et al*, 1998).

Combining both lines of evidence, it is therefore possible that pretreatment with KGF may protect the clonogenic cells (stem cells) of the hair follicle. We have therefore used X-irradiation as a highly controlled, site directed, model cytotoxic agent and compared the ability of the clonogenic cells of the hair follicle to survive and regenerate differentiated progeny with and without KGF pretreatment. In an attempt to try and elucidate a mechanism of action for this growth factor, the levels of apoptosis following radiation exposure and the possible involvement of the cell cycle regulatory protein p21^{waf1} were also measured.

MATERIALS AND METHODS

Mice Seven to eight week old male BDF1 mice were used throughout. At this age most follicles in the back skin are in the telogen (resting) growth phase (Silver *et al*, 1969 and confirmed in-house by histologic analysis). All mice were allowed free access to food and water and maintained on a 12 h

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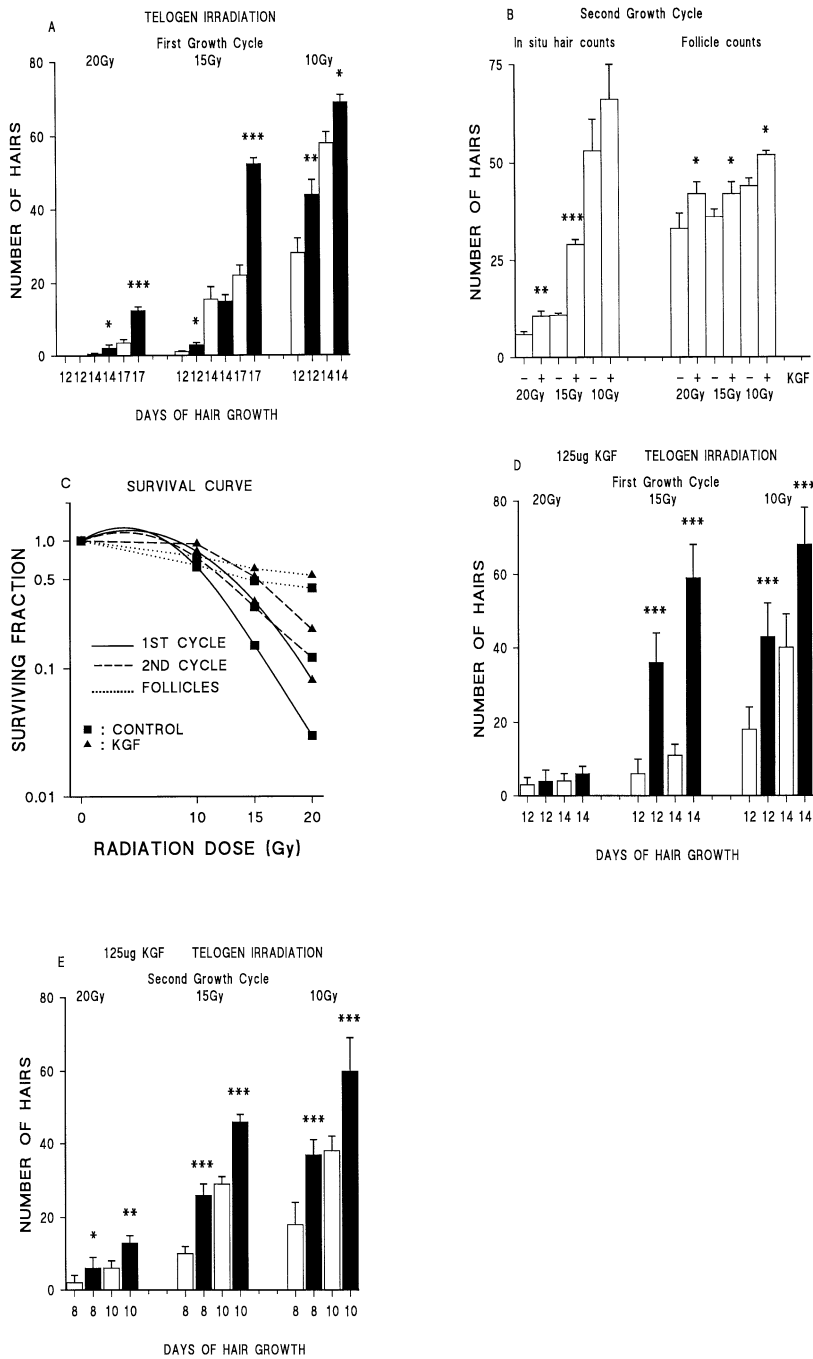


Figure 1. Effect of KGF on the ability of telogen hair follicles to survive irradiation. Pretreatment with either 12.5 or 125 µg KGF resulted in a statistically significant increase in hair and follicle survival through two growth cycles. (A) Resting hair follicles were treated with 12.5 µg KGF once a day, irradiated and immediately plucked. The number of hairs per 3 mm² were counted 12–17 d later (17 d following 10 Gy hairs were too long and too numerous to count). ■, KGF treatment; □, controls. (B) Ten days following a second plucking the regenerated hairs were counted (left-hand bars). Two days later hairs were too numerous to count and the skin was therefore excised and the number of follicles observed per 3 mm² on the dermal surface counted (right-hand bars). (C) Based upon the known number of hairs per mm² on the mouse dorsum a survival curve for both hairs and follicles following irradiation and KGF treatment is presented. Consistent protection can be seen. (D) Resting hair follicles treated with 125 µg KGF demonstrate a further increase in survival during the first hair cycle (10–12 d post-plucking). Further optimization of doses may yield even greater levels of protection. ■, KGF treatment; □, controls. (E) Resting hair follicles treated with 125 µg KGF sustained this same significant level of protection (see D) during the second hair growth cycle (8–10 d postplucking). ■, KGF treatment; □, controls.

light/dark cycle (lights on at 0700 GMT). All experiments were performed within the guidelines of the Animals (Scientific Procedures) Act 1986, U.K.

KGF Recombinant human KGF (a gift from Amgen, Thousand Oaks, CA) was given subcutaneously in the dorsum. KGF (12.5 µg, or 125 µg where stated, i.e., **Figure 1D, E**) was injected subcutaneously in 0.5 ml saline/bovine serum albumin for 3 d either once at 0900 or twice at 0900 and 21.00 h. An equal volume, equal temperature, vehicle-only control group were also injected as a control (also to control for injection trauma). Four mice per experimental group were examined in the first experiment, five mice per group in the second. 12.5 µg KGF is the dose that had previously been used to demonstrate successfully small intestinal clonogenic cell radioprotection (Farrell *et al*, 1998).

Irradiation A 1 cm diameter dorsal area of skin was irradiated on each mouse, the remainder of the mouse being protected by lead shielding. All mice were anesthetized prior to irradiation (and plucking, see below) using Ketamine and Rompun. Matched groups of KGF and vehicle controls

were given 10, 15, or 20 Gy using X-rays generated at 300 kV (HVL 2.3 mm Cu) at a dose rate of 0.52 Gy per min.

Protection protocols (see Fig 2) The first experiment was designed such that the mice were treated with KGF for 3 d. At 0900 on the fourth day the mice were anesthetized and irradiated. The dorsal hair was plucked immediately after irradiation in telogen, using OpSite plastic dressing (Smith and Nephew, Hull, U.K.) on anesthetized mice, and new hair follicles counted after 12–17 d growth. The most rigorous test of stem cell survival is the ability of stem cells to regenerate differentiated progeny – in this case a new hair. This assay therefore tests the ability of the stem cells that survive irradiation to repopulate and produce a new growing hair follicle capable of producing new hairs. Hair follicles were then replucked after 28 d growth and the regeneration examined 10–12 d later to provide a second more stringent test of clonogenicity (i.e., the stem cells must be able to generate the first postirradiation hair follicle and also survive catagen regression, reorganize, and form a second generation functional hair follicle).

Experiment 1: KGF treatment and irradiation of telogen follicles

7-8 weeks	KGF	X-ray/pluck	Assay 1	Replucked	Assay 2
DAY	123	4	16-21	32	42-44
	Telogen		Anagen 1		Anagen 2
			12-17 days growth		10-12 days growth

Experiment 2: KGF treatment and irradiation of anagen follicles

7-8 weeks	Pluck	KGF	X-ray	Pluck	Assay
DAY	0	9-11	12	35	45-47
	Telogen		Anagen 1		Anagen 2
				10-12 days growth	

Figure 2. Schematic representation of the two protocols used to assay hair follicle protection. KGF (12.5 µg) was administered for 3 d either daily or twice daily (both doses were tested in both protocols).

In the second experiment mice were plucked at the beginning of the experiment to initiate a new synchronous cycle of hair growth (Potten, 1972), and treated with KGF during the ninth to eleventh day of regrowth (mid-anagen). At 0900 on the 12th day the anagen follicles were irradiated (anagen again confirmed by histologic analysis). Five weeks following the irradiation mice were replucked and hair regrowth counted 10–12 d later.

In both cases the mice were killed at the time of the final hair count and the irradiated area of skin was removed, flattened on to paper to prevent curling, and fixed in Carnoy's fixative (60:30:10 ethanol/chloroform/propionic acid).

In situ hair counts The number of hairs observed in three 3 mm² random fields of the irradiated area of an anesthetized mouse were counted at ×40 magnification using a dissecting microscope.

Whole mount hair counts After fixation samples were stored in 70% ethanol at 4°C. Samples were prepared by rehydration and hydrolysis in 5 M HCl for 30 min followed by immersion in iced distilled water. The muscle and fat was stripped from the dermal surface and the remaining tissue stained in Schiff's reagent for 5–10 min. After quickly rinsing in distilled water the staining was stopped with a hydrogen sulfide rinse (0.5% sodium metabisulfite, 0.05 M HCl). The tissue was then washed in distilled water, dehydrated through a series of alcohols from 40% to 100% and then cleared in 50% ethanol–50% CitrocLEAR (HD Supplies, Aylesbury, Bucks, U.K.) for 30 min. After two changes of 100% CitrocLEAR the tissue was mounted dermal side uppermost in Xam (BDH-Merck, Leics, U.K.) on specially prepared built-up glass slides.

The number of follicles per mm² was then calculated using a Zeiss AxioHOME microscope at ×100 magnification. A minimum area of 10 mm² per mouse was counted. For this study all follicles were counted – underfur and guard hairs.

Apoptosis/mitosis counts As in the second set of regeneration experiments, 7–8 wk old mice were plucked and injected with KGF at 09.00 h during days 9–11 of anagen. On the fourth day mice were irradiated with 5 Gy X-rays. This dose was used because previous experiments have indicated that up to 5 Gy there is a concentration-dependent increase in apoptotic fragments, but this frequency then plateaus such that at higher doses the apoptotic response does not differ from 5 Gy for the subsequent 48 h (Potten, 1985).

KGF/vehicle matched groups (four mice per group) were killed after 2, 4, 12, and 24 h postirradiation. One control group (not injected, unirradiated) was killed 12 d postplucking (the time of irradiation).

After fixing in Carnoy's as described above, three nonserial 3 µm sections were cut along the cephalocaudal axis of the mouse. Haematoxylin and eosin stained sections were examined using the AxioHOME microscope and the total number of apoptotic fragments (the number of apoptotic cells is difficult to establish and hence fragments must be counted) and mitotic figures (late prophase to early telophase) counted in 25 good, full, longitudinal sections of follicles which included the dermal papillae. All slides were scored blind. All apoptotic fragments were counted, irrespective of their size or condensed chromatin content.

p21^{waf1} staining Mice were treated and 3 µm sections prepared as for the apoptotic counts above, except the samples were fixed in 4% formalin/phosphate-buffered saline. Sections were dewaxed in xylene, transferred to ethanol and endogenous peroxide blocked with 2% H₂O₂. After rehydrating, antigen retrieval was achieved by microwaving (650 W) in citrate buffer (pH 6) for 20 min. Following phosphate-buffered saline washing, sections were blocked in 10% normal goat serum in Tris-buffered saline/0.5% Tween for 45 min. Rabbit anti-p21 (Calbiochem, Cambridge, MA) was applied at 1:200 in the blocking solution at 4°C overnight. After washing, biotinylated goat antirabbit (Pierce and Warriner, Chester, U.K.; 1:200 in Tris-buffered saline/0.5% Tween with 5% normal mouse serum) was applied for 45 min at room temperature. After further washes avidin-peroxidase (Elite ABC Vectorstain, Vector Labs, Peterborough, U.K.) was applied for 30 min. Slides were washed, developed with diaminobenzidine tetrahydrochloride, washed again, dehydrated in 80% methanol, counterstained with thionine, dehydrated in ethanol and mounted in XAM. Cells were recorded as negatively stained, weakly or strongly stained. Slides were scored blind, and the same scorer examined the whole experiment.

Statistical analysis Comparisons were made using an independent-samples t test using the SPSS computer package. *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

RESULTS**KGF protects resting hair follicles from radiation damage**

Daily treatment of telogen hair follicles with 12.5 µg KGF resulted in a 2–3-fold (p ≤ 0.0005) increase in hair numbers 17 d after 15 and 20 Gy (10 Gy treated animals have too many hairs to be accurately counted *in situ* by 17 d). These “raw” data are shown in **Fig 1(A)**. Protection was maintained in both hairs and follicles following a second plucking (hair growth cycle), indicative of a clonogenic rather than a continued growth response (**Fig 1B**). On equivalent days postplucking fewer hairs can be seen in the first growth cycle than the second. This delay is due to cell killing, cell cycle arrest, and delayed regeneration immediately following irradiation. The second cycle is simply the response to plucking.

It is unlikely that the observed protective response is due to the telogen hairs being induced to enter early anagen by the injected KGF as there was no significant movement into growth (histologic analysis and appearance of bromodeoxyuridine labeled cells) 24 h after the last injection, i.e., the time of irradiation (data not shown). Of course this does not preclude the fact that proliferative differences may become apparent at a later stage and affect the repair/regeneration process.

Although the injection and irradiation sites are in close proximity it is unlikely that the two sites are the same (the sites of injection were not marked but were generally proximal to the irradiation site). It is therefore unlikely that the protective effects are localized just to the injection site, although whether they are present throughout the entire skin remains to be evaluated.

The results can be summarized as a hair survival curve (**Fig 1C**). There is a clear radiation induced loss of hairs and follicles. KGF induced protection can clearly be observed in both hairs or follicles during both hair cycles.

Data not presented indicate that an additional injection of KGF each day did not improve hair follicle protection.

In order to determine whether an increased dose of KGF could improve hair protection the single daily dose protocol was repeated using 125 µg KGF (10× higher). It can be seen from **Fig 1(D, E)**

that this afforded a further increase in hair survival in both the first and second hair growth cycles, with up to 6 fold protection possible in the first hair cycle.

KGF protects growing hair follicles from radiation damage

Owing to the nature of the assay one cannot analyze the protection of hairs treated during anagen during the same (first) hair growth cycle. One must therefore wait until the second when the level of hair protection is very similar to that observed in the second (equivalent) cycle following the telogen pretreatment. Daily pretreatment with either one or two doses of KGF increased hair number (survival) in the second cycle of hair growth at all three doses of radiation (mean value of 38% protection) (Fig 3A). Demonstration of protection by examination of the excised dermal surface (hair follicle number) was less convincing, with some effect detected in the single injection per day treatment group, but no protection in the twice daily treatment group (indeed a slight sensitization was apparent $p \leq 0.05$), Fig 3(B). As with the telogen follicle, KGF did not appear to move the cells into the opposite phase of the growth cycle (i.e., in this case into telogen). Bromodeoxyuridine labeling revealed no statistically significant change in S phase cells, although a trend towards reduced labeling index (S phase cells) was observed following KGF administration (15.4 ± 2.6 vs 18.7 ± 2.1 control BrdU labeled cells per follicle at the time of irradiation. This trend remained 2 d later – 3 d after the last injection of KGF – with 16.3 ± 1.6 cells BrdU labeled in the KGF treated follicles compared with 20.4 ± 3.0 in the controls).

KGF increases hair follicle survival by 14% Follicle protection factors can often exaggerate effects when few surviving clones (hairs) are present. For example, a 4 vs 2 survival ratio will give the same factor as 40 vs 20, even though far more hairs survive in the latter scenario. One can therefore also compare the actual number of hairs rescued as a measure of KGF effectiveness. Control BDF1 dorsal hair density was found to be 69.2 ± 3.8 per mm^2 . This observation is similar to the density observed on a DBA mouse (89 per mm^2) (Hendry *et al*, 1980) and a mean value of about 50 per mm^2 estimated from various publications (Potten, 1985). Using this figure, examination of telogen hair follicle survival following irradiation produced data comparable with that published several years ago (Dubravsky *et al*, 1976; Griem *et al*, 1979) (Fig 1C).

Following daily $12.5 \mu\text{g}$ KGF pretreatment and telogen irradiation the mean increase in the number of surviving hairs during the first hair cycle was 8.8 or 9.4 per mm^2 (one or two injections, respectively). This was maintained at 12.2 or 11.5 per mm^2 , respectively, during the second cycle. Hence, no effect of a second KGF dose could again be detected and an overall mean of 10.5 hairs were “rescued”. Following anagen irradiation a mean of 11.1 per mm^2 hairs were “rescued” in this second cycle. It is therefore fairly clear, that approximately 10 more hairs per mm^2 survive, equivalent to 14.5% of all the hairs. Increasing the dose of injected KGF 10 \times doubled this level of hair survival.

KGF further increases irradiation induced p21^{waf1} expression and levels of apoptosis in hair follicles, but does not appear to cause mitotic arrest

Following irradiation there was an increase in p21^{waf1} expression within 2 h (Fig 4). This persisted for over 24 h. The total number of p21 expressing cells during the 12 h following irradiation was increased by KGF pretreatment, although the KGF effect was no longer evident by 24 h. Cells were counted as either unlabeled, strongly labeled, or weakly labeled with anti-p21. Interestingly, the number of strongly labeled cells peaked earlier in the KGF treated animals, possibly indicating that KGF altered the kinetics or level of p21 expression.

Following irradiation there was a rapid drop in mitotic indices for both KGF and vehicle treated mice (Fig 5). Over the following 24 h this gradually returned back to control levels. There was no significant evidence of a KGF induced cell cycle arrest as might be suggested by the p21 result.

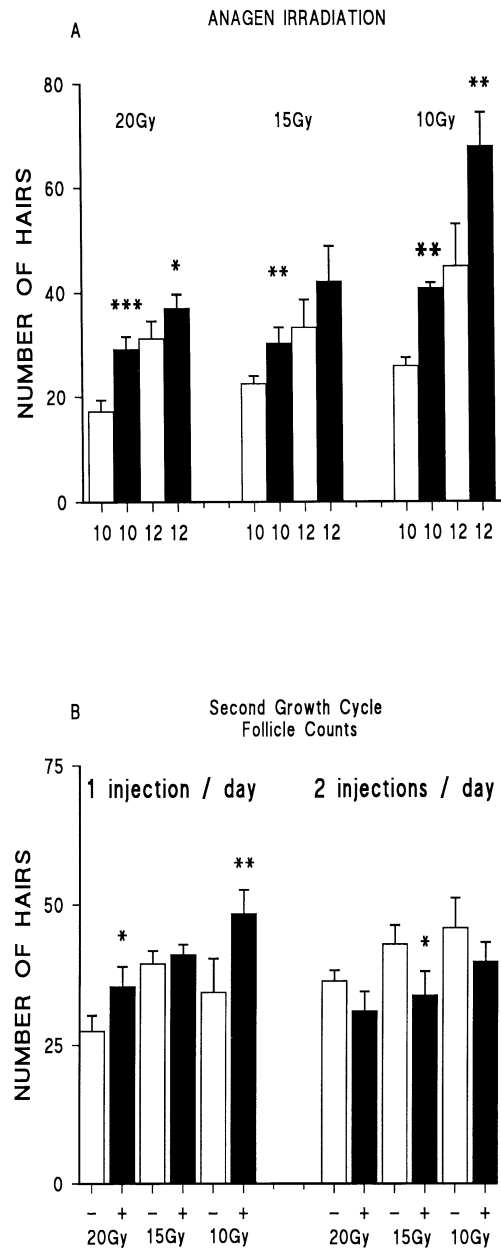


Figure 3. Effect of KGF on the ability of anagen hair follicles to survive irradiation. Pretreatment with $12.5 \mu\text{g}$ KGF resulted in a statistically significant increased hair survival in the subsequent hair cycle. (A) Hair counts 10 and 12 d following plucking after the completion of the anagen during which KGF treatment/irradiation occurred. (B) On the 12th day the irradiated area of skin was removed and prepared for follicle counts. ■, KGF treatment; □, controls.

Apoptotic fragments can easily be recognized at the base of the hair follicle, due to the chromatin condensation. A single apoptotic cell, however, can disperse into a number of fragments, some containing just cytoplasmic material (no chromatin). These are eosinophilic and can therefore also be identified. In this study the total number of fragments were counted, i.e., fragments containing either cytoplasmic or nuclear material. Counts were subdivided into two regions: The first spanning the bottom of the follicle to the base of the melanocytes (bulb midline); and a second area of equal size from midline upwards.

Although there was an increased number of apoptotic fragments during the 24 h following irradiation (peak at 12 h),

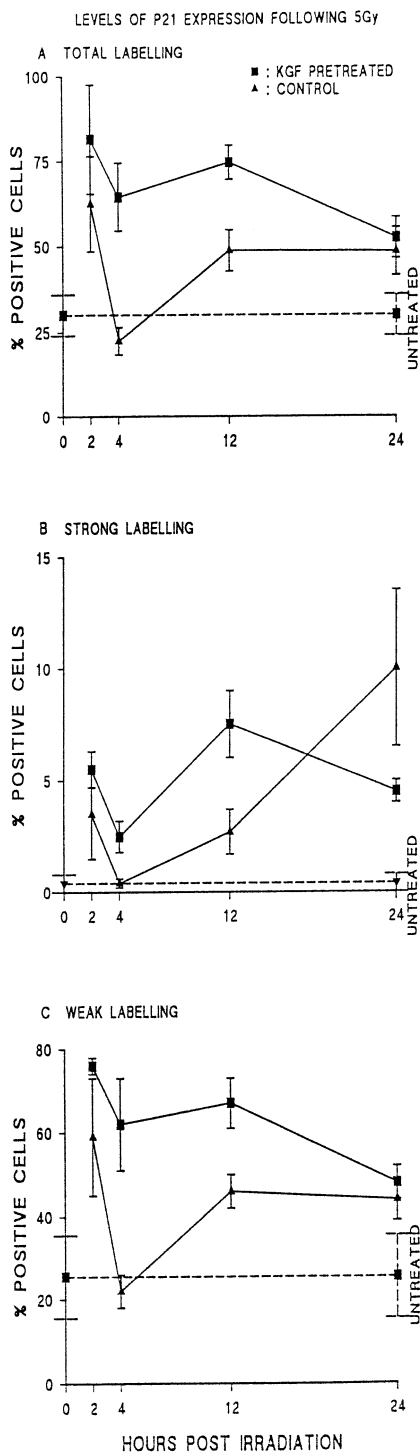


Figure 4. KGF pretreatment induced a significant increase in p21^{waf1} expression during the first 24 h post-irradiation. (A) Number of p21 positive cells over 24 h following 5 Gy, plus or minus KGF pretreatment. These were subdivided into strongly and weakly labeled cells (as determined by eye). (B) strongly positive cells; (C) weakly labeled cells.

KGF appeared to exert no protective effect (**Fig 6**). Indeed, a slight increase in the number of apoptotic fragments per follicle cross-section was apparent at the 12 h time point. Although most fragments were found in the lower region (containing the germinal matrix), this slight KGF sensitization effect at 12 h was more pronounced in the more mature region higher up the follicle (lower region 23% increase; upper region 127% increase).

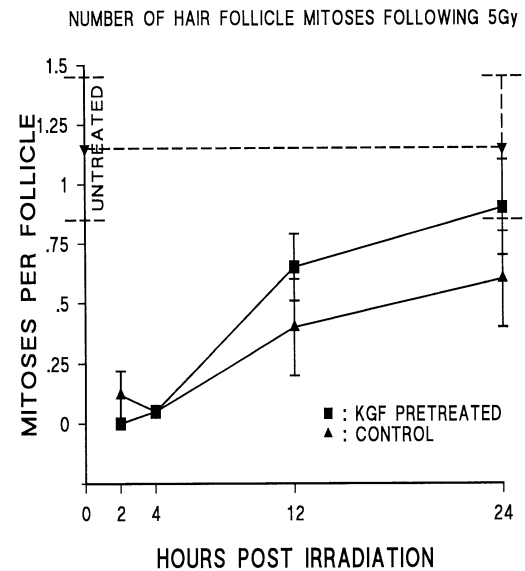


Figure 5. KGF pretreatment had no effect on the number of mitotic cells per follicle in the 24 h following exposure to 5 Gy radiation.

DISCUSSION

These results have brought together two previous observations that indicated first that KGF is a potent regulator of hair growth, and secondly is capable of protecting intestinal epithelial stem cells from chemotherapy and radiation induced death (Danilenko *et al*, 1995; Kahn *et al*, 1997; Farrell *et al*, 1998).

We have shown that increased numbers of hairs, and therefore the regenerative clonogenic cells, will survive lethal irradiation if pretreated with KGF. Pretreatment of telogen hairs with 12.5 μ g KGF resulted in an average of 2–3-fold more hairs growing during the first hair cycle following plucking, depending on radiation dose. Following regression in catagen significantly more hairs were again present in the second hair cycle, albeit at a lesser level of protection than in the first cycle. Inclusion of an extra dose of KGF (i.e., at 12 h intervals rather than 24 h over a 3 d period) did not improve the level of protection. Data from an experiment using a 10 \times higher dose of KGF indicated that the levels of protection can be further increased (6 \times more hairs surviving 15 Gy irradiation during the first hair cycle, 2.5 \times during the second). This level of protection could have a significant impact if replicated in the human scenario.

In both anagen and telogen treated follicles, the observed level of protection was less in the follicles than in the actual hair counts of the second cycle. This is probably due to errors inherent in follicle counting. In addition to counting surviving functional follicles, some sterile follicles, producing no hairs, will inevitably also be counted. Similarly any follicles containing multiple hairs may have their hair count reduced following irradiation, but such changes will not be detected at the level of the follicle. Alternative explanations could relate to the rate of keratinization or levels of hair retention. Despite these problems, however, which probably generate a less sensitive and less stringent assay than hair counts, 15%–25% more follicles were consistently present in the KGF treated animals compared with the controls. The only exception to this observation was in animals treated twice daily while in anagen. Here it appeared that KGF sensitized rather than protected the follicles. This was only significant at 15 Gy ($p < 0.05$), however. The explanation for this is unclear.

The fact that increased hair survival can be seen during the second hair cycle demonstrates that the factor is not simply modifying the rate of growth and differentiation of the hair (stimulating and therefore increasing the probability of the hair being longer and countable at an earlier stage). As it is the

clonogenic cells in the hair follicle that are responsible for regenerating the hair following this second plucking, this also suggests that KGF has enhanced the survival of this particular cell type. One possibility is that KGF administration may somehow increase the number of clonogenic cells per follicle prior to irradiation, thereby increasing both the growth rate of the follicle and the probability of at least one clonogenic cell per follicle surviving. Another contributing possibility is that KGF administration actually causes more clonogens to survive the plucking trauma, although this is not consistent with the observed protection of anagen follicles in which animals were not plucked until 24 d after the last KGF treatment. Whether KGF can increase hair survival by reducing fibrosis or modulating the dermal papilla directly also remains to be determined.

A slight stimulation of hair growth following irradiation might be suggested by the mitotic counts within the first 24 h, but this was not significant. This is not, however, a particularly sensitive assay of proliferation.

KGF pretreatment dramatically increased the expression of p21^{waf1} within the follicles. This protein is known to interrupt cell cycle progression by binding to the cdks and proliferating cell nuclear antigen (Xiong *et al*, 1992, 1993; Waga *et al*, 1994), allowing DNA repair to occur. p21 may therefore induce a more rapid onset or prolonged duration of cell cycle arrest. This may induce more efficient DNA repair and possibly a reduced level of apoptosis. A concomitant reduction in apoptosis within the follicle, however, was not observed. There may be a number of explanations for this. It is possible that the levels of p21 expression may have been insufficient to cause cell cycle arrest, and/or that the apoptosis observed is p53 independent (as observed in certain other tissues). This latter possibility will be addressed by repeating the investigation using p53^{-/-} mice.

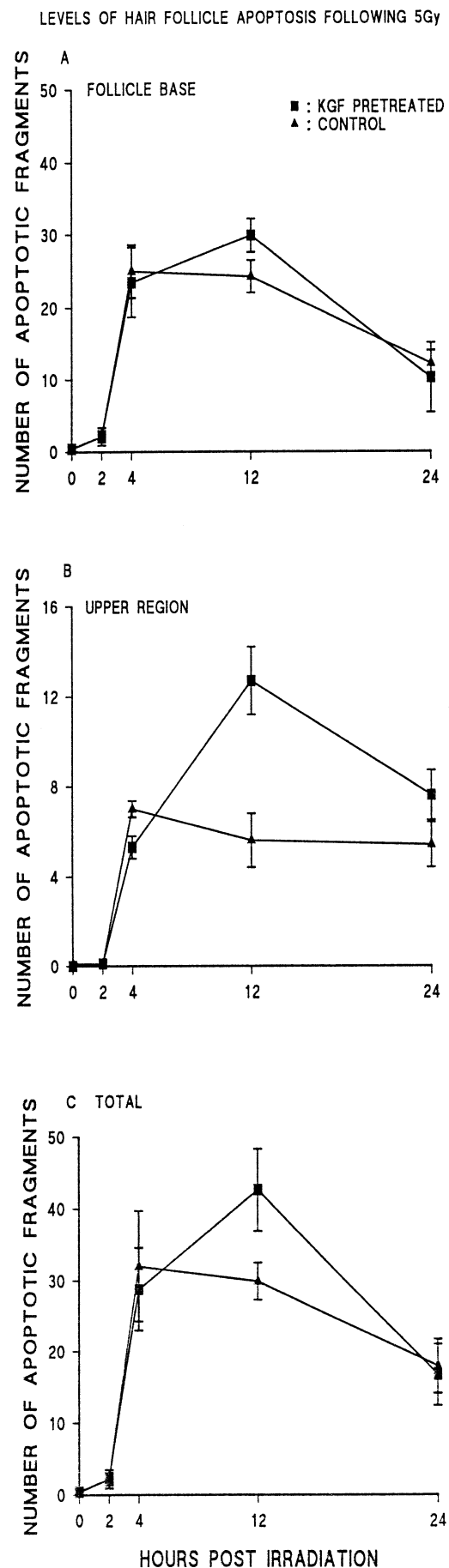
Another explanation may be the fact that it is not possible to count the absolute number of dying cells but only the number of fragments, and this may complicate the interpretation of the results. It has been demonstrated that following 5 Gy one cell can break up into six to seven fragments (Potten, 1985; Geng and Potten, 1990). KGF may alter the fragmentation or fragment engulfment kinetics and lead to an apparent change in the number of dying cells within the follicle.

Finally it must, of course, be borne in mind that the number of apoptotic fragments measured provides no information about the sensitivity of cells in the proliferative hierarchy within the follicle. Ultimately it is only the apoptotic sensitivity of the clonogenic cells that is relevant.

In conclusion KGF, or a synthetic analog, may have the clinical potential for reducing chemotherapy and/or radiation induced alopecia. The next step will be to investigate whether KGF can protect hairs from chemotherapy drug damage, whether KGF is effective when applied topically, and whether KGF can protect human hair follicles using skin xenografted on to immunocompromised mice.

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Figure 6. KGF treatment induced a significant increase in the number of apoptotic fragment 12 h post-irradiation (5 Gy), but at no other time point during the first 24 h post-irradiation. (A) Number of apoptotic fragments from follicle base to midline. (B) Number of fragments in the upper region (an area of equal size to that in part (A) but from the midline upwards). (C) Total number of apoptotic fragments per follicle.



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