

# Keratinocyte growth factor induces angiogenesis and protects endothelial barrier function

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## SUMMARY

**Keratinocyte growth factor (KGF), also called fibroblast growth factor-7, is widely known as a paracrine growth and differentiation factor that is produced by mesenchymal cells and has been thought to act specifically on epithelial cells. Here it is shown to affect a new cell type, the microvascular endothelial cell. At subnanomolar concentrations KGF induced in vivo neovascularization in the rat cornea. In vitro it was not effective against endothelial cells cultured from large vessels, but did act directly on those cultured from small vessels, inducing chemotaxis with an ED<sub>50</sub> of 0.02-0.05 ng/ml, stimulating proliferation and activating mitogen activated protein**

**kinase (MAPK). KGF also helped to maintain the barrier function of monolayers of capillary but not aortic endothelial cells, protecting against hydrogen peroxide and vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) induced increases in permeability with an ED<sub>50</sub> of 0.2-0.5 ng/ml. These newfound abilities of KGF to induce angiogenesis and to stabilize endothelial barriers suggest that it functions in microvascular tissue as it does in epithelial tissues to protect them against mild insults and to speed their repair after major damage.**

Key words: KGF, Angiogenesis, Vascular permeability

## INTRODUCTION

Keratinocyte growth factor (KGF; also called FGF-7; Aaronson et al., 1991) is a member of the large and still expanding fibroblast growth factor (FGF) family (Basilico and Moscatelli, 1992). KGF is distinguished from most other FGF family members by its strict paracrine mode of action. Produced predominantly by stromal cells, KGF acts specifically on epithelial cells, the only cell type in which the distinctly spliced receptor isoform, FGF-R2 IIIb, has been observed (Miki et al., 1992; Winkles et al., 1997). Within the FGF family, KGF most closely resembles FGF-10, sometimes termed KGF-2 (Emoto et al., 1997; Igarashi et al., 1998). KGF and FGF-10 both bind to the KGF receptor, stimulate keratinocytes but not fibroblasts, and hasten wound healing, but they have distinct expression patterns (Hattori et al., 1997; Ohuchi et al., 1997) and activity spectra as assessed in null mice (Guo et al., 1996; Min et al., 1998; Sekine et al., 1999). Whereas KGF is freely soluble when secreted and its activity is sensitive to heparin (Reich-Slotky et al., 1994; Ron et al., 1993), secreted FGF-10 associates with cells and/or the extracellular matrix and its activity is heparin independent (Igarashi et al., 1998).

In vitro KGF is a potent mitogen for a wide range of

epithelial derived cells and acts as an important mesenchymal mediator of their growth, differentiation and morphogenesis. KGF expression is often linked to tissue expansion and wound repair. Targeted overexpression of KGF in vivo leads to hyperplasia of epithelial tissues in a variety of organs (Guo et al., 1993; Kitsberg and Leder, 1996; Nguyen et al., 1996; Simonet et al., 1995). KGF expression is dramatically upregulated in cutaneous wounds (Werner et al., 1992) where it can speed the repair process (Pierce et al., 1994) and also in the hyperplastic skin disease psoriasis (Finch et al., 1997). KGF also stimulates the growth of hair (Danilenko et al., 1995) and the thickening of the gastrointestinal tract (Playford et al., 1998). In a variety of reproductive tissues circulating hormones regulate KGF expression, which often increases during periods of regulated or excessive cell growth (Koji et al., 1994; Pedchenko and Imagawa, 1998).

Angiogenesis, the growth of new capillaries from preexisting blood vessels, is essential to support the nutritional demands of tissues that are expanding or being repaired (Folkman, 1998). It occurs transiently during wound healing, in cycling reproductive organs and during anagen, the proliferative phase of hair growth (Detmar, 1996; Folkman and Shing, 1992; Iruela-Arispe and Dvorak, 1997). Persistent angiogenesis is essential for a myriad of pathological

conditions ranging from tumor growth to psoriasis (Folkman, 1995; Hanahan and Folkman, 1996). The observation that five other members of the FGF family have as one of their activities the ability to stimulate new blood vessel growth (Costa et al., 1994; Deroanne et al., 1997; Giordano et al., 1996; Yoshida et al., 1994), and the recognition that many KGF-linked processes are also angiogenesis-dependent, raises the possibility that KGF might also be an inducer of neovascularization. However, unlike most angiogenic factors, KGF is not mitogenic for endothelial cells, at least those cultured from large vessels (Rubin et al., 1989). No evidence for the expression of the KGF receptor has been found in cultured large vessel cells or in whole arteries (Rubin et al., 1989; Winkles et al., 1997). Here we examine the possibility that KGF may act exclusively on endothelial cells of the small vessels that are the source of angiogenic responses *in vivo* and show that KGF is indeed a potent direct-acting stimulator of angiogenesis.

KGF is also well known for its ability to protect the integrity of epithelial tissues that form essential barriers between the body and the environment. In the lung (Panos et al., 1995; Yano et al., 1996; Yi et al., 1996) and the gastrointestinal tract (Farrell et al., 1998) KGF ameliorates damage caused by a variety of toxic insults. Its protective activity in the lung may be due in part to its ability to maintain the barrier function of epithelial cells for it protects sheets of cultured cells against disruption by chemical or radiation injury (Savla and Waters, 1998; Waters et al., 1997), via a PKC and tyrosine kinase dependent process that is independent of its mitogenic activity.

The endothelial cells of the microvasculature also form an essential physiological barrier, but one that is more easily modulated by natural processes. The permeability of endothelial cells to large molecules can be increased by exposure to a variety of physiological agents like H<sub>2</sub>O<sub>2</sub> (Carbajal and Schaeffer, 1998), low calcium (Alexander et al., 1998), thrombin (Buchan and Martin, 1992), or vascular endothelial cell growth factor/vascular permeability factor (VEGF/VPF), an angiogenic factor that was first described as an *in vivo* inducer of vascular leakage (Senger et al., 1983). The purified VEGF/VPF induces increased vascular permeability *in vivo* (Keck et al., 1989) and in endothelial cell monolayers *in vitro* (Hippenstiel et al., 1998). Although inflammatory agonists may increase microvascular permeability by stimulating endothelial cell contraction (Morel et al., 1990; Shasby et al., 1982), it is unclear whether vascular leakage in response to VEGF/VPF occurs in a paracellular or intercellular fashion (Esser et al., 1998a,b). In our hands, KGF protected the barrier function of microvascular endothelial cell monolayers against disruption by either physiological VEGF/VPF or toxic H<sub>2</sub>O<sub>2</sub>. The direct action of KGF on endothelial cells and epithelial cells could provide a mechanism for coordinating the responses of these two cell types, both of which are mobilized to support tissue growth and repair.

## MATERIALS AND METHODS

### Reagents

Recombinant human KGF was kindly supplied by Amgen (Thousand Oaks, CA). Recombinant human aFGF, bFGF, VEGF and neutralizing antibodies targeted against human KGF and human bFGF were

purchased from R&D Systems (Minneapolis, MN). Antibodies were dialyzed extensively against PBS in Centricon microconcentrators (Amicon, Beverly, MA) prior to use. Heparin (sodium salt) isolated from porcine intestinal mucosa was obtained from Sigma (St Louis, MO).

### Rat cornea neovascularization assay

Female Fischer 344 rats (Harlan Industries, Indianapolis, IN), 120-140 grams body weight, were housed in the Northwestern University Center for Experimental Animal Resources. Research protocols were approved by the Institutional Animal Care and Use Committee and conducted in accordance with federal guidelines. The assay was performed as previously described (Polverini et al., 1991). Ethanol-based hydron solution (12%; Interferon Sciences, New Brunswick, NJ) was mixed with test samples and air-dried to form pellets of <5 µl that were implanted into the avascular corneal stroma of anesthetized rats, 1.0-1.5 mm from the limbus. After 7 days, vessels were perfused with colloidal carbon and corneal responses scored as positive when a vigorous ingrowth of vessels was seen sprouting from the limbus toward the pellet.

### Endothelial cell migration assay

Bovine adrenal capillary endothelial cells (generously provided by Dr J. Folkman, Children's Hospital, Boston, MA) were grown at 37°C in 7.5% CO<sub>2</sub> in DME containing 10% donor calf serum (Flow Laboratories, McLean, VA) and 100 µg/ml endothelial cell mitogen (Biomedical Technologies, Inc., Stoughton, MA) and used at passages 13 to 15. Human umbilical vein endothelial cells (HUVEC, Cell Systems, Seattle, WA) were cultured in DME, 10% fetal bovine serum and used prior to p15. Human dermal microvascular endothelial cells (HMVECs; Cell Systems, Seattle, WA) were grown at 37°C with 10% fetal calf serum (Gibco, Bethesda, MD) and the EGM-MV bullet kit (Clonetics Corp., San Diego, CA) and used at passages 6 to 8. Both cell strains were grown on culture dishes coated overnight with 0.1% gelatin (Difco Laboratories, Detroit, MI). Bovine aortic endothelial cells (BAEC) were isolated and cultured as previously described (Rastinejad et al., 1989) and used prior to passage 15.

Migration was assayed as previously described (Polverini et al., 1991). Confluent cells were serum deprived overnight in medium containing 0.1% BSA, harvested, washed, resuspended at approximately 8×10<sup>5</sup> cells/ml in this medium. 30 µl of cells were allowed to adhere to the lower surface of gelatinized polycarbonate membranes containing 4 ×10<sup>5</sup> uniform 5.0 µm pores per cm<sup>2</sup> (Nucleopore Corporation, Pleasanton, CA) in inverted, modified Boyden chambers. Cell attachment proceeded 1.0-1.5 hours prior to reinverting the chambers and adding test substances in control media to the opposite side of the chamber. Chambers were further incubated at 37°C for 3-4 hours. Membranes were removed from the chambers, fixed, stained (Diff-Quik, Baxter, McGaw Park, IL) and the number of cells that migrated to the top side of the membrane in 10 high powered (h. p.) fields was determined in quadruplicate for each test condition. In this assay, cells that attach near pores are more likely to migrate to the top of the chamber than cells that adhere further from pores. Typically 5-10% of plated cells are found to migrate across the membrane in the presence of an inducing agent. To compare experiments performed on different days, data was converted to percent maximum migration after subtracting background migration towards control media. In this case, background migration was set at 0% and positive control bFGF induced migration was normalized to 100%. For statistical analysis the student's *t*-test was applied to raw data where *n*≥4.

The directional component of KGF induced migration was measured using a checkerboard analysis. In this instance, cells were plated in the upper compartment of the Boyden chamber. Increasing concentrations of KGF were added in both the upper and lower compartments of the chamber in a checkerboard pattern to establish positive, null and negative concentration gradients across the

gelatinized membrane and cells were allowed to migrate for 4-5 hours. The number of cells migrating in 10 h. p. fields of six replicate wells was determined for each condition.

### Endothelial cell proliferation assay

Bovine capillary cells were plated at 10,000 cells/ml on gelatinized culture plates and allowed to adhere 24 hours. Cells were serum deprived overnight and the following day control cells were harvested to obtain a plating number. Experimental plates received 2% donor calf serum with or without growth factor or 5% donor calf serum daily for six consecutive days at which point the cells were harvested and counted. Each condition was determined in quadruplicate and the results are representative of separate experiments.

### Protection from peroxide or VEGF/VPF induced permeability

The permeability of bovine capillary or aortic endothelial cell monolayers to fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA) was evaluated by measuring the flux of tracer molecules across the monolayer using a transwell chamber (Casnocha et al., 1989). Prior to the experiments, cells were rinsed with Hanks' balanced salt solution (HBSS) supplemented with 0.5% BSA and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4. FITC-BSA (0.5%) in HBSS was added to the top well and HBSS with unlabelled BSA was added to the lower well. The plates were placed in a heated shaker with gentle rocking to prevent the formation of an unstirred boundary layer. Samples (5  $\mu$ l) were taken from the top and bottom chambers at 15, 60, 90, 165, and 255 minutes, diluted into 95  $\mu$ l of HBSS in a 96-well plate, and FITC-BSA (490 nm) concentrations measured using a plate reader spectrophotometer. Assuming the flux was due to diffusional transport, the permeability coefficient was estimated as previously described (Casnocha et al., 1989). The permeability of the membrane alone was evaluated in each experiment so that the contribution of the cell monolayer permeability could be separated from that of the membrane permeability. For experiments involving KGF, the medium in both the top and bottom chambers was replaced with medium containing KGF at 25 ng/ml, unless otherwise indicated, and 5% FBS. Studies in our lab have demonstrated that KGF is ineffective in this assay in medium without serum. The medium in all other wells was simultaneously replaced with medium containing 5% FBS.

### MAPK activity assay

Prior to induction, bovine capillary cells were serum deprived overnight in media containing 0.1% BSA. Cells were stimulated for 10 minutes at 37°C with fresh medium supplemented with serum or KGF and then lysed in ice-cold extraction buffer (150 mM NaCl, 20 mM Tris-Cl, pH 7.5, 5 mM EDTA, 0.5% Triton, 1% deoxycholic acid, 0.5 mM DTT, 50 mM  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM PMSF, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin; all from Sigma, St Louis, MO) for 15 minutes. MAPK was specifically immunoprecipitated using ERK 2 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Protein A-Sepharose beads (Pharmacia, Kalamazoo, MI). Immunoprecipitates were washed extensively in extraction buffer, then kinase buffer (50 mM Hepes, pH

7.5, 10 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{Na}_3\text{VO}_4$ , 12.5 mM  $\beta$ -glycerophosphate) and resuspended in 30  $\mu$ l kinase buffer containing 10  $\mu$ g myelin basic protein (MBP; Sigma, St Louis, MO) and 0.5  $\mu$ Ci [ $\gamma$ - $^{32}\text{P}$ ]ATP (ICN Biomedicals, Costa Mesa, CA). Reactions proceeded for 30 minutes at 30°C and were terminated by adding sample buffer and boiling for 5 minutes. Phosphorylated proteins were resolved by SDS-PAGE, visualized by autoradiography and quantified by scanning densitometry.

## RESULTS

### KGF induced corneal neovascularization

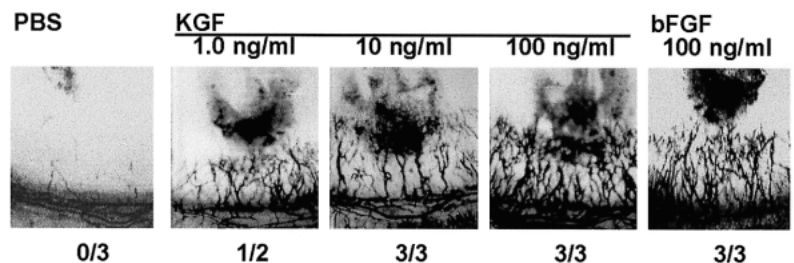
Samples of recombinant human KGF were implanted in the avascular rat cornea and angiogenic responses were assessed following the perfusion of vessels with colloidal carbon seven days later. Control implants yielded no positive responses, while pellets containing as little as 10 ng/ml of KGF consistently induced a robust angiogenic response (Fig. 1). Implants of 100 ng/ml KGF induced an even more vigorous ingrowth of limbal vessels, resembling the response to pellets containing 100 ng/ml of the classic angiogenic agent, bFGF.

### KGF directly stimulated capillary endothelial cell proliferation and migration

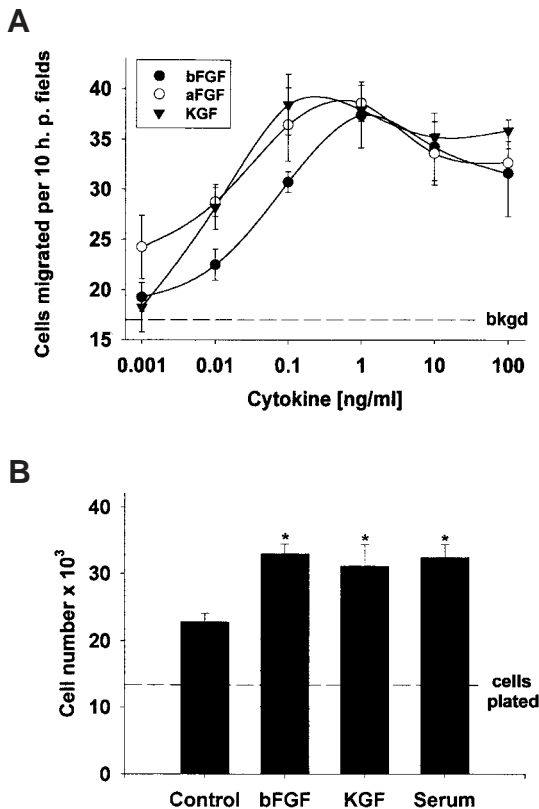
To determine if KGF was acting directly on endothelial cells that form new vessels, adrenal cortex derived capillary endothelial cells were examined for their ability to migrate towards KGF in a modified Boyden chamber. KGF stimulated endothelial cell migration through microporous gelatinized membranes in a dose-dependent manner with an  $\text{ED}_{50}$  of 0.05 ng/ml (2.5 pM) and a maximum at approximately 1-10 ng/ml (Fig. 2A). The slope of the KGF dose response curve and the magnitude of the response closely resembled the responses generated by angiogenic family members, aFGF and bFGF. Higher concentrations of all three factors did not induce additional cell migration. Structurally similar FGF-10 also stimulated the migration of bovine capillary endothelial cells with an  $\text{ED}_{50}$  of 0.05 ng/ml, approximately equal to that of KGF (data not shown).

Many although not all angiogenic factors induce the proliferation of endothelial cells (Bouck et al., 1996; Davis et al., 1996). KGF enhanced the proliferation of bovine capillary endothelial cells when it was used to supplement low serum containing media (Fig. 2B). After six days, KGF treated cells significantly outnumbered control cells and had expanded in numbers comparable to cells stimulated with bFGF or with 5% serum.

The migration stimulated by KGF was due to chemotaxis, directed movement up a concentration gradient and not simply chemokinesis. A checkerboard analysis showed that cells



**Fig. 1.** KGF stimulation of neovascularization. Slow release Hydron pellets containing the indicated concentrations of growth factors or PBS were implanted in the avascular cornea of the rat. Vigorous ingrowth of vessels towards the pellet at 7 days was scored a positive response. The number of positive responses/total corneas implanted is indicated below representative photos.



**Fig. 2.** KGF stimulation of capillary endothelial cell migration and proliferation. (A) Bovine capillary endothelial cells were allowed to migrate toward increasing concentrations of bFGF (●), aFGF (○) or KGF (▼). The background migration toward media containing 0.1% BSA (bkgd) is indicated. (B) Bovine capillary endothelial cells were fed control media in the absence or presence of 25 ng/ml KGF or 25 ng/ml bFGF, or 5% serum every day and cell growth measured after six days. \*A significant difference from control treated cells ( $P < 0.01$ ).

migrated above background levels only when moving up a concentration gradient toward KGF (Table 1).

To determine whether KGF-induced migration was a general phenomenon exhibited by all vascular endothelial cells or specific to a sub-population of cells, additional cell strains were examined for KGF sensitivity. KGF induced the migration of human dermal microvascular endothelial cells (Fig. 3A), but it was ineffective when used on cells cultured from large vessels including human umbilical vein endothelial cells and bovine aortic endothelial cells, although both cells migrated well towards bFGF (Fig. 3A) and VEGF (data not shown).

The observed activity was due to KGF and not a contaminant of the preparation because it was modulated by known, specific inhibitors of KGF. Migration toward KGF was sensitive to a KGF monoclonal antibody but resistant to one raised against bFGF (Fig. 3B). Neither antibody affected migration when tested alone or was able to neutralize the inappropriate growth factor. Heparin and heparan sulfate proteoglycans bind FGF family members directly and can modify their effects (Aviezer et al., 1994; Spivak-Kroizman et al., 1994), potentiating bFGF induced cell proliferation and blocking KGF mitogenic activity

**Table 1. KGF stimulation of chemotaxis**

	Control media	Upper compartment [KGF]		
		0.10 pg/ml	10 pg/ml	1000 pg/ml
Control media	23±1.7	26±2.2	29±1.9	25±1.7
0.10 pg/ml KGF	23±1.7	23±1.3	28±2.0	24±2.1
10 pg/ml KGF	30±2.8	27±2.1	27±2.4	26±1.3
1000 pg/ml KGF	41±1.5	39±1.4	37±2.4	28±1.5

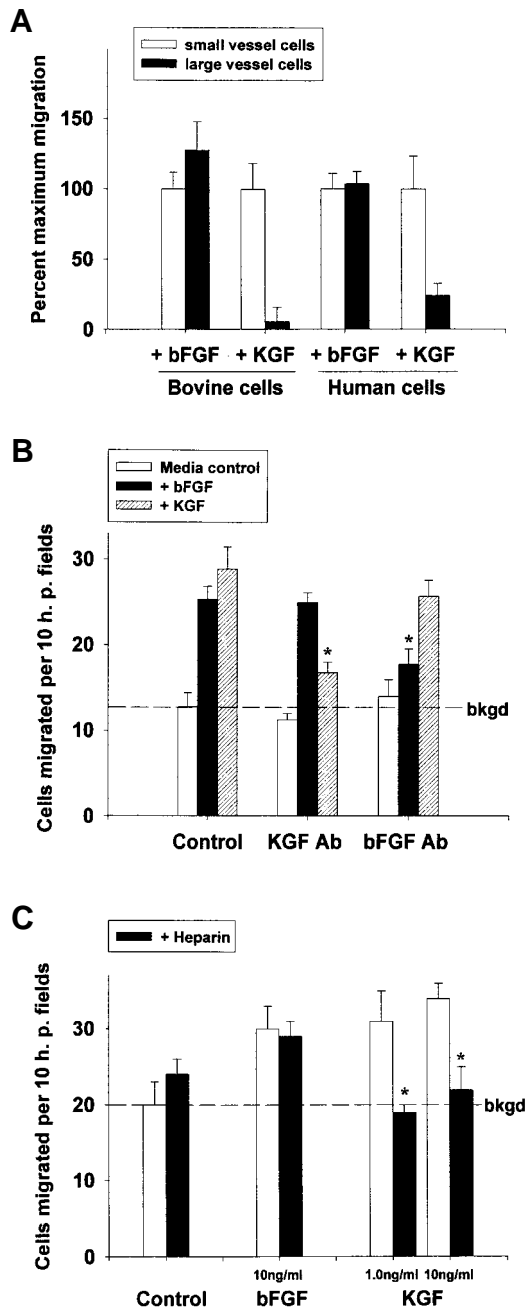
Checkerboard analysis of bovine capillary cells migrating from the upper compartment to the lower compartment of a Boyden chamber in the presence of a negative (above diagonals), positive (below diagonals) or neutral (between diagonals) gradient of KGF. Results are expressed as the mean number of migrated cells per 10 high powered (h. p.) fields ( $\pm$  the standard error;  $n=6$ ).

on epithelial cells (Miao et al., 1997; Ornitz et al., 1995; Reich-Slotky et al., 1994). Heparin also prevented KGF from inducing the migration of capillary endothelial cells although it had no inhibitory effect on bFGF induced migration or when tested alone (Fig. 3C).

### KGF stabilized the barrier function of capillary endothelial cells

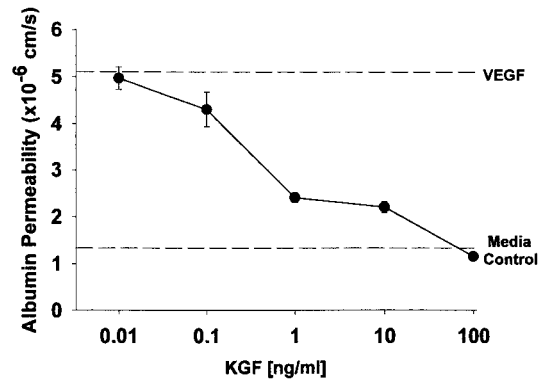
One of the many effects of KGF on epithelial cells is to help maintain barrier function in the face of injury in vivo (Farrell et al., 1998; Panos et al., 1995). In vitro this activity is reflected in its ability to block hydrogen peroxide induced permeability increases in epithelial cell monolayers (Waters et al., 1997). The endothelial cells of the vasculature also exhibit vital barrier functions in vivo that can be measured in vitro (Ferrero et al., 1995).

To determine whether KGF had a protective effect on endothelial cell barrier activity, cell monolayers were treated for 24 hours with increasing concentrations of KGF, and their permeability to albumin was measured in the presence of VEGF/VPF, a cytokine that induces increased permeability in endothelial cells in vivo and in vitro (Esser et al., 1998b; Hippenstiel et al., 1998; Keck et al., 1989). The permeability of resting capillary endothelial cell monolayers increased in a dose dependent manner in response to VEGF/VPF with maximum permeability reached at 10 ng/ml (data not shown), a concentration chosen for further study. Under similar experimental conditions, VEGF/VPF treated capillary cells form the cytoplasmic structures commonly associated with transcellular flux of plasma proteins (Esser et al., 1998b). KGF effectively antagonized the VEGF/VPF increase in vascular permeability with an ED<sub>50</sub> of 0.25 ng/ml (12.5 pM) and maximum protective effect achieved at approximately 25-100 ng/ml (Fig. 4). Treating endothelial cells for 24 hours with 25 ng/ml KGF had no effect on the basal level of capillary or aortic endothelial cell permeability (see Fig. 5). Barrier protection was not a characteristic of all FGF family members because bFGF, a potent stimulator of endothelial cell migration and mitogenesis, was unable to block VEGF/VPF induced



**Fig. 3.** Sensitivity of KGF induced migration to endothelial cell type, anti-KGF antibodies and heparin. (A) Bovine cells cultured from small vessels of the adrenal gland or from the large aorta, and human cells cultured from dermal capillaries or from the large umbilical vein were allowed to migrate towards 10 ng/ml of bFGF or KGF. (B) Bovine capillary endothelial cells were allowed to migrate toward 10 ng/ml bFGF (filled bars) or 10 ng/ml KGF (hatched bars) in the absence or presence of neutralizing antibodies against bFGF or KGF (each used at 5  $\mu$ g/ml). \*A significant difference from positive controls ( $P < 0.02$ ). (C) Migration towards bFGF or KGF in the absence (open bars) or presence (filled bars) of 20  $\mu$ g/ml heparin. Control shows migration towards heparin alone. \*A significant difference from KGF without heparin ( $P < 0.03$ ). Standard errors are shown.

vascular permeability (data not shown). Despite their opposing effects upon vascular permeability, both KGF and VEGF/VPF



**Fig. 4.** KGF antagonism of VEGF-induced increases in vascular permeability. Bovine capillary cells were pretreated for 24 hours with the indicated concentrations of KGF and their permeability to FITC-conjugated albumin was measured in the presence of 10 ng/ml VEGF. Media control indicates permeability without treatment. VEGF permeability was measured in the presence of VEGF alone.

are potent stimulators of angiogenesis. When tested together in an endothelial cell migration assay KGF and VEGF/VPF effects were additive, not antagonistic (data not shown).

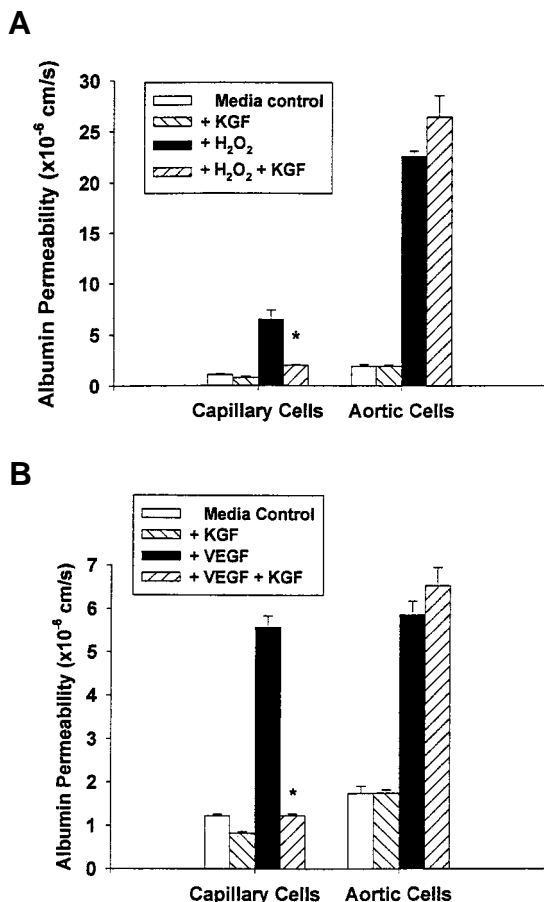
KGF was also able to protect endothelial cell monolayers from disruption by 0.5 mM  $H_2O_2$  (Fig. 5A), a concentration known to induce actin reorganization and membrane blebbing in large vessel cells (Huot et al., 1998). As was the case in the angiogenesis assays, KGF was effective only against cells cultured from the microvasculature and was unable to provide significant protection to monolayers of aortic cells against either  $H_2O_2$  or VEGF (Fig. 5A,B).

### KGF activated MAPK

Endothelial cells are not known to have epithelial-type KGF receptors, although most efforts to identify them have used large vessel cells which also do not respond to KGF in barrier retention or angiogenesis assays. When we investigated microvascular cells, no classical KGF receptor was detected in crosslinking experiments using bovine capillary endothelial cells (kindly performed in the laboratory of J. Rubin). Northern blots designed to specifically detect message for the KGF receptor gave a strong signal using mRNA from epithelial cells of the human lung, but did not reveal a similar band in bovine capillary or aortic cell strains (data not shown). Similarly, when primers designed to amplify specifically the KGF receptor, FGF-R2 IIIb, were used (Parrott and Skinner, 1998), they amplified the expected size RT-PCR fragment from two keratinocyte cell strains, but not from the microvascular endothelial cells shown here to respond to KGF. Control RT-PCR amplification of actin fragments proceeded well in all samples tested (data not shown).

In an effort to demonstrate that KGF did transduce signals within capillary endothelial cells despite the absence or low levels of the classical, epithelial-type KGF receptor, MAPK activity was investigated. When serum deprived endothelial cells were treated for 10 minutes with 20 ng/ml KGF, activation of MAPK was nearly equivalent to MAPK induction in cells treated with 20% serum (Fig. 6). No additional MAPK activation was observed when KGF was tested at



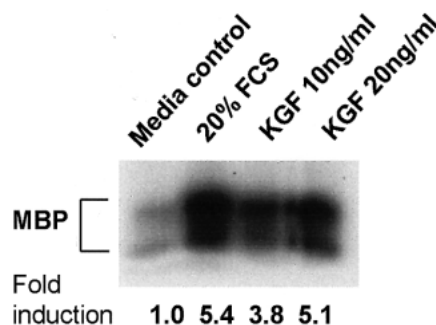


**Fig. 5.** Sensitivity of KGF barrier protection to endothelial cell type. (A) Bovine capillary or aortic endothelial cells were left untreated (media control) or were pretreated for 24 hours with 25 ng/ml KGF and their permeability to FITC-conjugated albumin was measured in the presence or absence of 0.5 mM H<sub>2</sub>O<sub>2</sub>. \*A significant difference from H<sub>2</sub>O<sub>2</sub> treated cells without KGF ( $P < 0.003$ ). (B) Bovine capillary or aortic endothelial cells were left untreated (media control) or were pretreated for 24 hours with 25 ng/ml KGF and their permeability to FITC-conjugated albumin was measured in the presence or absence of 10 ng/ml VEGF. \*A significant difference from VEGF treated cells without KGF ( $P < 0.01$ ).

concentrations as high as 100 ng/ml or when capillary endothelial cells were separately treated with 20 ng/ml bFGF (data not shown).

## DISCUSSION

At low nM concentrations previously shown to induce mitogenesis in keratinocytes, KGF proved to be a potent inducer of angiogenesis. It both activated capillary endothelial cells in vitro and induced neovascularization in vivo, identifying it as a direct acting angiogenic factor and providing the first evidence that this cytokine can target non-epithelial cells. KGF is the sixth FGF family member to exhibit angiogenic activity, but the only one whose effects on the vascular endothelium are restricted to capillary cells. This restriction explains the previously observed failure of KGF to



**Fig. 6.** KGF induction of MAPK activity in capillary endothelial cells. Bovine capillary endothelial cells were treated for 10 minutes with serum or with KGF prior to the generation of cell extracts and addition of ERK2 antibody. The ability of immunoprecipitated MAPK to phosphorylate myelin basic protein (MBP) was measured. Fold induction of MAPK activity is quantified below the gel autoradiogram.

promote mitogenesis in cells from large vessels (Rubin et al., 1989; Winkles et al., 1997). Structurally similar FGF-10 shown here to induce capillary endothelial cell migration may also be an angiogenic factor. If borne out, this new function would raise the intriguing possibility that some of the embryonic defects seen in FGF-10 deletion mutants (Min et al., 1998; Sekine et al., 1999) are the result of improper signaling within the developing vasculature.

In keratinocytes, KGF induces mitogenesis by binding to a cell surface tyrosine kinase receptor, FGF-R2 IIIb, that induces phospholipase C- $\gamma$  signaling and MAPK activation (Ishii et al., 1995; Liang et al., 1998; Shaoul et al., 1995). It is unclear whether or not FGF-R2 IIIb also mediates the effects of KGF on microvascular endothelial cells. Heparin binding growth factors of the FGF family can enter cells and influence gene expression in the absence of high affinity receptors via lower affinity interactions with cell surface heparan sulfates (Quarto and Amalric, 1994; Roghani and Moscatelli, 1992; Rusnati et al., 1993). However, the stimulation of mitogenesis, such as seen here in endothelial cells, has so far been associated only with interaction of ligand with high affinity receptors. The fact that nanomolar concentrations of KGF were sufficient to induce MAPK activation and biological responses in capillary endothelial cells also suggests that a high affinity receptor is involved. Some of our observations indicate that the receptor for KGF on endothelial cells may be similar to the one on epithelial cells. The activities induced by KGF in endothelial cells (proliferation, chemotaxis and barrier maintenance) mimicked KGF-induced activities observed in epithelial cells and KGF activity on both cell types was sensitive to heparin.

Despite these similarities, the FGF-R2 IIIb isoform specific for KGF interactions in epithelial cells was not detectable in reactive endothelial cells when examined by crosslinking experiments, northern blot, or RT-PCR. It is possible that microvascular endothelial cells possess very low levels of FGF-R2 IIIb. Alternatively, KGF may act on endothelial cells via a different, yet unidentified splice variant of the family of FGF receptor genes. This possibility is supported by the surprisingly vigorous and rapid MAPK activation induced by KGF in capillary endothelial cells where KGF was as effective as bFGF. This would not be expected if KGF was acting through

its typical epithelial cell receptor because in cells expressing the cloned FGF-R2 IIIb, KGF activation of MAPK is quite weak compared to that stimulated by the interaction of bFGF with its transfected receptor (Shaoul et al., 1995).

Although KGF clearly can stimulate angiogenesis by acting directly on endothelial cells, it can also act indirectly by inducing epithelial cells to synthesize and release a variety of pro-angiogenic molecules. KGF can enhance epithelial cell secretion of pro-angiogenic proteases including uPA, MMP-9 and MMP-13 (Putnins et al., 1995; Sato et al., 1995; Uitto et al., 1998), potentially making the matrix a more favorable environment for a sustained angiogenic response (Brown et al., 1997). Like many other angiogenic factors, KGF also induces keratinocytes to secrete the angiogenic factor VEGF (Frank et al., 1995). The ability of KGF to protect the endothelial cell barrier function against VEGF/VPF disruption suggests that it may act in vivo to counteract the vascular permeability enhancing features of VEGF without diminishing the angiogenic response. The co-expression of KGF and VEGF/VPF by fibroblasts in a simulated wound environment supports such a possibility (Iyer et al., 1999).

Efforts to understand the ability of KGF to protect lung tissue from chemical and radiation damage and to ameliorate the accompanying edema have focused on epithelial cells known to be sensitive to KGF (Guery et al., 1997; Guo et al., 1998; Panos et al., 1995; Savla and Waters, 1998; Waters et al., 1997; Yi et al., 1996, 1998). The demonstration that capillary cells are also stimulated by KGF and that this cytokine maintains the endothelial barrier function suggests that the vasculature may also be a crucial target of KGF in protection against the pulmonary edema that is often attributable to small vessel leakage (Hurley, 1982).

Viewing KGF as directly affecting endothelial cells in addition to its documented activities on epithelial cells makes it possible to reinterpret several observations in the literature. The high levels of KGF that are secreted by cells in the dermis of psoriatic skin have been presumed to stimulate the growth of basal and suprabasal cells beyond the basement membrane (Finch et al., 1997). One must now consider the possibility that in addition to stimulating the epithelial cells, KGF induces the dermal angiogenesis that accompanies psoriasis, contributing to its progressive pathology. Prostate tumor cells expressing the KGF receptor grow more slowly in vivo whether or not the receptor is kinase active (Feng et al., 1997). This finding can be explained if this inactive receptor is sequestering angiogenic KGF and thereby reducing the vigor of the angiogenic response upon which the growth of all tumors depends. Recent evidence suggests that the mesenchymal expression of angiogenic activity may be essential for tumor progression (Fukumura et al., 1998) and fibroblast-produced KGF factor is well positioned to contribute to such angiogenic activity.

In addition, there are a number of examples where KGF may be contributing both to epithelial growth and differentiation and to the angiogenesis that accompanies and supports it, such as in wound healing (Pierce et al., 1994; Werner et al., 1992), the maturation of the ovarian follicle (Parrott and Skinner, 1998), in the luteal phase of the menstrual cycle (Koji et al., 1994), in normal hair growth (Danilenko et al., 1995), and in hyperplastic abnormalities and the growth of tumors (Finch et al., 1997; Kitsberg and Leder, 1996). The next challenge will be to dissect this matrix of KGF functions and evaluate their

individual contributions to the many physiological and pathological processes with which KGF has already been associated.

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