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Basic fibroblast growth factor: Lysine 134 is essential for its neuroprotective activity

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Abstract

Basic fibroblast growth factor (bFGF) is a heparin-binding growth factor known to cause cell proliferation, angiogenesis and neuroprotection. We have performed site-directed mutagenesis to identify the amino acids that are essential for heparin/growth factor interaction and for neuroprotection. Binding to heparin-acrylic beads was markedly reduced when lysine in position 134 of bFGF was replaced by alanine. Wildtype (wt)-bFGF was shown to protect rat primary cultures of embryonic hippocampal neurons against damage caused by staurosporine and to reduce the infarct size in mice after focal cerebral ischemia. These neuroprotective effects of wt-bFGF could not be shown for the mutant bFGF(K134A). Furthermore, phosphorylation of Akt and ERK1/2 was significantly reduced in cultured neurons treated with bFGF(K134A) indicating diminished intracellular signaling compared to neurons treated with wt-bFGF. In conclusion, lysine at position 134 of bFGF is essential for bFGF to bind heparin, then to interact with its receptor and, subsequently, to protect neurons against damage.

Keywords: bFGF; Growth factor; Neuroprotection; Site-directed mutagenesis

Various proteins require binding of heparin for activity, e.g. most of fibroblast growth factors (Klagsbrun and Shing, 1985), VEGF (Keyt et al., 1996), human fibronectin (Ingham et al., 1990) and aspartic acid protease BACE1 (Beckman et al., 2006). An essential role of heparin binding was also demonstrated in context with acidic and basic fibroblast growth factors. It has been shown that heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation (Spivak-Kroizman et al., 1994; Venkataraman et al., 1996).

Basic fibroblast growth factor (bFGF) is an important modulator of cell growth and differentiation under both physiological and pathological conditions. Five isoforms of bFGF can be distinguished and are translated. Four high molecular weight forms (22, 22.5, 24 and 34 kDa) are predominantly located in the cell nucleus while the low molecular weight 18 kDa protein is found in the cytosol (Florkiewicz and Sommer, 1989; Arnaud et al., 1999; Delrieu, 2000; for review see Sorensen et al., 2006). bFGF is exclusively composed of antiparallel β-strands. The continuous β-meander forms a barrel closed by the amino- and carboxyl-terminal strands (Zhang et al., 1991). The surface of the molecule is rich in positively charged amino acids, particularly arginine and lysine. This contributes to the unusually high isoelectric point of 9.6. Domains and sites for bFGF-heparin binding (Moy et al., 1997; Thompson et al., 1994), bFGF dimerization (Facchiano et al., 2003; Herr et al., 1997), bFGF receptor binding (Facchiano et al., 2003; Springer et al., 1994; Reich-Slotky et al., 1995) and phosphorylation of serine and threonine residues by protein kinase A and protein kinase C (Feige et al., 1989) have been identified. The ability of bFGF-autophosphorylation and/or ATP-binding in context with neuroprotection was described previously (Klumpp et al., 2006).

Thompson et al. (1994) identified the amino acid residues on the surface of the mature bFGF protein important for heparin binding. These amino acids were predominantly located

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between K128 and K144. Thompson et al. used a nine amino acid N-terminal truncated variant. Therefore, the numbering of the amino acids of bFGF in their publication is different from ours. To make the data comparable we have adjusted their original numbering to ours by adding nine to their numbers, respectively. The linear sequence in bFGF from amino acid number 128 to 144 accounts for 76% of the binding free energy, the residues R129 and K134 were in direct contact with sulphate anions. Li et al. (1994) replaced K128, R129, K134 and K138 of bFGF with glutamine and determined the heparin binding capacity by quenching of intrinsic fluorescence: R129Q and K134Q had no significant impact, whereas bFGF carrying the mutations K128Q and K138Q showed a 10-fold lower heparin binding affinity. Here we describe that the single amino acid exchange K134A in bFGF results in loss of neuroprotection both in cultured neurons and in a mouse model of focal cerebral ischemia.

1. Experimental procedures

1.1. Expression and purification of wt-bFGF and his-bFGF

Escherichia coli TG1 cells carrying the plasmid pCytexp1-bFGF (Seeger and Rinas, 1996) were used for overexpression of bFGF (accession number P09038, human). Plasmid pET16b::bFGF was constructed to overexpress his-bFGF in *E. coli* BL21(DE3) according to standard procedures. For overexpression of pCytexp1-bFGF, the temperature was increased from 37 to 42 °C at OD600 = 0.7 and incubation continued for 4 h. Cells were collected by centrifugation (3000 × *g*, 15 min, 4 °C), resuspended in phosphate buffer (0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.5) and frozen at −80 °C. The cell suspension was sonicated after thawing, and bFGF was isolated from the soluble extract (20,000 × *g*, 30 min, 4 °C). Fast protein liquid chromatography was performed in phosphate buffer using heparin–sepharose CL-6B (V = 1 ml; flow rate 0.5 ml/min) as reported (Seeger and Rinas, 1996). After loading and a washing step with phosphate buffer including 1 M NaCl, bFGF was eluted with phosphate buffer containing 2 M NaCl. In contrast, bFGF(K134A) was already eluted with 0.2–0.5 M NaCl. Protein containing fractions were pooled (2–3 ml) and dialyzed against 25 mM Tris–HCl, pH 7.5, containing 0.1% 2-mercaptoethanol. Glycerol (10%) was added, and bFGF was stored frozen in aliquots at −80 °C.

1.2. Site-directed mutagenesis

Site-directed mutagenesis was performed according to the protocol of the QuikChange site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) using pCytexp1-bFGF or pET16b::bFGF as template and the following primer pairs: 5′-CTGGTACGTTGCTCTGGCCCGTACCGGTCAGTACAA-3′ and 5′-TTGTACTGACCGGTACGGGCCAGAGCAACGTACCAG-3′ (K128A); 5′-CTGAAACGTACCGGTCAGTATGCATTGGGTTCGAAAACCGGT-3′ and 5′-GGACCGGTTTTCGAACCCAATGCATACTGACCGGTACGTTTCAG-3′(K134A); 5′-GTACAAACTGGGTTCGGCCACCGGTCCGGGTCAG-3′ and 5′-CTGACCCGGACCGGTGGCCGAACCCAGTTTGTAC-3′ (K138A); 5′-CCGGTCCGGGTCAGGCGGCCATCCTGTTCCTGCC-3′ and 5′-GGCAGGAACAGGATGGCCGCCTGACCCGGACCGG-3′ (K144A); 5′-CCTGGTACGTTGCTCTGAAGGCCACCGGTCAGTACAAACTGG-3′ and 5′-CCAGTTTGTACTGACCGGTGGCCTTCAGAGCAACGTACCAGG-3′ (R129A). bFGF derivatives were subcloned in *E. coli* TG1 and BL21(DE3), respectively. Correctness of each mutation was verified by sequence analysis of the DNA fragment encompassing the mutation (Seqlab, Göttingen, Germany).

1.3. [γ-^{32}P]ATP-labeling of bFGF

bFGF (1–5 μg) was incubated in a volume of 15 μl containing 25 mM Tris–HCl, pH 7.5, 0.1 mM $MgCl_2$, 1 mM dithiothreitol and 1 μM ATP including 6 μCi [γ-^{32}P]ATP (GE Healthcare, Munich, Germany) (Klumpp et al., 2006). Reactions were stopped after incubation for 10–30 min at 37 °C by adding 5 μl sample buffer (15 mM Tris–HCl, pH 6.8, 4% SDS, 2% 2-mercaptoethanol, 8 M urea, 10% sucrose, 10 mM EDTA, 0.01% bromphenol blue). Proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide mini-gel electrophoresis, the gels were dried, and finally, phosphorylation was detected by autoradiography or phosphoimaging.

1.4. Determination of neuroprotection

Brain hippocampal pieces from E19 fetuses of Sprague–Dawley rats (Charles-River, Sulzfeld, Germany) were treated with trypsin, the cells were dissociated by gentle trituration and seeded at a density of 2.5 × 10^4 cells/cm^2 onto polyethyleneimine-coated 35 mm dishes (1 ml medium) as described (Mattson et al., 1993). Cells were cultured at 37 °C in humidified atmosphere containing 5% CO_2 in modified Eagle's Medium supplemented with 10% fetal calf serum. After cell attachment (4 h), the plating medium was replaced by Neurobasal™ medium with B27 supplement. Culture medium was changed after 5 days just prior to the experiments. Cultures consisted of ≥95% neurons as revealed immunohistochemically by detection of neurofilament NF160 (data not shown). The growth factor bFGF (2.5 ng/ml) or heparin (from porcine intestinal mucosa, molecular mass ∼6,000; Sigma, Taufkirchen, Germany) was added to the cells 1–2 h prior to induction of apoptosis by staurosporine (200 nM). After 20 h, cells were fixed (4% formalin), incubated with the DNA fluorochrome Hoechst 33258 (10 μg/ml, 20 min, 37 °C), washed, and the cellular and nuclear morphology were analyzed using fluorescent microscope Axiovert 100 Zeiss (Jena, Germany). Apoptotic cells exhibited the typical nuclear features, including shrunken nuclei and condensed chromatin.

Cell death was also induced in neurons by oxygen and glucose deprivation (OGD). The culture medium was changed from Neurobasal™, containing 25 mM glucose, to Locke's medium without glucose, and the cells were placed in a humidified gas-tight chamber flushed with 5% CO_2/95% N_2 at 37 °C. After 4 h of OGD, Locke's medium was replaced by the initially removed Neurobasal™ medium and cells were further incubated for reoxygenation (16 h) in 5% CO_2/95% air prior to fixation and staining with Hoechst 33258 as described above.

1.5. Focal cerebral ischemia in mice

Male Naval Medical Research Institute (NMRI) mice (25–30 g) (Charles-River, Sulzfeld, Germany) were kept under controlled light and environmental conditions (12 h light/dark circle, 23 ± 1 °C, 55 ± 5% relative humidity) and had free access to food (Altromin) and water. Middle cerebral artery occlusion (MCAO) was performed in 10 animals (25–30 g) per group according to the method described previously (Junker et al., 2002). After the mice were anesthetized with tribromoethanol (350 mg/kg), a hole was drilled in the skull to expose the left MCA. The stem of the MCA and both branches were permanently occluded under visual control by electrocoagulation. Body temperature was maintained at 37 ± 0.5 °C with a heating lamp during the surgical procedure. After MCAO, rectal temperature was controlled every 30 min, and normothermia (37 ± 0.5 °C) was maintained by keeping the mice at an environmental temperature of 30 °C for 6 h. In our experiments bFGF (4 μg/kg) was administered intraventricularly 15 min before MCAO. Control animals received vehicle only (0.9% saline). Two days after MCAO, the mice received 0.5 ml of a 1% neutral red solution intraperitoneally. Thirty min later, brains were removed and the unstained tissue on the brain surface was determined as infarct area (mm^2) by means of an image analyzing system (Kontron).

1.6. Immunoblot analysis

Proteins electroblotted onto nitrocellulose membranes were incubated overnight with mouse α-tubulin antibody (1:2500) (Sigma, Taufkirchen, Germany), rabbit anti-FGF-2 (1:1000) (Oncogene, San Diego, USA), rabbit anti phospho-Akt (1:1000) (Cell signaling, Temekula, USA), and rabbit anti phospho-Erk1/2 (1:2000) (Cell signaling, Temekula, USA). Secondary antibodies (1:2500) (GE Healthcare, Munich, Germany) were added for 1 h.

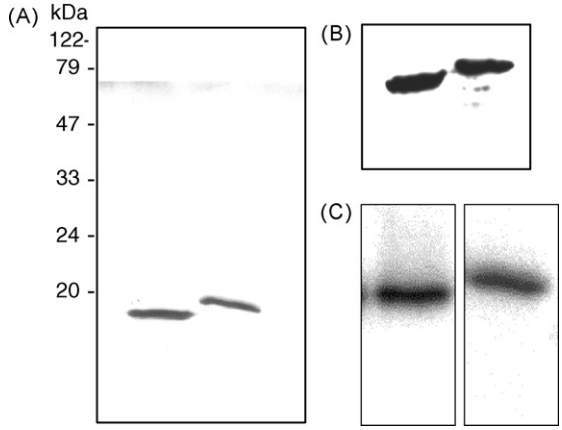

Fig. 1. Purity, identity and labeling of wt-bFGF (left lane, respectively) and his-bFGF (right lane, respectively). (A) Silver staining (1 μg), (B) Western blot analysis (2 μg) and (C) [γ-^{32}P]ATP-labeling with incubations (2 μg bFGF, respectively) carried out for 15 min as described in Section 1. Proteins were separated on a 15% SDS-PAGE minigel.

1.7. Heparin binding assay

Ten microliters of heparin-acrylic beads (Sigma, Taufkirchen, Germany) were incubated with 1 μg bFGF in 40 μl PBS (0.01 M phosphate buffered saline; pH 7.4) for 2 h at 37 °C. The beads were washed 5 times with PBS or PBS containing 2 M NaCl. bFGF was removed from the beads by boiling in sample buffer and then applied to SDS-PAGE. Proteins were visualized by silver staining or Western blotting with anti-FGF-2 antibody (aa 40–63; Oncogene, San Diego, USA).

1.8. Statistics

Cell culture experiments were performed on at least four separate dishes, with measurements of each culture performed in triplicate. Values were expressed as means ± S.D. Differences between experimental groups were evaluated by analysis of variance (ANOVA) followed by a Scheffé test for multiple comparisons.

2. Results

2.1. bFGF and his-bFGF exhibit ATP-labeling and neuroprotection

The growth factor bFGF used in these studies was heterologously expressed in *E. coli* and purified on Heparin-sepharose (wt-bFGF). Due to the low binding capacity of bFGF-mutants with defects in the heparin binding domain, a his-tagged variant of bFGF was also expressed and purified upon Ni-NTA-agarose (his-bFGF).

First, purity and identity of wt-bFGF and his-bFGF were determined (Fig. 1A and B). Silver staining of wt-bFGF and his-bFGF revealed no contamination by other proteins (Fig. 1A). Antibodies directed against bFGF were used to prove identity of the recombinant growth factor protein (Fig. 1B). It has been reported that bFGF, BDNF and NGF undergo autophosphorylation and/or ATP-binding and this binding of phosphate was shown to be essential for neuroprotection (Klumpp et al., 2006). Here we demonstrate that labeling of his-bFGF by [γ-^{32}P]ATP is the same as for

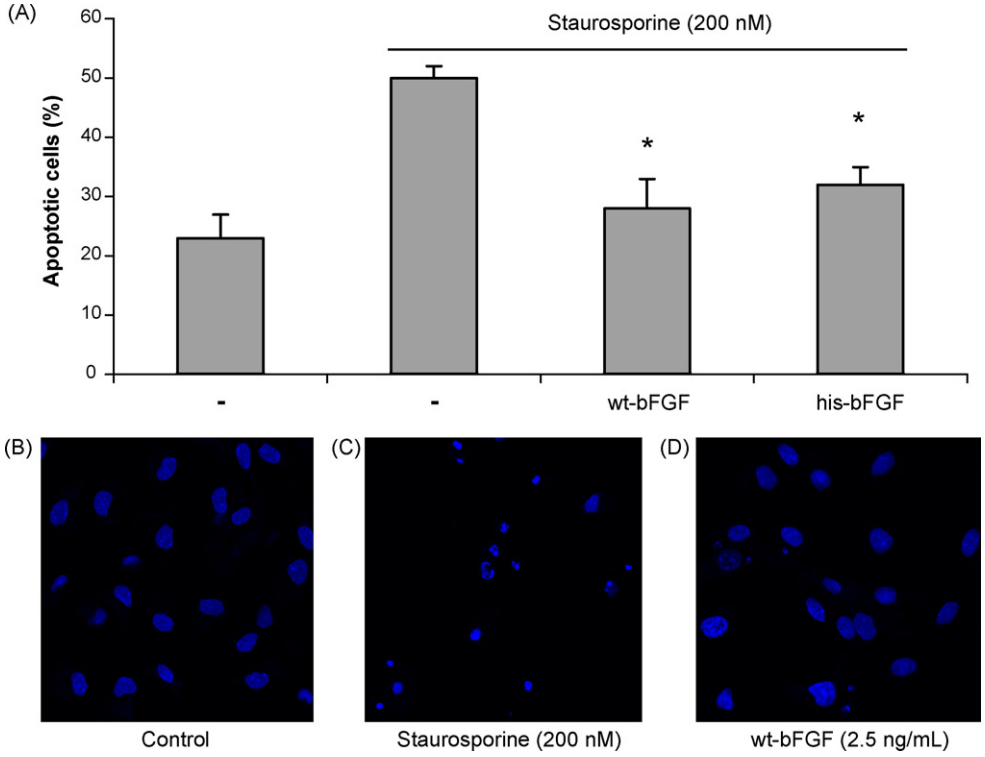

Fig. 2. Neuroprotection by bFGF. (A) wt-bFGF and his-bFGF protected rat primary cultures of embryonic hippocampal neurons against damage caused by staurosporine. Apoptosis was induced by staurosporine (200 nM) for 20 h and apoptotic cells were determined after staining with Hoechst 33258. Neurons were incubated with 2.5 ng/ml bFGF. (B–D) Non-apoptotic and apoptotic cells visualized with a fluorescent microscope after incubation with Hoechst 33258. $^{*}P < 0.001$ compared to staurosporine treated cells.

wt-bFGF (Fig. 1C). We used primary cultures of rat embryonic hippocampal neurons to examine whether ATP-labeled his-bFGF is as neuroprotective as ATP-labeled wt-bFGF. Neuroprotection by the growth factor was shown after damaging the cultured neurons with staurosporine (Fig. 2A–D). The wt-bFGF and its his-tagged variant revealed similar neuroprotective effects. This indicates that the artificial his-tag did not interfere with [γ-^{32}P]ATP-labeling or affect heparin- and receptor-binding.

2.2. bFGF(K134A) lacks neuroprotection

In order to study the relevance of distinct amino acids within the heparin-binding domain of bFGF (amino acids 128–144; Thompson et al., 1994), site-directed mutagenesis was carried out both with wt-bFGF and his-bFGF to exchange all positively charged amino acids within this domain. Five different single amino acid mutants of bFGF (K128A, R129A, K134A, K138A and K144A) were constructed, expressed and purified. [γ-^{32}P]ATP-labeling was determined to decide whether a loss of ATP-labeling is correlated with a loss in neuroprotection. All five mutants exhibited [γ-^{32}P]ATP-labeling similar to wt-bFGF and his-bFGF, respectively (data not shown).

Staurosporine-treated embryonic hippocampal neurons were used to study the neuroprotective activity of mutated bFGF proteins. Fig. 3A shows that bFGF carrying the mutation K128A, or R129A, or K138A or K144A protected cultured neurons as effectively as wt-bFGF. In contrast, neither bFGF(K134A) nor his-bFGF(K134A) protected the cells against degeneration (Fig. 3A). After incubation with bFGF(K134A), the number of apoptotic cells remained unchanged compared to untreated controls. Thus, the amino acid substitution K134A located within the heparin-binding domain of bFGF resulted in a complete loss of neuroprotective activity.

Next, we injected wt-bFGF intraventricularly at a dose of 4 μg/kg to mice, 15 min before permanent MCAO. As expected, wt-bFGF significantly reduced the infarct size by 14% compared to controls. Injection of bFGF(K134A), however, had no effect (Fig. 3B). Overall, the mutation K134A abolished the neuroprotective activity of bFGF as demonstrated both in cell culture and in the mouse model.

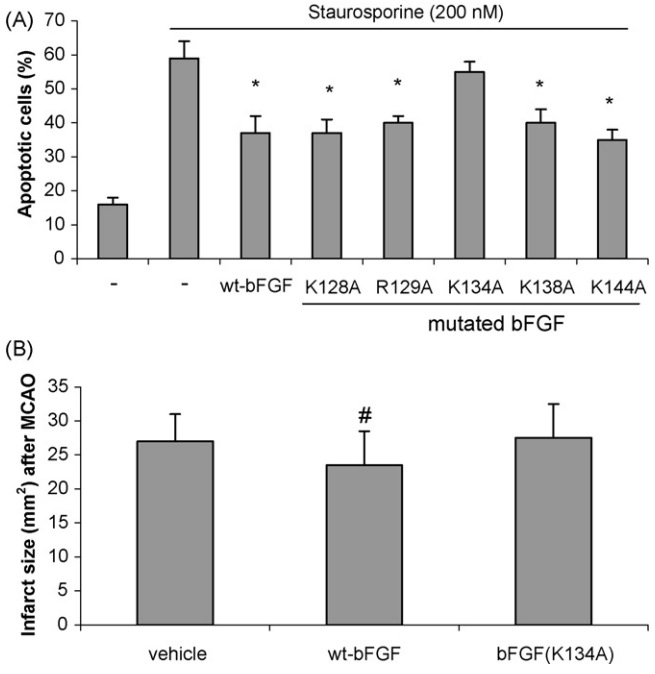

Fig. 3. Replacement of lysine 134 in bFGF against alanine abolished the neuroprotective effect. (A) Neuroprotection of wt-bFGF and of mutated growth factor proteins bFGF(K128A), bFGF(R129A), bFGF(K138A) and bFGF(K144A) – but not of bFGF(K134A) – in embryonic hippocampal primary cells treated with staurosporine. Apoptosis was induced by staurosporine (200 nM) for 20 h and apoptotic cells were determined after staining with Hoechst 33258. Embryonic hippocampal cells were incubated with 2.5 ng/ml of bFGF or mutated bFGF proteins, respectively. $^{*}P < 0.001$ compared to staurosporine treated cells. (B) Cerebroprotection by wt-bFGF in mice. Four micrograms per kilograms bFGF and bFGF(K134A) were administered intraventricularly 15 min before MCAO. Control animals received vehicle only (0.9% saline). Two days after MCAO, mice received 0.5 ml of a 1% neutral red solution intraperitoneally. Brains were removed 30 min later and the unstained tissue on the brain surface was determined as infarcted area (mm^2) by means of an image analyzing system (Kontron). $^{\#}P < 0.05$ compared to control.

2.3. Akt and ERK1/2 signaling is not observed with bFGF(K134A)

After binding of bFGF to heparin and to FGFR-1, signal cascades were initiated and the MAP-kinases ERK1 and ERK2 as well as protein kinase B (Akt) were phosphorylated (Desire et al., 2000). To study such activation, antibodies specific against the phosphorylated forms of ERK1/2 and Akt were used. Western blot analysis demonstrated that wt-bFGF was able to increase phosphorylation of ERK1/2 as well as phosphorylation of Akt (Fig. 4).

These effects could be observed from 5 min up to 2 h after administration (data not shown). In contrast, the levels of phospho-ERK1/2 and phospho-Akt were not increased after adding bFGF(K134A) to the neuronal cultures (Fig. 4). Our results suggest that lysine at position 134 of bFGF is decisive for intracellular signaling.

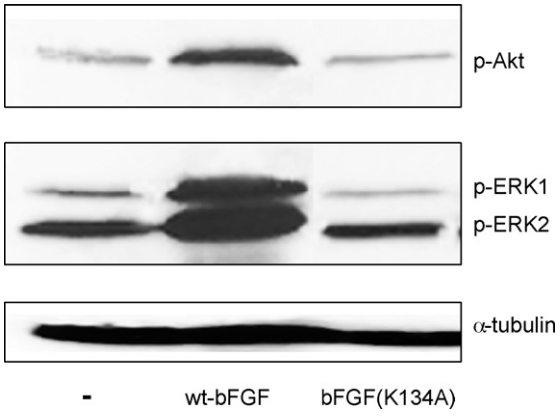

Fig. 4. bFGF and its downstream signaling pathways. Western blot analysis of phospho-ERK1/2 and phospho-Akt in rat primary cultures of hippocampal neurons 1 h after incubation with wt-bFGF or bFGF(K134A). Twenty micrograms of cell proteins were used and analyzed with antibodies against phospho-Akt (1:1000) and phospho-ERK1/2 (1:2000).

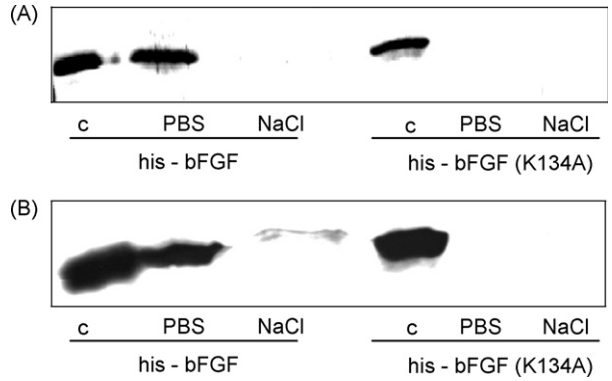

Fig. 5. Heparin binding capacity of his-bFGF compared with that of his-bFGF(K134A). Ten microliters of heparin-acrylic beads were incubated for 2 h at 37 °C with 2 μg his-bFGF or his-bFGF(K134A), respectively. Heparin-acrylic beads loaded with growth factor were washed five times with PBS or PBS containing 2 M NaCl. The washed beads were boiled with sample buffer for 10 min and run on a 15% SDS-PAGE minigel. (A) bFGF was visualized by silver staining and (B) Western blot analysis. For control (c), beads loaded with growth factor were analyzed by SDS-PAGE without washing-steps.

2.4. Impaired heparin binding capacity of bFGF(K134A)

We studied the ability of his-bFGF as well as his-bFGF(K134A) to bind to heparin using heparin-acrylic beads. After washing with phosphate buffered saline (PBS) the beads still retained bFGF. Two M NaCl was necessary to dissociate his-bFGF from heparin. In contrast, his-bFGF(K134A) was almost quantitatively eluted from the beads with PBS alone as revealed by protein staining and western blot analysis (Fig. 5). The data demonstrate that the binding affinity of his-bFGF(K134A) to heparin was markedly reduced compared to that of his-bFGF.

2.5. Neuroprotection by heparin

Ishikawa and Kitamura (1999) described that heparin itself exhibited cell-protective activity in glomerular cells damaged by staurosporine as well as H_2O_2. Furthermore, heparin activated various members of anti-apoptotic pathways, e.g. PI3K, ERK1/2 and JNK (Hills et al., 2006).

In order to test the neuroprotective ability of heparin, primary cultures of rat hippocampal cells were incubated with 0.1–10 μg/ml heparin. Two hours later neuronal cells were treated with staurosporine to induce apoptosis. Fig. 6 shows a concentration-dependent neuroprotective effect of heparin. Neuroprotection by heparin was also observed in untreated controls (Fig. 6). Ten micrograms per milliliters heparin significantly reduced the number of apoptotic cells in controls and staurosporine-damaged cells (Fig. 6A). A similar neuroprotective effect of heparin could be obtained in primary cultures of rat hippocampal cells upon oxygen and glucose deprivation (Fig. 6B).

Compared to the average concentration of heparin in blood (0.1 μg/ml), the concentration required for neuroprotection (10 μg/ml) is unphysiologically high. Because of the low amount (2.5 ng/ml) of wt-bFGF and bFGF(K134A) applied in our experiments, we can exclude effects of heparin liberated due to mutations with bFGF.

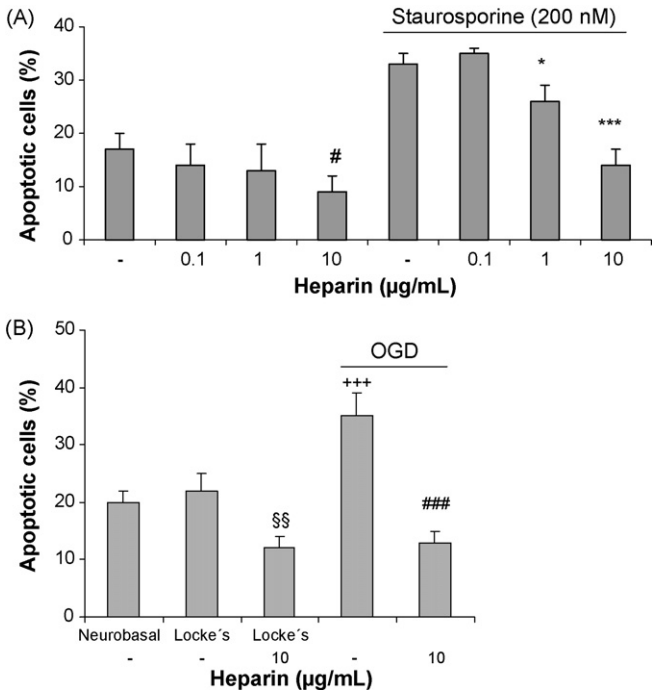

Fig. 6. Neuroprotection by heparin in primary cultures of rat embryonic hippocampal neurons. (A) Apoptosis was induced by staurosporine (200 nM) for 20 h and apoptotic cells were determined after staining with Hoechst 33258. The cultured neurons were incubated with 0.1–10 μg/ml heparin 2 h before induction of apoptosis. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to staurosporine-treated cells, $^{\#}P < 0.01$ compared to untreated cells. (B) Cells were incubated with heparin (10 μg/ml) 2 h before induction of apoptosis by OGD. After 4 h of OGD cells were further incubated (16 h) for reoxygenation. $^{\S\S}P < 0.01$ compared to Locke's control without heparin added; $^{+++}P < 0.001$ compared to Locke's control without heparin added; $^{\#\#\#}P < 0.001$ compared to OGD control.

3. Discussion

We could demonstrate that the amino acid substitution K134A located within the heparin-binding domain of the growth factor bFGF reduced its neuroprotective effect significantly. This was observed with primary cultures of hippocampal neurons and in the mouse model of focal cerebral ischemia. The loss of neuroprotection was based on a markedly reduced heparin binding capacity of the mutant bFGF(K134A) compared to wildtype bFGF. This is in line with results obtained by Thompson et al. (1994) demonstrating that the amino acids N36, R129, K134 and Q143 together account for 56% of the total bFGF-heparin binding free energy. They exchanged target residues for the interaction between bFGF and heparin against alanine. Binding parameters were determined amongst others for K128A, R129A, K134A, K138A, K144A and the double mutant R129A/K134A. The most dramatic change of the thermodynamic dissociation constant K_d for one of those mutants was observed with the double mutant, in which the binding affinity for heparin decreased 36-fold relative to wt-bFGF. A 17-fold decrease in

affinity was found for the single-site mutant bFGF(K134A) and, therefore, was the most dramatic change in K_d for any single-site mutant. This fits well with our data of the drastically reduced heparin binding capacity of bFGF(K134A). It also might explain, why the single-site mutant K134A revealed reduced neuroprotection in cultured neurons whereas all the other mutations (K128A, R129A, K138A, K144A) within the heparin-binding domain of bFGF did not.

Bellosta et al. (2001) demonstrated that only lysine 188 of FGF-4 and lysine 198 of FGF-4 match the appropriate amino acids in bFGF (K134 and K144, respectively). Besides that the heparin binding residues differ between FGF-4 and bFGF.

Li et al. (1994) exchanged amino acids in bFGF associated with the binding of heparin by glutamine instead of alanine and could not observe reduced heparin binding of bFGF(K134Q) compared to wt-bFGF. Instead, they found a slight increase in heparin binding of the K134Q mutant (about five-fold) compared to wt-bFGF. This is in contrast to the results obtained by Thompson et al. (1994) and also not in line with our results described here. Li et al. (1994) considered the residues K128 and K138 as most important for heparin binding. These mutants revealed a decrease in heparin binding to about 10% of that of wt-bFGF. The heparin binding of the double-mutant K128Q/K138Q decreased to about 1% of that of wt-bFGF. In a next step, they investigated the biological relevance of those mutations. A significant reduction in biological activity to a level of 10% compared to wt-bFGF (investigated by plasminogen activator-inducing activity in endothelial cells) could be demonstrated for the double-mutant K128Q/K138Q. The single-site mutants K128Q, K134Q, and K138Q did not affect the biological activity. In our studies, however, the neuroprotective activity of bFGF(K134A) was markedly reduced compared to that of the wild-type. This obvious discrepancy between Li's studies and our data presented here might be attributed to details as follows: (i) the test systems were different. We checked for the neuroprotective activity of bFGF in neuronal cell cultures whereas Li et al. (1994) focused on plasminogen activator-inducing activity in endothelial cells; (ii) we had replaced lysine against alanine whereas Li introduced a glutamine instead. Thus, we were dealing with differently charged amino acids with differently free energy binding profiles.

In conclusion, recombinant bFGF protected cultured neurons against damage caused by staurosporine and reduced the infarct size after MCAO in mice. In contrast, bFGF(K134A) did not reveal neuroprotection in any of these models. These observations open new insights into growth factor-heparin interaction and possibly pave the way to develop non-antibody based drugs able to inhibit vasculogenesis and angiogenesis in diseases initiated by bFGF.

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