

# Ultrastructural immunolocalization of basic fibroblast growth factor in endothelial cells: morphologic evidence for unconventional secretion of a novel protein

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**Abstract** Basic fibroblast growth factor (bFGF) is one of the most potent angiogenic factors. Unlike many other growth factors, bFGF lacks a classic peptide sequence for its secretion. Recent studies suggest that there is an unconventional secretory pathway for this growth factor. The aim of this study was to identify the specific location of bFGF in endothelial cells and to find morphologic evidences concerning its synthesis, storage and release from endothelial cells. The capillaries in hippocampus, adrenal gland, kidney, peripheral nerves as well as the vessels in connective tissues were analysed by using immunogold labeling techniques at electron microscope level. Results show that endogenous bFGF is mainly located in the nuclei of endothelial cells. Slight immunoreactivity is found in the cytoplasm. Immunolabeling is notably absent in pinocytotic vesicles, Golgi complexes, endoplasmic reticulum, nuclear membrane and intercellular junctions. These results provide morphologic evidence suggesting that endothelial cells might export bFGF via unique cellular pathways that are clearly distinct from classical signal peptide mediated secretion and/or release of this protein could be directly through mechanically induced disruptions of these cells. The current study support the recent hypothesis related with unconventional secretory pathway for bFGF as some other “cargo” proteins.

**Keywords** Endothel · Basic fibroblast growth factor · Immunocytochemistry · Cargo proteins

## Introduction

Basic fibroblast growth factor (bFGF) is a very potent mitogenic and angiogenic polypeptide, which induces cell proliferation and neovascularization in an autocrine and/or paracrine fashion (Baird et al. 1986; Villaschi and Nicosia 1993; Seghezzi et al. 1998; Tomanek et al. 1998). This growth factor is a morphogenic modulator in kidney vessel development (Kloth et al. 1998; Takahashi et al. 1998). It has mitogenic activity for endothelial cells (Gospodarowicz 1985; Schweigerer et al. 1987), stimulates tube formation (Sato and Rifkin 1988; Maffucci et al. 2009), migration (Biro et al. 1994) and protease production by these cells (Gospodarowicz 1990; Mignatti et al. 1989). Nehls et al. (1998) present evidence that endothelial cell-derived basic fibroblast growth factor serves as a chemotactic signal for other cells to migrate along a preestablished capillary-like structure. Tengood et al. (2011) recently have demonstrated the importance of this factor at the integration of angiogenesis. Besides its critical role in a variety of physiological processes including embryonic development (Cines et al. 1998) and wound healing (Kinsella et al. 1997; Ono et al. 2004; Akasaka et al. 2004; Katsuno et al. 2011); there are evidences showing its importance in some pathologic conditions like various inflammatory disorders (Folkman 1995; Yeh et al. 1999; Katsuno et al. 2011) and carcinogenesis (Bijnsdorp et al. 2011). Chen et al. (2004) emphasized the relationship between angiostasis and bFGF for therapeutic interventions. Its therapeutic potential for soft tissue engineering is also being discussed as another issue (Kato et al. 2010).

Basic fibroblast growth factor secretion has also been shown to be a marker of malignant transformation. Several reports have demonstrated increased production of bFGF in patients with cancer (Tanimoto et al. 1991; Ohta et al.

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1995; Yamamoto et al. 1999; De Jong et al. 1998; Barton et al. 1997; Dellacano et al. 1997). It is believed that overexpression and release of bFGF may stimulate endothelial proliferation and neovascularization, which contribute to the process of tumour development (De Jong et al. 1998; Emoto et al. 1997; Yamazaki et al. 1997).

Even though the reports showing synthesis of bFGF by endothelial cells (Rifkin and Moscatelli 1989; Vladovsky et al. 1987); the mechanism of its secretion is unclear because it lacks the signal peptide sequence for secretion (Burgess and Maciag 1989). It has been suggested that intracellular bFGF may be released in response to cell damage associated with tissue injury or tumour necrosis (Muthukrishnan et al. 1991). However, studies on bovine papilloma virus 1 transfected murine cells have showed that bFGF is released from transformed cells (Kandel et al. 1991; Christofori and Hanahan 1994). Albuquerque et al. (1998) also reports bFGF releasing in endothelial cell culture. These results suggest there might be other mechanism(s) for secretion and/or release of this protein.

Nickel (2007) propose that there is an unconventional secretory pathway for bFGF. He hypothesizes that bFGF appears to be secreted by direct translocation across the plasma membrane in an ATP- and membrane-potential-independent manner. Its translocation across the membrane is a diffusion-controlled process in which cell surface heparan sulfate proteoglycans function as an extracellular molecular trap that drives directional transport of bFGF. It has been supported in another recent study (Wegehingel et al. 2008).

Immunoreactivity for bFGF has been studied in vitro with cultures of endothelial cells (Biro et al. 1994; Muthukrishnan et al. 1991; Eguchi et al. 1992; Schechter et al. 1996; Yu et al. 1993). In addition, immunostaining for bFGF in endothelial cells from normal human and rat tissues has been demonstrated at the light microscope level (Casscells et al. 1990; Cordon-Cardo et al. 1990). However, no detailed correlative *in vivo* study about its expression patterns on subcellular level has not been published. This is important, since the precise location of bFGF may provide morphological evidence about the mechanism of synthesis, storage and release of this growth factor in endothelial cells. Answering these questions will certainly provide an overview of bFGF participation in several biological processes judged to be relevant to clinical haematologists and investigators of vascular biology.

To gain insight into these questions; we undertook the present study. We have previously examined the localization of basic fibroblast growth factor in fibroblasts and peripheral nerves (Aktas and Kayton 2000; Kayton and Aktas 2000). In this study; our aims were (1) to identify the specific location of bFGF in endothelial cells, (2) to determine the distribution of bFGF in capillaries of different

types of tissues, (3) to find morphologic evidences about the mechanism(s) by which bFGF might be released to extracellular sites. We examined capillaries within (1) the adrenal gland and the kidney, since these organs have great capacity for regeneration, (2) the hippocampus known to contain bFGF in the central nervous system and (3) the supporting tissues throughout the body, which play a major role after wounding. We used post-embedding immunogold labeling technique, since detergents, which are used during the pre-embedding labeling, causes destruction of the ultrastructure of the cells and gold labeling techniques allows to show the location of proteins precisely.

## Materials and methods

Archival blocks from sciatic nerve, hippocampus, adrenal gland and kidney were chosen for the study. The blocks had been prepared from the tissues of normal adult rats. Rats had been perfused with 4% paraformaldehyde/1–5% glutaraldehyde. Both osmicated and non-osmicated resin blocks were used for the study. Spurr's resin was embedding medium for all tissues.

Resin blocks were sectioned at 10–25 micron thickness to specify the regions of blocks for examination under electron microscope. These semi-thin sections were placed on slides. The slides were stained with toluidine blue (1%), rinsed with double distilled water, dried and cover-slipped. After staining, sections were examined under light microscope. Regions, which contained capillaries, were selected for further ultra-thin sectioning and examining under transmission electron microscope.

Ultrathin sections were cut at 70–90 nm thickness. They were collected on 200-mesh nickel grids previously coated with Coat-Quick "G" (Electron Microscopic Sciences) and then immunolabeled with either polyclonal (F3393-Sigma) or monoclonal antibodies (F6162-Sigma, C3316-ZymoGenetics Inc.) specific for bFGF using a two-step immunogold labeling method. All immunogold steps were carried out in 70- $\mu$ l reagent drops on parafilm (American National Can; Greenwich, CT) at room temperature. The sections were first treated with hyaluronidase at 2 mg/ml in 0.1 M acetate buffer, pH 5.2 for 15 min to unmask antigens, as reported previously (Casscells et al. 1990; Qu et al. 1995, 1998; Aktas and Kayton 2000). After two 5-min washes in Tris-buffered saline (TBS), pH 7.5, containing 0.1% Triton X-100, nonspecific binding sites were blocked by incubation for 20 min in blocking solution (1–2% crystalline BSA, 0.05% Tween-20, and 0.05% NaN<sub>3</sub>, with 1% normal goat serum in TBS, readjust pH to 7.2–7.3). The aim of this step was to exclude non-specific labeling and to produce highly specific results by blocking unreacted aldehyde groups and non-specific binding sites (Roth et al. 1989).

Then the grids were incubated for 1 h at room temperature with a goat anti-rabbit IgG antibody conjugated with 5-, 10- or 20-nm gold particles (Goldmarck Biologicals; Philips, NY) in TBS buffer, pH 8.2. After several washes in TBS and *d*H<sub>2</sub>O, the grids were air-dried and then counterstained with aqueous uranyl acetate.

In control groups, the specific primary antibodies were either omitted or were replaced by irrelevant IgGs from the same species (Rabbit anti-Histamine, anti-fibroblast 5B5). Preabsorption of the primary antibodies with recombinant bFGF was also applied for confirmation of the staining specificity.

## Results

Examination of immunolabeled tissue specimens from hippocampus, adrenal gland, kidney and supporting tissues around these organs and sciatic nerves from different normal adult rats demonstrate the following findings.

### Supporting tissues

At the electron microscope level, immunogold labeling demonstrates that bFGF is mainly located in the nuclei of endothelial cells (Fig. 1a, b, 2c). Slight immunoreactivity found in the cytoplasm. Immunolabeling is notably absent in the pinocytotic vesicles, Golgi complexes, endoplasmic reticulum, nuclear membrane, and intercellular junctions (Fig. 1a, b, 2c). There is also labeling on the collagen bundles (Fig. 1a).

### Adrenal gland

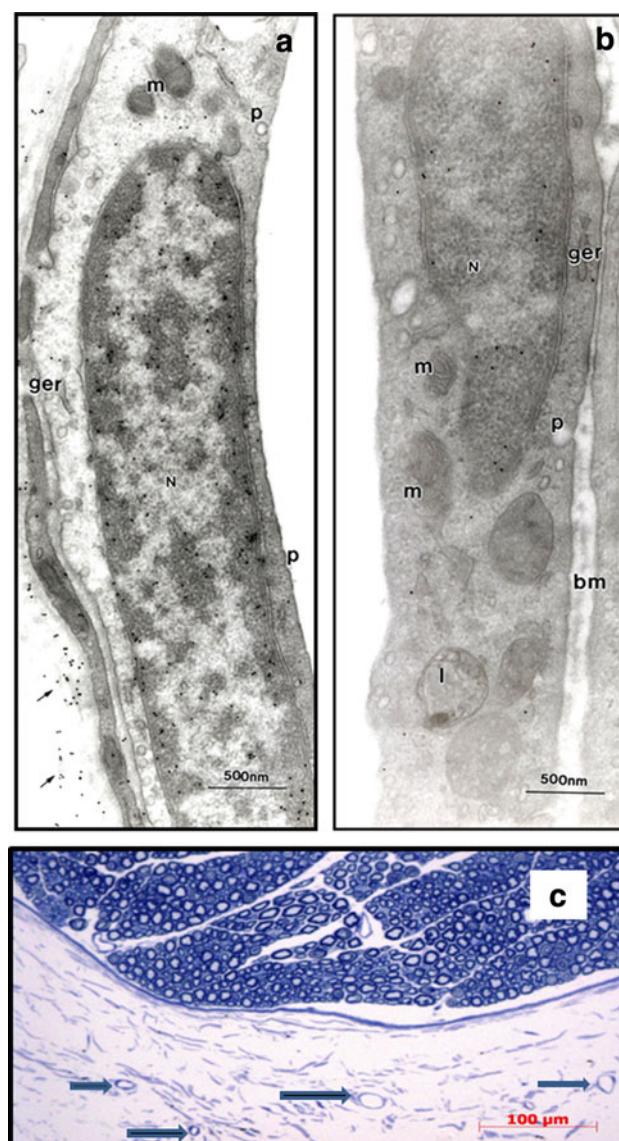
There is significant immunolabeling in the nuclei (Fig. 2a). Cytoplasmic reactivity for bFGF is higher than the reactivity of capillaries in hippocampus. Capillaries from three zones of adrenal cortex (zona glomerulosa, zona fasciculata and zona reticularis) show same immunolabeling pattern. The capillaries in the capsule of the adrenal glands also show similar staining pattern except lower labeling intensity in the cytoplasm.

### Hippocampus

Capillaries in hippocampal tissue specimens show same immunolabeling pattern. Distinct labeling is seen in the nuclei of the capillaries. Only a few gold particles can be seen in the cytoplasm (Fig. 2b).

### Kidney

Heavy immunolabeling in the nuclei and slight reactivity in the cytoplasm are seen in the endothelium of the glomeruli

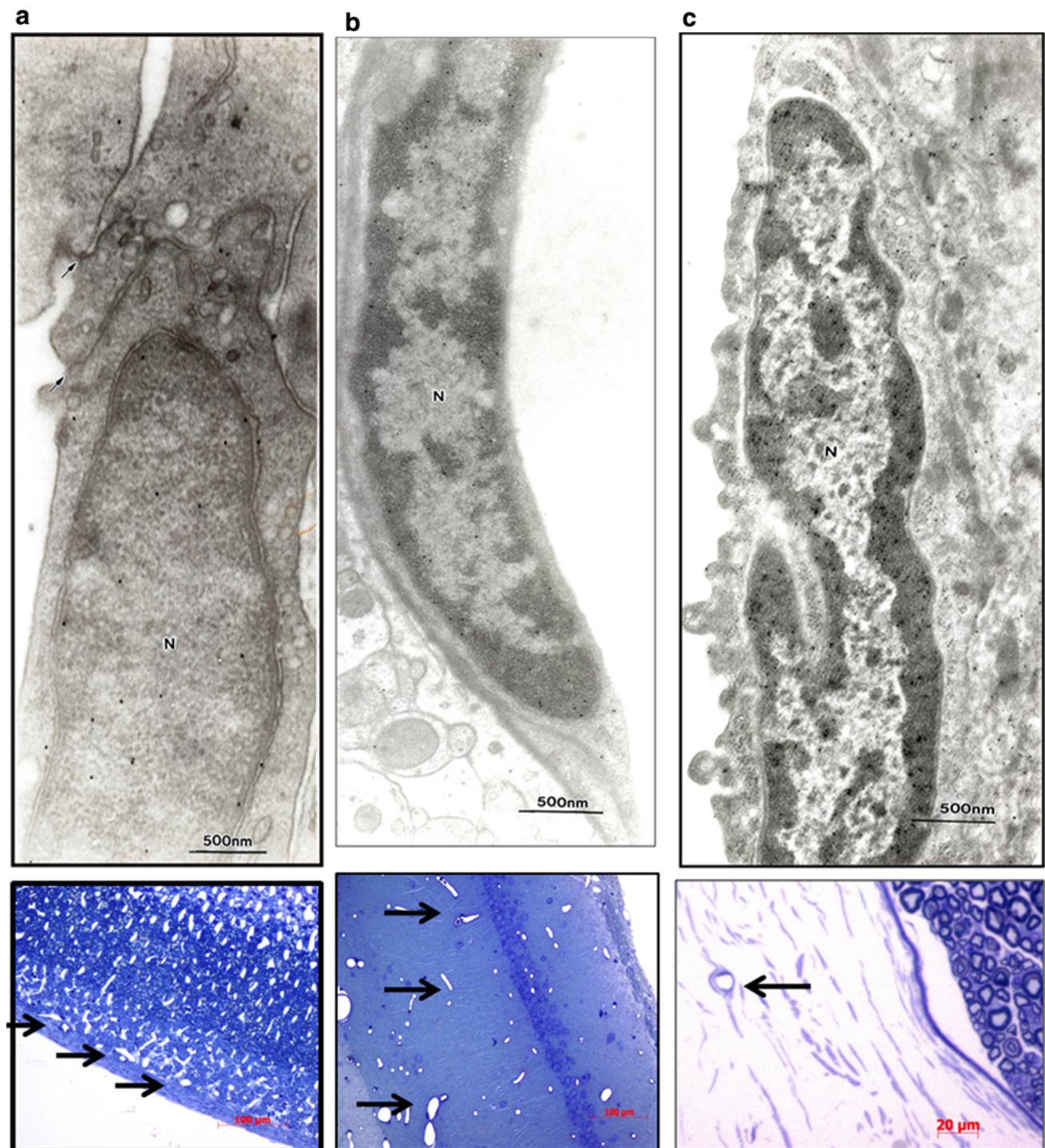


**Fig. 1** Immunogold labeling of two endothelial cells capillaries in supporting tissue around the rat sciatic nerve (a and b): Positive staining for bFGF is localized predominantly in the nucleus. Note the lacking of immunoreactivity in the cytosol and cytoplasmic organelles. In addition, note the immunolabeling in the bundles of collagen fibres beneath the cell (Primary antibody, F3393; Secondary antibody, 10 nm gold labeled IgG). The semi-thin section shows capillaries (arrows) in loose connective tissue around sciatic nerve (c). Selected area from this section of the block were used for ultrathin sectioning

(Fig. 3a) and in the other capillaries within the cortical area. The intensity of labeling in the cytoplasm is similar to the one in the adrenal glands.

### Confirmation of the specificity of bFGF staining

All antibodies (one monoclonal and two polyclonal) give similar patterns of immunostaining (Figs. 1, 3a). Both osmicated and non-osmicated tissue specimens show the



**Fig. 2** Immunoreactivity in endothelial cells in capillaries in adrenal gland (a), hippocampus (b) and sciatic nerve (c) of a normal adult rat. There is strong positive labeling in the nuclei of endothelium. Note that staining of the cytosol is minimal. (Primary antibody, F3393; Secondary antibody, 10 nm gold labeled IgG for Fig. 2 and Fig. 3/

primary antibody, F6162; secondary antibody, 20 nm gold labeled IgG for Fig. 4. Light microscopic pictures of semi thin sections under each electron micrograph demonstrates the areas selected for examining under electron microscope. Arrows indicate some capillaries in these tissues

same immunolabeling pattern. The only difference is that there is higher labeling intensity in non-osmicated tissues. Cells in the same tissues and tissues from different animals also show the similar pattern of immunolabeling.

Omission of primary antibody, substitution of a bFGF-specific antibody with an irrelevant antibody from the same species as the specific antibody abolished the immunogold particle localization (Fig. 3b). Preabsorption of the primary

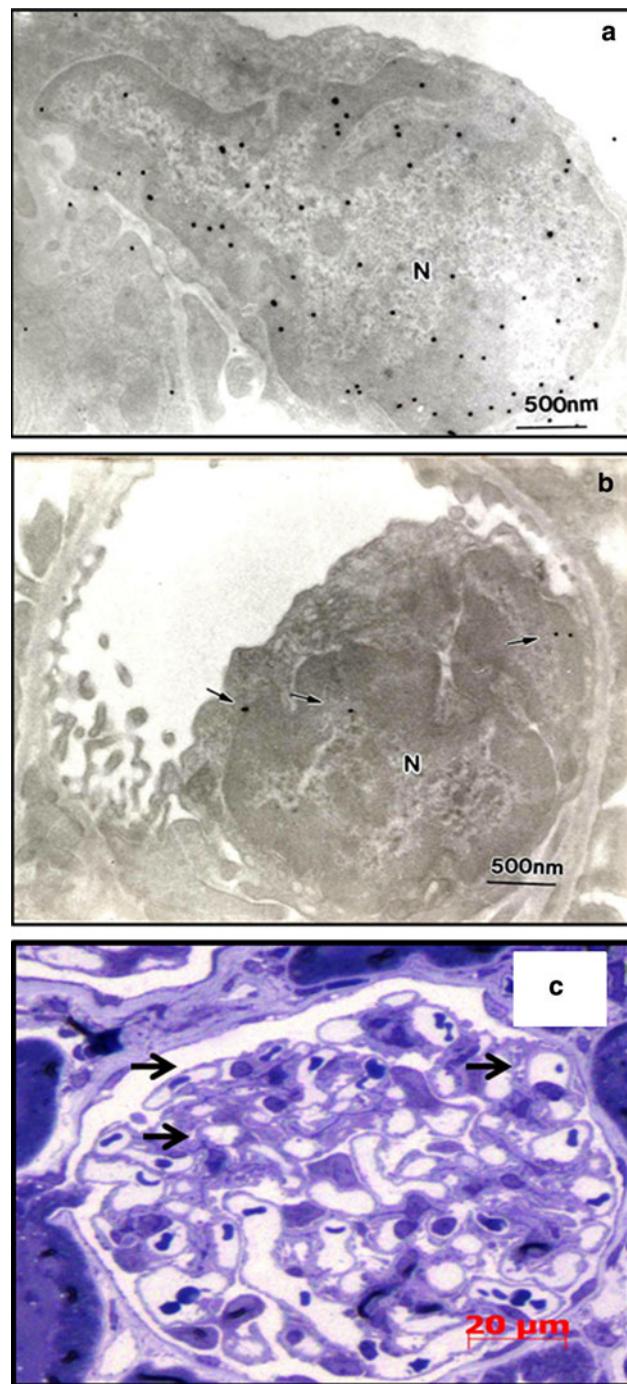
antibodies with recombinant bFGF greatly reduced the positive labeling intensity (data not shown).

## Discussion

We undertook the present study to investigate the expression patterns of bFGF in endothelial cells, in search of its possible autocrine and paracrine effects. This article presents morphological evidence supporting the hypothesis of unconventional secretion of bFGF.

The majority of extracellular proteins are exported from cells through the classical endoplasmic reticulum (ER)/Golgi-dependent secretory pathway (Lee et al. 2004). They first translocate into the lumen of the ER and then undergo vesicular transport through the Golgi complex to the cell surface. In the case of soluble factors, an N-terminal signal peptide directs secretory proteins into this pathway (Osborne et al. 2005). bFGF has been described as a novel protein since it has not a secretory signal sequence (Mignatti and Rifkin 1991; Mignatti et al. 1992). Then; it came as a great surprise when certain soluble extracellular proteins were discovered that do not possess this signal (Cleves 1997; Hughes 1999; Nickel 2003; Prudovsky et al. 2003). The existence of unconventional mechanisms was supported by the demonstration that brefeldin A, a drug that blocks membrane trafficking through the ER/Golgi complex (Lippincott-Schwartz et al. 1989; Misumi et al. 1986), does not affect their secretion rates (Hughes 1999; Nickel 2003).

Unconventional secretory mechanisms have been revealed for several biomedically important factors, including bFGF (Nickel 2003; Prudovsky et al. 2003). Backhaus et al. (2004) have found that membrane translocation of bFGF does not require protein unfolding. Schäfer et al. (2004) described the unconventional secretory mechanism for bFGF. Diverse mechanisms have been proposed to explain these unconventional secretory processes, including lysosomal secretion, plasma membrane shedding, release in exosomes as well as secretion through transporters that reside in the plasma membrane (Nickel 2005). Multiple mechanisms have even been proposed for individual unconventionally secreted proteins. Nickel (2007) discusses a new model for the molecular mechanism of bFGF secretion based on recent data. Key aspects of this model are: (1) direct translocation of growth factor from the cytoplasm across the plasma membrane in the absence of transport vesicles; (2) the independence of membrane translocation from ATP hydrolysis or a membrane potential; (3) diffusion-controlled membrane translocation process; and (4) an extracellular molecular trap formed by membrane-proximal heparan sulfates that ensures directional transport of into the extracellular space.



**Fig. 3** Ultrastructural immunolocalization of bFGF endothelial cells in glomerular capillaries in kidney (a). Gold particles are mainly located in the nuclei of the endothelial cell. (Primary antibody, C3316; Secondary antibody, 20 nm gold labeled IgG). Immunostaining has been greatly reduced when endothelial cells has been labeled by an irrelevant antibody as a control (b). Only a few gold particles can be seen (arrows) in the cell. (Primary antibody, VGF; Secondary antibody, 10 nm gold labeled IgG). Semi-thin section of a glomerulus in kidney (c) indicates regions containing endothelial cells (arrows), which were chosen for immunostaining for electron microscopic observations

The presence of bFGF in blood vessels has been previously documented on experimental data in vitro and at light level in vivo (Biro et al. 1994; Muthukrishnan et al. 1991; Eguchi et al. 1992; Yu et al. 1993; Cordon-Cardo et al. 1990). However; the present study is the first document examining its subcellular location in endothelial cells in different tissues by using immunogold labeling technique at electron microscope level in vivo. Previous endothelial cell culture studies validate our TEM findings. Summary of comparison of our results with these reports have been summarized below.

Our study indicates that endogenous bFGF is mainly located in the nuclei of endothelial cells. Muthukrishnan et al. (1991) found nuclear immunoreactivity in endothelial cell cultures. It is likely that nuclear localization of bFGF is in some way associated with the proliferative or activated state (like other growth-related proteins found in nuclei i.e. p53, Int-2, cyclin, statin, abl, sis, SV40 large T antigen). Basic fibroblast growth factor can act as a self-stimulating growth factor for capillary endothelial cells. Gu and Kay (1998) have suggested that nuclear binding of bFGF may mediate the long-term effect (e.g. changes in chromatin structure to allow transcription and replication of genes). Nuclear, cytoplasmic, and extracellular matrix fractions of normal and modulated corneal endothelial cells were separated in their study. Basic fibroblast growth factor isoforms were further purified by heparin-Sepharose column. The molecular sizes of the isoforms were determined by immunoblot analysis, using a specific antibody directed against bFGF. The rapidly growing cells showed strong staining of bFGF in the nucleus, whereas cytoplasmic and extracellular matrix staining was weak. When modulated cells reached confluence, the staining of bFGF in the nuclei remained strong, whereas extracellular matrix staining was significantly increased. Immunoblot analysis of the subcellular fraction showed that the 24-kDa bFGF was predominantly present in the nucleus, whereas the 18-kDa form was the major molecule in cytoplasmic and extracellular fractions in normal and modulated cells.

Cytoplasm exhibits only a slight reactivity in all tissue samples. The cytoplasmic immunolabeling intensity both in the kidney and in the adrenal gland is higher than the intensity in the hippocampus. Immunostaining for bFGF is present in the cytoplasm of endothelial cell cultures in some previous studies (Villaschi and Nicosia 1993; Yu et al. 1993). Yu et al. (1993) suggest that the nuclei of endothelial cells contain 24 and 26 kDa forms of bFGF, in contrast to the 18-kDa form usually found in the cytoplasm in vitro. There are two investigations (Biro et al. 1994; Muthukrishnan et al. 1991) which report that intracellular bFGF levels increase in the cytoplasm during migration, whereas non-migrating endothelial cells exhibit minimal cytoplasmic bFGF. Schechter et al. (1996) has reported that

immature capillaries contained bFGF within the cytoplasm while immunostaining for bFGF was absent or minimal in adult capillaries of anterior pituitary from adult animals. We believe that differences between the stages of angiogenesis may cause different immunolabeling intensity. Gu and Kay's study (1998) support this hypothesis.

Tessler and Neufeld (2005) studied the intracellular localization of basic fibroblast growth factor (bFGF) in BHK-21 cells transfected with an expression vector containing the complementary DNA (cDNA) of the human bFGF (pbFGF) gene. The intracellular location of bFGF was determined using indirect immunofluorescence. The antibodies used were polyclonal antibodies directed against either recombinant human bFGF or recombinant *Xenopus*: bFGF. The nuclei of transfected cells that produce bFGF, but not the nuclei of untransfected cells, were labeled strongly by the antibodies. The nuclear staining was totally abolished when anti-bFGF antibodies preadsorbed with bFGF were used. Several types of endothelial cells known to produce bFGF were also stained in their nuclei by the antibodies.

We did not find immunoreactivity in the basement membrane as mentioned in some studies (Biro et al. 1994; Cordon-Cardo et al. 1990). However, using electron microscope we observe immunolabeled bundles of collagen fibres close to the basal membranes (Fig. 1) that might explain this discrepancy.

Whereas the mechanism of bFGF secretion remains unknown, our present data at electron microscope level supports several conclusions: This protein can not be secreted via a traditional secretory pathway; since there is no morphologic evidence showing its presence in cytoplasmic organelles, such as granular endoplasmic reticulum, Golgi apparatus, and pinocytotic vesicles. Endogenous bFGF release from endothelial cells to the extracellular matrix may result from cell lysis, or secretion via a nontraditional mechanism. According to Vlodavsky et al. (1987), endothelial cells synthesize bFGF, most of which remains cell-associated and some of which is deposited and sequestered in the subendothelial extracellular matrix. Renko et al. (1990) investigated subcellular location of bFGF in culture cells. Analysis of bFGF distribution by immunofluorescence using an antibody that recognized all forms of bFGF indicated both cytoplasmic and nuclear localization but failed to reveal any growth factor in structures representing secretory vesicles in their study. Therefore, they concluded that bFGF has a distribution inconsistent with that of a secretory protein.

Schechter et al. (1996) suggest that immature endothelial cells in anterior pituitary release bFGF by focal fragmentation of blebs and cytoplasmic processes. We could not observe significant labelling in cytoplasmic processes. Muthukrishnan et al. (1991) and Mc Neil et al. (1989) show bFGF release via plasma membrane disruptions of endothelial cells, thus making it a strong candidate for a

“wound hormone”. Albuquerque et al. (1998) report bFGF releasing in endothelial cell culture. Mignatti et al. (1989) show transfected 3T3 cells release bFGF via a mechanism of exocytosis, independent of the ER–Golgi system. Florkiewicz et al. (1995) report that an energy dependent non-ER/Golgi pathway directs the export of bFGF in bFGF-transfected COS-1 cells. Basic fibroblast growth factor appears to be exported, through some currently undefined mechanism, to the cell surface. Presumably this export process (as observed in several experimental models) confers an exquisite regulatory ability to the target cell, enabling it to control the growth factor’s activity by restricting the growth factor to the local environment and thus limit its actions to where it may be needed most. Hughes et al. (1999) report that unconventionally secreted proteins do not localize to the ER/Golgi complex and consistently lack posttranslational modifications added in these compartments. Nickel call these proteins as “cargo” proteins and describes their potential export routes for unconventional secretion (2003). He suggests that bFGF probably reach the extracellular space by direct translocation across the plasma membrane and it uses distinct transport system. Zehe et al. (2006) report that “an unconventional export machinery” is essential for secretion of this protein and a functional interaction between bFGF and heparin sulphate proteoglycans is required for the export of this growth factor from mammalian cells.

Further studies need to clarify how this protein is released and/or secreted from mature endothelial cells *in vivo*. Definition of the cellular steps of expression of bFGF, that direct microvascular assembly, promises to identify therapeutic targets for repair and adaptive remodeling of vessels. These findings may also justify anti-angiogenesis strategies based on the neutralization of bFGF in patients with tumours, since angiogenesis is essential process for tumour progression and metastasis.

## References

- Akasaka Y, Ono I, Yamashita T, Jimbow K, Ishii T (2004) Basic fibroblast growth factor promotes apoptosis and suppresses granulation tissue formation in acute incisional wounds. *J Pathol* 203(2):710–720
- Aktas RG, Kayton RJ (2000) Ultrastructural immunolocalization of basic fibroblast growth factor in fibroblasts and extracellular matrix. *Histochem Cell Biol* 113:227–233
- Albuquerque MLC, Akiyama SK, Schnaper HW (1998) Basic fibroblast growth factor release by human coronary artery endothelial cells is enhanced by matrix proteins, 17 $\beta$ -estradiol, and a PKC signaling pathway. *Exp Cell Res* 245:163–169
- Backhaus R, Zehe C, Wegehingel S, Kehlenbach A, Schwappach B, Nickel W (2004) Unconventional protein secretion: membrane translocation of FGF-2 does not require protein unfolding. *J Cell Sci* 117(Pt 9):1727–1736
- Baird A, Esch F, Mormede P, Ueno N, Ling N, Bohlen P et al (1986) Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. *Rec Prog Horm Res* 42:143–205
- Barton DPJ, Cai A, Wendt K, Young M, Gameo A, De Cesare S (1997) Angiogenic protein expression in advanced epithelial ovarian cancer. *Clin Cancer Res* 3:1579–1586
- Bijnnsdorp IV, Capriotti F, Kruijt FA, Losekoot N, Fukushima M, Griffioen AW, Thijssen VL, Peters GJ (2011) Thymidine phosphorylase in cancer cells stimulates human endothelial cell migration and invasion by the secretion of angiogenic factors. *Br J Cancer* 104(7):1185–1192
- Biro S, Yu Z, Fu Y, Smale G, Sasse J, Sanchez J et al (1994) Expression and subcellular distribution of basic fibroblast growth factor are regulated during migration of endothelial cells. *Circ Res* 74:485–494
- Burgess WH, Maciag T (1989) The heparin binding (fibroblast) growth factor family of proteins. *Annu Rev Biochem* 58:575–606
- Casscells W, Spir E, Sasse J, Klagsburn M, Allen P, Lee M et al (1990) Isolation, characterization, and localization of heparin-binding growth factors in the heart. *J Clin Invest* 85:433–441
- Chen CH, Poucher SM, Lu J, Henry PD (2004) Fibroblast growth factor 2: from laboratory evidence to clinical application. *Curr Vasc Pharmacol* 2(1):33–43
- Christofori G, Hanahan D (1994) Molecular dissection of multi-stage tumorigenesis in transgenic mice. *Semin Cancer Biol* 5(1):3–12
- Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP et al (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* 91(10):3527–3561
- Cleves AE (1997) Protein transports: the nonclassical ins and outs. *Curr Biol* 7(5):R318–R320
- Cordon-Cardo C, Vlodavsky I, Haimovitz-Freidman A, Hicklin D, Fuks Z (1990) Expression of basic fibroblast growth factor in normal human tissues. *Lab Invest* 63:832–840
- De Jong JS, Diest PJV, Valk PVD, Baak JPA (1998) Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I: an inventory in search of autocrine and paracrine loops. *J Pathol* 184:44–52
- Dellacano FR, Spiro J, Eisma R, Kreutzer D (1997) Expression of basic fibroblast growth factor and its receptors by head and neck squamous carcinoma tumor and vascular endothelial cells. *Am J Surgery* 174:540–544
- Eguchi K, Migita K, Nakashima M, Ida H, Terada K, Sakai M et al (1992) Fibroblast growth factors released by wounded endothelial cells stimulate proliferation of synovial cells. *J Rheumatol* 19(12):1925–1932
- Emoto A, Nagakawa M, Wakabayashi Y, Hanada T, Naito S, Nomura Y (1997) Induction of tubulogenesis of microvascular endothelial cells by basic fibroblast growth factor from human SN12C renal cancer cells. *J Urol* 157:699–703
- Florkiewicz RZ, Majack RA, Buechler RD, Florkiewicz E (1995) Quantitative export of FGF-2 occurs through an alternative, energy dependent, non-ER/Golgi pathway. *J Cell Physiol* 162:388–399
- Folkman J (1995) Clinical applications of research on angiogenesis. *N Engl J Med* 333:1757–1763
- Gospodarowicz D (1985) Biological activity *in vivo* of pituitary and brain fibroblast growth factor. In: Ford RJ, Maizel ANY (eds) *Mediators in cell growth and differentiation*. Raven Press, New York, pp 109–134
- Gospodarowicz D (1990) Fibroblast growth factor: chemical structure and biological function. *Clin Orthop* 257:231–248
- Gu X, Kay EP (1998) Distribution and putative roles of fibroblast growth factor-2 isoforms in corneal endothelial modulation. *Invest Ophthalmol Vis Sci* 39:2252–2258

- Hughes RC (1999) Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim Biophys Acta* 1473(1):172–185
- Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan D (1991) Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. *Cell* 66(6):1095–1104
- Kato H, Suga H, Eto H, Araki J, Aoi N, Doi K et al (2010) Reversible adipose tissue enlargement induced by external tissue suspension: possible contribution of basic fibroblast growth factor in the preservation of enlarged tissue. *Tissue Eng Part A* 16(6):2029–2040
- Katsuno A, Aimoto T, Uchida E, Tabata Y, Miyamoto M, Tajiri T (2011) The controlled release of basic fibroblast growth factor promotes a rapid healing of pancreaticojejunal anastomosis with potent angiogenesis and accelerates apoptosis in granulation tissue. *J Surg Res* 167(1):166–172
- Kayton RJ, Aktas RG (2000) Electron microscopic immunolocalization of basic fibroblast growth factor-like molecules in peripheral nerves. *Histochem Cell Biol* 114:413–419
- Kinsella MG, Tsoi CK, Jarvelainen HT, Wight TN (1997) Selective expression and processing of biglycan during migration of bovine aortic endothelial cells: the role of endogenous basic fibroblast growth factor. *J Biol Chem* 272(1):318–325
- Kloth S, Gerdes J, Wanke C, Minuth WW (1998) Basic fibroblastic growth factor is a morphogenic modulator in kidney vessel development. *Kidney Int* 53:970–978
- Lee, MC, Miller EA, Goldberg J, Orci L, Schekman R (2004) Bidirectional protein transport between the ER and Golgi. *Annu Rev Cell Dev Biol* 20:87–123
- Lippincott-Schwartz J, Yuan LC, Bonifacino JS, Klausner RD (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* 56:801–813
- Maffucci T, Raimondi C, Abu-Hayyeh S, Dominguez V, Sala G, Zachary I et al (2009) Phosphoinositide 3-kinase/phospholipase Cgamma1 pathway regulates fibroblast growth factor-induced capillary tube formation. *PLoS One* 4(12):e8285
- Mc Neil PL, Muthukrishnan L, Warder E, D'Amore PA (1989) Growth factors are released by mechanically wounded endothelial cells. *J Cell Biol* 109:811–822
- Mignatti P, Rifkin DB (1991) Release of basic fibroblast growth factor, an angiogenic factor devoid of secretory signal sequence: a trivial phenomenon or a novel secretion mechanism? *J Cell Biochem* 47(3):201–207
- Mignatti P, Tsuboi R, Robbins E, Rifkin DB (1989) In vitro angiogenesis on the human amniotic basement membrane: requirement for basic fibroblast growth factor-induced proteases. *J Cell Biol* 108:671–682
- Mignatti P, Morimoto T, Rifkin DB (1992) Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum–Golgi complex. *J Cell Physiol* 151(1):81–93
- Misumi Y, Misumi Y, Miki K, Takatsuki A, Tamura G, Ikebara Y (1986) Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J Biol Chem* 261:11398–11403
- Muthukrishnan L, Warder E, Mc Neil P (1991) Basic fibroblastic growth factor is efficiently released from a cytosolic storage site through plasma membrane disruptions of endothelial cells. *J Cell Physiol* 148:1–16
- Nehls V, Herrmann R, Huhnken M (1998) Guided migration as a novel mechanism of capillary network remodeling is regulated by basic fibroblast growth factor. *Histochem Cell Biol* 109:319–329
- Nickel W (2003) The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur J Biochem* 270(10):2109–2119
- Nickel W (2005) Unconventional secretory routes: direct protein export across the plasma membrane of mammalian cells. *Traffic* 6(8):607–614
- Nickel W (2007) Unconventional secretion: an extracellular trap for export of fibroblast growth factor 2. *J Cell Sci* 120(Pt 14):2295–2299
- Ohta T, Yamamoto M, Numata M, Iseki S, Tsukioka Y, Miyashita T et al (1995) Expression of basic fibroblast growth factor and its receptor in human pancreatic carcinomas. *Br J Cancer* 72(4):824–831
- Ono I, Yamashita T, Hida T, Jin HY, Ito Y, Hamada H et al (2004) Combined administration of basic fibroblast growth factor protein and the hepatocyte growth factor gene enhances the regeneration of dermis in acute incisional wounds. *Wound Repair Regen* 12(1):67–79
- Osborne AR, Rapoport TA, van den Berg B (2005) Protein translocation by the Sec61/SecY channel. *Annu Rev Cell Dev Biol* 21:529–550
- Qu Z, Liebler JM, Powers MR, Galey T, Ahmadi P, Huang X et al (1995) Mast cells are a major source of basic fibroblast growth factor in chronic inflammation and cutaneous hemangioma. *Am J Pathol* 147:564–573
- Qu Z, Kayton RJ, Ahmadi P, Liebler JM, Powers MR, Planck SR et al (1998) Ultrastructural immunolocalization of basic fibroblast growth factor in mast cell secretory granules: morphological evidence for bFGF release through degranulation. *J Histochem Cytochem* 46(10):1119–1128
- Prudovsky I, Mandinova A, Soldi R, Bagala C, Graziani I, Landrisina M, Tarantini F, Duarte M, Bellum S, Doherty H, Maciag T (2003) The non-classical export routes: FGF1 and IL-1alpha point the way. *J Cell Sci* 116(Pt 24):4871–4881
- Renko M, Quarto N, Morimoto T, Rifkin DB (1990) Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J Cell Physiol* 144(1):108–114
- Rifkin DB, Moscatelli D (1989) Recent developments in the cell biology of basic fibroblast growth factor. *J Cell Biol* 109:1–6
- Roth J, Taatjes DJ, Warhol MJ (1989) Prevention of non-specific interactions of gold labeled reagents on tissue sections. *Histochemistry* 92(1):47–56
- Sato Y, Rifkin DB (1988) Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. *Cell Biol* 107:1199–1205
- Schäfer T, Zentgraf H, Zehe C, Brügger B, Bernhagen J, Nickel W (2004) Unconventional secretion of fibroblast growth factor 2 is mediated by direct translocation across the plasma membrane of mammalian cells. *J Biol Chem* 279(8):6244–6251
- Schechter J, Pattison A, Pattison T (1996) Basic fibroblast growth factor within endothelial cells during vascularization of the anterior pituitary. *Anat Record* 245:46–52
- Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC, Gospodarowicz D (1987) Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. *Nature* 325:257–259
- Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES et al (1998) Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol* 141:1659–1673
- Takahashi T, Huynh-Do U, Daniel TO (1998) Renal microvascular assembly and repair: power and promise of molecular definition. *Kidney Int* 53(4):826–835

- Tanimoto H, Yoshida K, Yokozaki H, Yasui W, Nakayama H, Ito H et al (1991) Expression of basic fibroblast growth factor in human gastric carcinomas. *Virchows Arch B Cell Pathol* 61(4):263–267
- Tengood JE, Ridenour R, Barodsky R, Russell AJ, Little SR (2011) Sequential delivery of basic fibroblast growth factor and platelet-derived growth factor for angiogenesis. *Tissue Eng Part A* 17(9–10):1181–1189
- Tessler S, Neufeld G (2005) Basic fibroblast growth factor accumulates in the nuclei of various bFGF producing cell types. *J Cell Physiol* 145(2):310–317
- Tomanek RJ, Doty MK, Sandra A (1998) Early coronary angiogenesis in response to thyroxine: growth characteristic and upregulation of basic fibroblast growth factor. *Circ Res* 82: 587–593
- Villaschi S, Nicosia RF (1993) Angiogenic role of endogenous basic fibroblast growth factor released by rat aorta after injury. *Am J Pathol* 143(1):181–190
- Vladovsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R, Sasse J et al (1987) Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc Nat Acad Sci* 84:2292–2296
- Wegehingel S, Zehe C, Nickel W (2008) Rerouting of fibroblast growth factor 2 to the classical secretory pathway results in post-translational modifications that block binding to heparan sulfate proteoglycans. *FEBS Lett* 582(16):2387–2392
- Yamamoto T, Umeda T, Yokozeki H, Nishioka K (1999) Expression of basic fibroblast growth factor and its receptor in angiosarcoma. *Am J Acad Dermatol* 41:127–129
- Yamazaki K, Nagao T, Yamaguchi T, Saisho H, Kondo Y (1997) Expression of basic fibroblast growth factor (FGF-2)-associated with tumour proliferation in human pancreatic carcinoma. *Virchows Arch* 431:95–101
- Yeh C, Peng H, Huang T (1999) Cytokines modulate integrin  $\alpha_v\beta_3$ -mediated human endothelial cell adhesion and calcium signalling. *Exp Cell Res* 251:57–66
- Yu Z, Biro S, Fu Y, Sanchez J, Smale G, Sasse J et al (1993) Localization of basic fibroblast growth factor in bovine endothelial cells: immunohistochemical and biochemical studies. *Exp Cell Res* 204:247–259
- Zehe C, Engling A, Wegehingel S, Schäfer T, Nickel W (2006) Cell-surface heparan sulfate proteoglycans are essential components of the unconventional export machinery of FGF-2. *Proc Natl Acad Sci USA* 103(42):15479–15484