Expression of angiogenic factors in neurofibroma

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Abstract

We studied the expression of angiogenic factors (vascular endothelial growth factor, basic fibroblast growth factor, platelet derived growth factor and hepatocyte growth factor) in cutaneous neurofibroma which arose in on patients with neurofibromatosis-1. Immunohistochemical staining and the reverse transcribed polymerase chain reaction (RT-PCR) method demonstrated vascular endothelial growth factor and basic fibroblast growth factor to be highly expressed in neurofibroma cells at both protein and mRNA level, thus suggesting that vascular endothelial growth factor and basic fibroblast growth factor contribute to both the angiogenesis and hypervascularity of neurofibroma.

Introduction

The development of a tumor is well known to require oxygen and nutrients, which are supplied through neovascularization. Angiogenesis is not a passive process: it is driven by the production of tumor and/or host derived angeogenic factors(1). Several factors participating in the development of microvasculature have been identified, and the most important ones include; transforming growth factor (2) and (3), tumor necrosis factor (4), acidic and basic fibroblast growth factor (FGF) (5, 6), platelet-derived growth factor-B (PDGF-B) (7) and hepatocyte growth factor (HGF) (8). VEGF is an endothelial cell specific mitogen which induces angiogenesis and vascular permeability in vivo. Of the various angiogenetic factors described so far, there is mounting evidence that VEGF may be a prime regulator of normal and tumor angiogenesis (9-11). Anti-VEGF strategies including the use of neutralizing antibodies (12), antisense oligonucleotide (13) and dominant-negative receptors (14) have also demonstrated tumor growth to be angiogenesis-dependent and while tumor angiogenesis is VEGF-dependent. It selectively acts on the endothelial cells that express VEGF receptor; fms-like tryrosine kinase-1 (flt-1) or KDR/ flk-1, while other angiogenetic factors, such as basic FGF, act ubiquitously. Basic FGF was the first angiogenic cytokine to be identified (15). Basic FGF stimulates endothelial cell proliferation in vitro, induces angiogenesis in vivo and is also frequently present at sites of capillary growth (16, 17). PDGF-B is also one of several known growth factors since it is involved in the regulation of endothelial cell proliferation, migration and cord formation (18). PDGF-B has been

reported to be highly expressed in several types of carcinoma (19). HGF is a mesenchymal-derived mitogen, isolated first from the sera of patients with hepatic failure (20). HCG has mitogenic, motogenic, and morphogenic functions in vitro in various types of epithelial cells (21). Recent studies have also indicated HGF to be a powerful inducer of angiogenesis (22, 23).

Neurofibroma is a typical hypervascular tumor, which occasionally causes a large amount of bleeding during an operation. Intra-tumoral bleeding in large diffuse plexiform-type neurofibromas is a life-threatening problem for patients with neurofibromatosis-1 (NF-1). However, the relationship between the vascularity of neurofibroma and the expression of angiogenetic factors has not yet been investigated. In the present study, we describe the expression of VEGF, basic FGF, PDGF-B and HGF in neurofibroma, because these substances directly induce the proliferation of endothelial cells (24).

Materials and Methods

Sibjects and tissue samples: Five neurofibroma samples were obtained during a surgical resection from five independent patients with NF-1 and two diffuse-type neurofibroma samples were obtained from two different patients with NF-1. The samples were each divided into three pieces, which were then subjected to fixation in formalin for immunohistochemical examination, RNA extraction for RT-PCR and cell culture, respectively.

Immunohistochemical staining: Consecutive 4 μm sections were cut from each paraffin-embedded

study block. The sections were immunostainted for factor VIII, VEGF, basic FGF, PDGF-B and HGF. Immunohistochemical staining was performed using the immunoperoxidase technique. The antibodies used included a mouse monoclonal antibody at a 1: 100 dilution for VEGF (sc-7296, Santa Cruz Biotechnology, CA, USA) and a rabbit polyclonal antibody at a 1: 100 dilution for basic FGF (sc-79, Santa Cruz Biotechnology), a rabbit polyclonal antibody (PC21, Calbiochem, MA, USA) at a 1: 10 dilution for PDGF-B, a rabibit polyclonal antibody (7500P47, Immunobiological Laboratory, Gunma, Japan) at a 1: 10 dilution for HGF and a rabbit polyclonal antibody (A0082, Dako Co., CA, USA) at a 1: 250 dilution for factor VIII.

Microvessel density count: The number of microvessels was recorded by counting the capillaries or small venules that were positively stained for factor VIII in a 40 x microscopic field (0.331 mm^2), while selecting several of the most vascularized areas, and five of these counts were used to be determine the microvessel density for each case.

RT-PCR analysis of the transcript of the angiogenetic fators: The resected sample tissue specimens (2.0 g in weight) were homogenized with a Polytron (Kinematica-Ag, Lucerne, Switzerland) homogenizer at high speed for one minute in 10 ml of guanidium thiocyanate and phenol (Isogen RNA extraction kit, Nippon Gene Co., Tokyo, Japan). First-strand cDNA was synthesized by using a Ready to Go T-primed first-strand kit (Pharmacia Biotech, NJ, USA). PCR amplification was performed using 1 U of Taq polymerase (Amasham Life Science, OH, USA) according to the protocol described in the

GeneAmp DNA amplification kit (Perkin-Elmer, CA, USA). In the amplification process, a 5 μl aliquot of the first-strand cDNA pool was denatured for 1 min at 94°C, followed by 27 amplification cycles of denaturation for 0.5 min at 94°C, anealing for 0.5 min at 56°C, and extension for 1 min at 72°C. At the end of the 27 cycles, an additional extension was carried out at 72°C for 10 min. The primers used in the PCR consited of the following: 5'-CTCGCCTTGCTGCTCTACCTC-3' (forward primer for VEGF), 5'-AAGCTGCCTCGCGCAAGGCCC-3' (reverse primer for VEGF), 5'-TCACCACGCTGCCCGCCTTGCCCG-3' (forward primer for basic FGF), 5'-GTATAGCTTTCTGCCCAGGTCCTG-3' (reverse primer for basic FGF), 5'-AATCGCTGCTGGGCGCTCTTCCTG-3' (forward primer for PDGF-B), 5'-GGCTGCAAGGGTCTCCTTCAGTGC-3' (reverse primer for PDGF-B), 5'-GCACGACAGTGTTTCCCTTCTCG-3' (forward primer for HGF), 5'-TGGATTGGCGCATCCACGGCCGGG-3' (reverse primer for HGF). To normalize the amount of mRNAs, the G3PDH mRNA, which is known to be a housekeeping gene, was amplified using the same cDNA pools as those described above and then subjecting them to agarose gel electrophoresis.

Cell culture: The central portion of the cutaneous neurofibroma was cut into small tissue pieces (1 x 1 x 1 mm) and then was cultured in a culture medium consisting of Dulbecco's modified Eagle medium (Gibco BRL, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum, 12 mM Hepes, 2

mM L-glutamine, 0.03 mM 2-ME, 100 mg/ml penicillin and streptomycin and 50 mg/ml gentamycin. After 7 days, the spreading cells from tissue pieces were harvested and then subjected to a second culture. These second culture cells were used for VEGF secretion assay. Dermal fibroblasts primarily cultured from the non-tumor dermal portion of the patients with NF-1 were used as controls.

VEGF secretion by the neurofibroma-derived cells: The amount of secreted VEGF in the culture medium of the neurofibroma-derived cells and dermal fibroblasts was measured using the enzyme-linked immunosorbent assay (ELISA) method. The supernatants were assayed for VEGF using the human VEGF ELISA kit (Immunobiolaboratory, Gunma, Japan). The mean value was determined from three independent experiments, and all measurements were performed in duplicate for each experiment. The amounts of VEGF were assessed after correcting for the number of cells.

Results

Microvessel counts: The mean vessel counts by factor VIII staining for neurofibroma, diffuse-type neurofibroma, neurilemmoma and normal dermais are shown in Table 1. The microvessel density in the neurofibromas and diffuse-type neurofibromas was significantly higher than that in neurilemmomas and normal dermis. No significant difference was observed in the microvessel count between the nerofibromas and diffuse-type neurofibromas.

Immunohistochemical staining: Immunohistochemical staining for VEGF and basic FGF showed a

strong immunoreactivity in diffuse-type neurofibroma cells, while that for PDGF-B and HGF showed either only a faint degree of immunoreactivity or none at all (Fig.1 a-d, Fig.2 a,b). Neurofibroma cells showed the same immunoreactivity for VEGF, basic FGF, PDGF-B and HGF as diffuse-type neurofibroma cells (data not shown). In addition, neurilemmoma cells showed only a faint immunoreactivity for VEGF (Fig. 2 c).

RT-PCR analysis of the transcript of the angiogenetic fators: Fig. 3 shows the results of the electrophoreic analysis of PCR products. The levels of VEGF and basic FGF mRNA expression in neurofibroma were thus shown to be higher than that in the normal dermis (Fig. 3 lane 1-4), while the PDGF-B mRNA expression in the normal dermis was higher than that in neurofibroma (Fig. 3 lane 5, 6). HGF mRNA was scarcely detected in either the neurofibroma or the normal dermis (Fig. 3 lane 7, 8).

Secretion of VEGF from neurofibroma-derived cells: After a 96-hour culture, VEGF secretion from neurofibroma-derived cells in the culture supernatants was much higher (378 ± 49 ng/ml/10^6 cells) than that from normal dermal fibroblasts (36 ± 7.9 ng/ml/10^6 cells) (Fig. 4).

Discussion

Angiogenesis is well known to be essential for the progression and invasion of solid tumors in addition to normal tissue development and wound healing (24). For the neovascularization of tumors a critical number of its cells have to switch to the angiogenic phenotype. The angiogenic activity of

tumors arises from the tumor cell itself in the form of the release of such angiogenic molecules as VEGF (25). Up to now, several angiogenic factors have been identified, including: FGF, VEGF, PDGF-B and HGF.

A common abnormality manifested in neurofibromatosis-1 is the development of peripheral nerve tumors called neurofibromas, which contain abundant capillaries and small vessels. Sheela S et. al. reported that neurofibroma-derived Schwann-like cells promote angiogenesis in the chicken chorioallantoic membrane model system (26). Takamiya Y et. al. reported that AGM-1470, a fungal-derived synthetic inhibitor of angiogenesis, effectively inhibited the growth of human neurofibromas implanted into mice (27). However, the angiogenic molecule which contributes the most to angiogenesis in neurofibroma has yet to be elucidated. We demonstrated that both protein and mRNA of VEGF and basic FGF are strongly expressed in conventional and diffuse-type neurofibroma tissue while PDGF-B and HGF were not detected. Furthermore, neurilemmoma, which commonly arises from neurofibromatosis-2 and does not contain any abundant vessels, was also shown to express a small amount of VEGF protein. These findings strongly suggest VEGF and basic FGF to be major potent angiogenesis-promoting factors in the pathogenesis of neurofibroma. Both factors possibly act independently because there is still no evidence indicating that VEGF and basic FGF interact with each other. Basic FGF may therefore also contribute to the proliferation of neurofibroma cells because basic FGF is known to be a mitogen for mesenchymal cells (28).

Figure legends

Table 1

Microvessel counts in a 40x microscopic field (0.331 mm^2). (NF: neurofibroma, DNF: diffuse-type neurofibroma, NL: neurilemmoma, a-c: independent patients with neurofibromatosis-1.)

Figure 1

Low magnification photographs of immunohistochemical staining for angiogenic factors. a: VEGF (x 40), b: basic FGF (x 40), c: PDGF-B (x 40), d: HGF (x 40). A dotted line indicates the boundary between the normal dermis and neurofibroma tissue.

Figure 2

High magnification photographs (x 100) of immunohistochemical staining for VEGF and basic FGF. a: VEGF expression in neurofibroma, b: basic FGF expression in neurofibroma, c: VEGF expression in neurilemmoma.

Figure 3

Upper line of photographs; the expression of mRNA in the angiogenic factors (VEGF, basic FGF, PDGF-B and HGF) in either neurofibroma or normal dermis tissue analyzed by RT-PCR. (lane 1: VEGF

in normal dermis, lane 2: VEGF in neurofibroma, lane 3: basic FGF in normal dermis, lane 4: basic FGF in neurofibroma, lane 5: PDGF-B in normal dermis, lane 6: PDGF-B in neurofibroma, lane 7: HGF in normal dermis, lane 8: HGF in neurofibroma) Lower line of photographs; G3PDH mRNA as a control.

Figure 4

The secretion of VEGF from neurofibroma-derived cells and dermal fibroblasts. After a 96-hour culture, the VEGF concentration in the culture supernatants was determined by ELISA.

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