Circulating Growth Factor Levels Are Associated with Tumorigenesis in Neurofibromatosis Type 1

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ABSTRACT

Purpose: Neurofibromatosis type 1 (NF1) is characterized by systemic development of neurofibromas. Early clinical diagnosis can be ambiguous, and genetic diagnosis can be prohibitively difficult. Dysregulation of a number of growth factors has been suggested to be a mechanism of pathogenesis. This study was performed to assess the contribution of circulating growth factors for diffuse tumorigenesis and the diagnostic value of circulating growth factor identification in serum.

Experimental Design: The growth stimulation of neurofibroma-derived cells by serum from NF1 patients was tested, and serum growth factor levels in a cohort of NF1 patients (n = 39) between the ages of 7 and 70 years were analyzed.

Results: Concentrations of midkine (MK) and stem cell factor, but not epidermal growth factor, were substantially increased in serum of NF1 patients when compared with healthy controls. Within the NF1 group, MK levels increased dramatically at puberty from an average of 0.79 ng/mL in patients <18 years to 1.18 ng/mL in patients >18 years old. Stem cell factor and MK concentrations above a defined threshold in serum of NF1 patients are of diagnostic benefit for 96% of patients in the cohort tested. Furthermore, serum from NF1 patients enhanced proliferation of human neurofibroma-derived primary Schwann cells and endothelial cells substantially better than normal serum.

Conclusions: Enhanced circulating growth factor levels contribute to diffuse tumorigenesis in NF1 and may provide the basis for molecular diagnosis.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a genetic disorder of the nervous system affecting approximately 1 in 3,000 individuals, and about half of these are spontaneous cases (1, 2). NF1 has an autosomal dominant mode of transmission, variable expressivity, and nearly complete penetrance (3, 4). NF1 is associated with mutations in the tumor suppressor gene NF1, which encodes for the Ras-GTPase-activating protein neurofibromin (5–7). Abnormalities of neurofibromin lead to a plethora of clinical phenomena, including café-au-lait macules, Lisch nodules, axillary freckling, and the characteristic feature of multiple cutaneous neurofibromas. Patients may also have plexiform neurofibromas, malignant peripheral nerve sheath tumors (MPNSTs), benign astrocytomas, and optic pathway gliomas. There are a host of other neurologic, orthopedic, hematologic, dermatologic, and systemic disorders associated with NF1 (reviewed in ref. 8).

Diagnosis relies on the early clinical signs of NF1 (9), which may appear within the first 2 years of life and tend to be melanogenic abnormalities such as café-au-lait macules. Congenital plexiform neurofibromas are frequently present in NF1 children and develop with distinct growth properties and fluctuating growth rates (10). In contrast, cutaneous neurofibromas generally start to appear at puberty and increase in number with age. The number and size of cutaneous neurofibromas vary widely between patients, and the presence of thousands of these benign tumors is not rare.

Due to the difficulties of direct NF1 mutational screening and the intrinsic ambiguities of clinical diagnosis, the need for a biochemical diagnosis of NF1 remains. This problem may be addressed by a defined profile of circulating proteins detectable in serum of patients. The basis of such a matrix is likely formed by secreted proteins specifically up-regulated in tumors of NF1 or induced as a consequence of NF1 haploinsufficiency (11–13). Paracrine growth stimulation of tumor cells by neurons and fibroblasts has also been suggested (14, 15). Among others, stem cell factor (SCF), midkine (MK), and epidermal growth factor (EGF)-regulated pathways have been implicated in the stimulation of neurofibroma growth (16–20).

SCF is required for the development of migratory cells such as hematopoietic stem cells, germ cells, and melanocytes (21). SCF and its tyrosine kinase receptor, Kit, are both up-regulated in neurofibromas and neurofibromin-deficient Schwann cells, whereas expression in normal Schwann cells...
is undetectable (22). Dysregulation of the SCF-Kit loop in NF1 modulates the growth of neurofibroma cells, mast cells, and hematopoietic cells (23, 24). Similarly, EGF receptor expression is up-regulated in neurofibromas, and activation by EGF strongly stimulates proliferation of neurofibromin-deficient Schwann cells and MPNST-derived cells in vitro (25, 26).

The M, 13,000 growth and angiogenic factor MK stimulates proliferation of neurofibroma-derived cells and endothelial cells in vitro and is up-regulated with loss of neurofibromin in Schwann cells, neurofibromas, and MPNSTs. It has also been detected in symptomatic and asymptomatic epidermis of patients with NF1, but not in healthy individuals (11, 20). MK is normally expressed in the neuroectoderm during midgestation, and it is down-regulated in most adult tissues (27). It has been shown to play a role in neural migration, as well as neural differentiation, maturation, and survival (28–32). MK has also been found to be up-regulated in a variety of cancers (reviewed in refs. 27 and 33), including tumors of the nervous system (34, 35), and is thought to mediate tumorigenesis and angiogenesis (36, 37). MK has been detected by enzyme-linked immunosorbent assay (ELISA) in the serum of patients with hepatocellular carcinoma, gastric carcinoma, and lung carcinoma, whereas MK levels in normal serum were low (38, 39).

We investigated the utility of the neurofibroma-derived secreted factors MK, SCF, and EGF in serum of NF1 patients for their potential role in multifocal tumorigenesis and as diagnostic markers. We report increased growth factor serum levels in NF1 patients and a marked MK up-regulation during puberty, a critical stage of pathogenesis. Furthermore, we demonstrate enhanced stimulation of endothelial and Schwann cell proliferation by serum of NF1 patients.

MATERIALS AND METHODS

Patient Selection. Patients were accrued at Klinikum Nord Hamburg-Ochsenzoll and at Massachusetts General Hospital. All NF1 patients were clinically diagnosed according to National Institutes of Health criteria (8, 9). The age of NF1 patients (n = 39; 18 males and 21 females) ranged from 7 to 70 years (average age, 28.4 years). Patients with NF1 or neurofibromatosis type 2 or patients diagnosed for any tumor were excluded from the control group (n = 35). The control group was age- and gender-matched to the NF1 group (age range, 5–66 years; average age, 33.7 years; 18 females and 17 males). Patient accrual and serum collection were guided by institutional review board-approved protocols.

The NF1 cohort was initially unselected with respect to the patient’s tumor phenotype. Clinical evaluation of the tumor phenotype within the NF1 group was subsequently performed. The tumor burden of NF1 patients was quantified by counting the number of cutaneous neurofibromas in defined body areas. The size of these tumors and total tumor mass were not determined. The presence or absence of plexiform neurofibromas was recognized by accompanying features such as pigmentation and hair abnormalities and documented by magnetic resonance imaging (MRI). MPNSTs were diagnosed by MRI and confirmed by histopathology. Astrocytoma and optic pathway glioma were diagnosed by MRI but not confirmed by histo-

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Abbreviations: CNF, cutaneous neurofibroma; ND, not detected; POA, postoperative astrocytoma; PNF, plexiform neurofibroma; Mamma-Ca, mammary carcinoma.

* No plexiform neurofibromas were diagnosed.

Table 1 Tumor diagnosis, age, and sex of NF1 patients included in this study

pathology, except for one pilocytic astrocytoma for which postoperative histopathology confirmed the diagnosis (Table 1, case 27).

Serum Collection and Enzyme-Linked Immunosorbent Assay. Venous blood (1–10 mL) was drawn, and serum was subsequently prepared within a 2-hour period and stored in aliquots at −80°C until use. It was critical to prepare serum immediately after blood draws for accurate MK quantification. MK ELISA was performed essentially as described previously (38). Briefly, 10 μL of serum were diluted in 100 μL of peroxidase-conjugated MK-antibody solution, of which 50 μL were added to each well of a MK antibody-coated 96-well plate and incubated for 60 min. Wells were washed extensively (1% Tween in PBS), 100 μL of substrate solution (0.5 mg/mL tetramethylbenzidine in DAKO S 1600) were added, and plates were incubated for 30 minutes. The reaction was stopped with 2 N sulfuric acid, and A450 nm was measured using a multwell
were measured in triplicates. Detection limit was 0.05 ng MK/mL serum. For EGF and SCF, ELISA kits were purchased from Biosource (Camarillo, CA) and R&D Systems (Minneapolis, MN), respectively. Analyses were performed according to the manufacturers’ instructions.

Statistics. Statistical significance was evaluated using Student’s t test and Mann-Whitney U tests for unpaired comparisons. Correlations were tested using the Spearman rank correlation coefficient. Statistical calculations were performed using StatView (Abacus, Berkeley, CA).

Proliferation Assays. Primary human umbilical vein endothelial cells (HUVECs) were obtained from Glyco Tech Corp (Rockville, MD). Primary Schwann cells from a neurofibroma of a NF1 patient were cultivated under standard conditions to >80% purity (40). Primary cells from passage < 5 were used for proliferation assays, and 5,000 cells were plated in triplicates into 12-well tissue culture inserts (0.45-μm pore size; Transwell; Costar, Cambridge, MA) in cell-specific complete growth medium for Schwann cells or endothelial cells, respectively. After 24 hours, medium in the inserts was replaced with serum-free Dulbecco’s modified Eagles’s medium, and inserts were placed into wells filled with Dulbecco’s modified Eagle’s medium and 50% heat-inactivated serum from NF1 patients and serum from healthy control subjects as indicated in Figs. 1 and 3, respectively. Cells were fixed at day 2 and day 4 in 4% paraformaldehyde and stained in 0.1% crystal violet solution. Five independent fields at fixed positions were counted in each insert using an inverted microscope at a magnification of ×100. All assays were performed in triplicates, and serum samples were tested separately. In a parallel assay, tripary blue exclusion was used to determine the numbers of dead cells at days 2 and 4. The identity of the Schwann cells was verified by immunohistochemistry using an anti-S100 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (40).

RESULTS

Serum from NF1 Patients Enhances Human Umbilical Vein Endothelial Cell and Schwann Cell Proliferation. To test directly whether serum from NF1 patients contains circulating factors that may stimulate growth of neurofibromas, serum from NF1 patients and healthy controls was tested for its effects on HUVEC and neurofibroma-derived primary Schwann cell proliferation. To investigate the effects of the different serum samples, we used Transwell cell culture inserts to separate the cells from the serum by a permeable membrane. Cells were exposed to the different serum samples for 2 and 4 days. Six matched serum samples were individually tested from each group. There was a significantly increased proliferation of both HUVECs and Schwann cells that were exposed to serum from NF1 patients compared with serum from healthy subjects at day 4 (Fig. 1A; P < 0.0002 for both HUVECs and Schwann cells, unpaired t test). No significant difference was found on day 2 (data not shown). The integrity of the Schwann cell population was verified by S100 immunostaining (Fig. 1B and C). The number of dead cells did not differ between groups during the culture period.

Midkine and Stem Cell Factor Serum Concentrations Are Increased in NF1 Patients. Increased cell proliferation of neurofibroma cells may be supported by circulating growth factors. To investigate this possibility, we tested the serum levels of SCF, EGF, and MK. MK and SCF are overexpressed in neurofibromas, and all three factors were shown to stimulate Schwann cell proliferation in vitro.

MK levels in serum of NF1 patients were significantly elevated compared with serum from healthy controls (P < 0.0002, unpaired t test; Fig. 2A). The mean serum concentration in the NF1 group (n = 39) was 1.1 ng/mL (median, 1.12 ng/mL), whereas in the control group (n = 35), the mean MK concentration was 0.78 ng/mL (median, 0.738 ng/mL). Both groups are age- and gender-matched. No correlation between MK levels and gender was found in either group.

Measurement of SCF serum levels in the same cohorts of NF1 patients and healthy subjects revealed increased serum concentrations in the NF1 group (P = 0.0004, unpaired t test; Fig. 2B). No correlations between SCF levels, gender, or age were found in either group. SCF concentrations in patients with glioblastoma multiforme are not different from those in healthy individuals (data not shown).

EGF receptor is expressed in most neurofibroma-derived cells, and EGF has been implicated in promoting neurofibroma growth (26). However, EGF is not overexpressed in neurofibromas. Analysis of serum EGF concentration in the NF1 and healthy subject groups did not reveal any correlation of EGF serum concentrations with diagnosis of NF1, age, gender, or number and type of tumors (data not shown).

Stem Cell Factor and Midkine Serum Concentrations as Diagnostic Markers for NF1. Combining MK and SCF serum concentrations resulted in a slight but significantly better differentiation between NF1 patients and healthy subjects (P <
Surrogate Markers in NF1

SCF serum concentrations of 95% (37 of 39) of NF1 subjects by using threshold levels of NF1 and healthy individuals. Diagnosis of NF1 was confirmed published data (38) and chosen to maximize differentiation between NF1 patients and healthy individuals. Diagnosis of NF1 was confirmed 0.0001; Fig. 2

An age-dependent increase of SCF serum concentrations was not found. In contrast, SCF serum concentrations were significantly different from serum concentrations in healthy subjects in all age groups (P = 0.0445 for <18 years; P = 0.0048 for >18 years; Fig. 3C). As for the undivided cohort (Fig. 2C), the differentiation between NF1 and healthy subjects increases slightly but significantly when MK and SCF are combined for analysis in the age group ≤18 years (P = 0.0038; data not shown).

High Midkine Serum Levels Stimulate Endothelial Cell Proliferation. To test whether differential MK levels in serum from NF1 patients modulate proliferation of Schwann cells and endothelial cells, NF1 serum samples were divided into two groups: (a) serum from NF1 patients with low MK levels (<0.7 ng/mL) and a low number of cutaneous neurofibromas, and (b) serum from NF1 patients with high MK levels (>1.1 ng/mL) and a high number of cutaneous neurofibromas (n = 3 for each group). There was no significant variation in the SCF serum concentrations in the samples used. Proliferation of HUVECs and primary Schwann cells was determined in 12-well cell culture inserts to separate the cells from medium containing 50% serum. Cells were counted after 4 days (Fig. 3D). There was significantly increased proliferation of HUVECs that were exposed to serum from NF1 patients with high MK levels (P = 0.041, unpaired t test), whereas the difference in Schwann cell number did not reach statistical significance at day 4 (Fig. 3D).

Association of Midkine Serum Levels and Tumors in NF1 Patients. The number of cutaneous neurofibromas generally increases with age, and increased systemic MK levels in NF1 may reflect an increased number of NF1-expressing tumor cells. However, correlation of MK serum levels with the number of cutaneous neurofibromas did not reach statistical significance, although there is a tendency for a large number of cutaneous neurofibromas to be accompanied by elevated MK serum levels (R² = 0.31; Table 1).

MK levels appeared to be increased in patients with optic pathway gliomas and MPNSTs (Table 1). In the NF1 age group <18 years (n = 10), three patients were diagnosed by MRI with optic pathway glioma. All three patients had above average MK serum concentrations accompanied by a low count of cutaneous neurofibromas. Interestingly, one patient (case 27), who had a pilocytic astrocytoma removed 3 years earlier, showed MK levels that did not differ from those of the (>18) age group. The preoperative MK serum concentration is not known for this patient.

Both NF1 patients with MPNST had MK serum levels elevated above average in their age group (>18). The presence of a plexiform neurofibroma had no effect on MK serum levels.

Midkine Serum Concentrations in NF1 Increase Sharply at Early Adulthood. Surprisingly, we observed a biphasic pattern of MK concentrations in NF1 patients with a relatively sudden age-dependent increase in MK levels, which was not found in the control group (Fig. 3A and B). The average MK level in NF1 patients <18 years of age was 0.789 ng/mL (n = 10). This corresponded to the mean MK level in the control group, but it was significantly lower than the mean MK level in the NF1 patients >18 years of age (n = 29; 1.184 ng/mL; P = 0.0054, unpaired t test). The correlation between MK serum levels and age is statistically significant at R² = 0.46 (Fig. 3B). The sharp increase of MK serum levels in NF1 patients coincides with onset of cutaneous neurofibroma development around puberty.

An age-dependent increase of SCF serum concentrations was not found. In contrast, SCF serum concentrations were significantly different from serum concentrations in healthy subjects in all age groups (P = 0.0445 for <18 years; P = 0.0048 for >18 years; Fig. 3C). As for the undivided cohort (Fig. 2C), the differentiation between NF1 and healthy subjects increases slightly but significantly when MK and SCF are combined for analysis in the age group ≤18 years (P = 0.0038; data not shown).

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Both NF1 patients with MPNST had MK serum levels elevated above average in their age group (>18). The presence of a plexiform neurofibroma had no effect on MK serum levels.
No associations were found between SCF or EGF serum concentrations and tumor burden or tumor type in the NF1 group.

**DISCUSSION**

Tumors express and secrete a variety of growth factors, which may ultimately be found in the serum of cancer patients (13). Besides their potential benefit for serum-based tumor diagnosis and classification, these circulating growth factors may also promote systemic tumor growth and are potential therapeutic targets. Targeting of circulating heparin-binding proteins by pentosan polysulfate has indeed shown efficient serum depletion of these factors (41). The reduced potency of pentosan polysulfate-treated serum to stimulate tumor cell growth in vitro provides strong support for the concept of paracrine tumor growth stimulation by circulating proteins. On the other hand, unspecific targeting of heparin-binding proteins may deplete serum of coagulation factors and angiogenesis inhibitors secreted by primary tumors (42). Thus, to implement more specific targeting, knowledge of the nature of circulating factors in a specific disease context is required.

The secreted heparin-binding growth factor MK is expressed in the epidermis and tumors of NF1 patients (11, 20), making it a candidate for molecular diagnosis and also a potential therapeutic target. Similarly, SCF is expressed and secreted by neurofibromin-deficient Schwann cells (22). This study identifies both MK and SCF as circulating serum factors elevated in NF1 patients. Elevated MK serum concentrations in NF1 patients are only found at adulthood, whereas MK levels in younger patients are not different from those of healthy controls. Because the biphasic mode of MK serum concentrations was not observed in healthy controls, it is unlikely that MK up-regulation simply reflects changes in hormone status. Whether the previously observed aberrant regulation of MK expression in NF1 (11, 20) is sensitive to hormonal changes remains to be studied.

The increase of MK serum levels coincides with the disseminated development of cutaneous neurofibromas in NF1, which most often begin to appear in early puberty. The MK concentrations do not strictly correlate with the number of cutaneous neurofibromas in our unselected NF1 cohort. Additional studies are under way to more accurately determine total cutaneous neurofibroma mass by measuring the size and depth of infiltration of each individual tumor in addition to tumor numbers in a selected cohort of patients.

The argument that MK and SCF may contribute to systemic tumor growth is supported by earlier data that show that both factors stimulate proliferation of MPNST-derived cells, whereas MK also stimulates proliferation of endothelial cells (20, 37, 43). Here, we show that proliferation of neurofibroma-derived primary human Schwann cells is markedly increased by serum from NF1 patients. Part, but not all, of this stimulation is associated with elevated MK concentrations. Additional factors present in NF1 serum are necessary to account for the observed Schwann cell stimulation, whereas MK seems to be a significant factor to stimulate endothelial cells. It is surprising that a 2-fold difference in serum concentrations of only two factors significantly affects cell proliferation in vitro. It has been suggested, however, that small changes in the
expression of oncogenes may have a strong effect on tumor growth and can perhaps explain the periodic growth bursts seen in NF1 (12, 44).

Together, the current data suggest that circulating growth factors may stimulate the growth of cutaneous neurofibromas, whereas the number of these tumors may be determined by other genetic or epigenetic mechanisms (45, 46). It is unlikely that any circulating factor alone is sufficient to enhance proliferation of neurofibroma cells in a physiologic situation. We propose that a matrix of circulating serum factors, in addition to SCF and MK, is crucial to reach a threshold sufficient for systemic tumor growth stimulation and may be identifiable (12). Several other serum factors are candidates with clinical relevance as both circulating tumor stimulators and diagnostically useful markers in NF1. Increased serum levels of fetal antigen 1 and nerve growth factor have been demonstrated in NF1 patients (47–49). Interestingly, and similar to MK, higher fetal antigen 1 serum levels in children with NF1 were only observed in the context of cerebral and spinal pathology (47). Nonpeptidic serum factors such as progesterone and docosahexenoic acid may also be associated with tumor growth (50, 51). A broader approach to identify a NF1-specific serum protein matrix may help to understand the fluctuating growth patterns typical for tumors in NF1.

MK serum concentrations appeared not to be influenced by the presence of plexiform neurofibromas. However, the two NF1 patients in the cohort with MPNST showed extremely high MK serum concentrations. Prospective monitoring of MK serum levels will establish whether a further elevation may indicate the development of MPNSTs from preexisting plexiform neurofibromas. Similarly, the presence of an optic glioma in three NF1 patients correlated with exceptionally high serum MK levels in their age group, corroborating earlier studies showing increased MK expression in astrocytoma (35). This finding may suggest that the presence of astrocytomas in young patients with NF1 is associated with increased MK serum levels. However, to establish a diagnostic relationship, it will be necessary to analyze a larger patient population with defined and confirmed astrocytoma diagnosis.

In conclusion, we have used existing knowledge of growth factor and receptor expression in neurofibromas to identify MK and SCF as two components of a matrix of secreted proteins detectable in serum of NF1 patients that may have pathogenic, diagnostic, and prognostic significance. Concentration changes of SCF, MK, and other unidentified factors may precede measurable regression or stable disease in patients. Using these surrogate markers may be useful to assess the efficacy of innovative treatment approaches.

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George A. Mashour, Pablo Hernández Driever, Melanie Hartmann, et al.


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