Midkine lacking its last 40 amino acids acts on endothelial and neuroblastoma tumor cells and inhibits tumor development

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Abbreviations: MDK, Midkine; MDKΔ81-121, Midkine lacking 81 to 121 residues; PTN, Pleiotropin; ALK, Anaplastic Lymphoma Kinase; RPTP, Receptor-like Protein Tyrosine Phosphatase; LRP, LDL-receptor-Related Protein; HBGF, Heparin-Binding Growth Factor; HUVEC, Human Umbilical Vein Endothelial Cell; SMC, Smooth Muscle Cell; CHOP, C/EBP Homologous Protein
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Abstract

Midkine (MDK) is a member of a new family of neurotrophic factors considered as rate-limiting growth and angiogenic factors implicated in the onset, invasion and metastatic process of neuronal tumors including neuroblastoma. We showed that all neuroblastoma cell lines highly expressed MDK indicating that it is a critical player in tumor development, which may henceforth represent an attractive therapeutic target. We showed that the knockdown of MDK expression by siRNA led to a marked and significant decrease in neuroblastoma (IGR-N91 and SH-SY5Y) cell proliferation \textit{in vitro}. Using a new strategy, we then evaluated the antitumor effect of a truncated MDK protein, lacking the C-terminal 81-121 portion of the molecule (MDK$_{\Delta81-121}$), which may act as a dominant-negative effector for its mitogenic, angiogenic and tumorigenic activities by heterodimerizing with the wild type protein. \textit{In vitro} studies showed that MDK$_{\Delta81-121}$ selectively inhibited MDK-dependent tumor cells and was able to strongly reduce endothelial cell proliferation and migration and to induce ER stress-mediated apoptosis.

We then investigated the effects of MDK$_{\Delta81-121}$ \textit{in vivo} using electrotransfer of a plasmid encoding a secretable form of MDK$_{\Delta81-121}$ into \textit{tibialis cranialis} muscles of nude mice. We showed that MDK$_{\Delta81-121}$ dramatically inhibited (up to 91\%) tumor development and growth. This inhibition was correlated with the detection of the MDK$_{\Delta81-121}$ molecule in plasma and the suppression of intratumor neovascularization. Our findings demonstrate that MDK inhibition is a tractable therapeutic target for this lethal pediatric malignancy.
Introduction

Over the last decade, a number of new antiangiogenic agents have demonstrated their ability to retard tumor growth and some of them are physiological angiostatic factors that are derived upon proteolytic cleavage of native proteins. In this field, we previously evaluated antiangiogenesis and antitumor activities of a 38-kDa internal fragment of plasminogen, a 24-kDa noncollagenous domain of collagen type IV α-chains and a 20-kDa fragment of collagen XVIII, called respectively angiostatin, canstatin and endostatin (1–3). All these fragments are known to silence cytokine-induced angiogenesis pathways by binding to integrin receptors and triggering mitochondrial apoptosis (4–6). They specifically target endothelial cells, do not possess antineoplastic activities and should therefore be administered in combination with chemotherapy or radiotherapy to achieve a major clinical response (4,7,8). Thus, the development of new anticancer strategies targeting both endothelial and neoplastic compartments should be advantageous. In this field, we already designed truncated proteins that are dominant-negative effectors of native growth factors specific to endothelial and tumor cell proliferation. They are capable of silencing the native protein by forming inactive heterodimeric molecules (9). In particular, we demonstrated that a truncated Pleiotrophin (PTN), lacking the C-terminal 111-136 portion of the molecule (PTNΔ111-136), acted as a dominant-negative mutant that selectively inhibited in vitro the proliferation of a PTN-dependent MDA-MB-231 breast tumor and endothelial cells by silencing the VEGF-A and HIF1α pathways and breast tumor growth in vivo by 81% (10).

In the present study, we assessed the antiangiogenesis and antitumor activities of a new dominant-negative mutant derived from the Midkine (MDK) growth factor. MDK is a 121 amino acid protein that like PTN belongs to the Heparin-Binding Growth
Factor (HBGF) family and is constitutively active when it forms homodimers that are stabilized by heparin (11). Although MDK is composed of two major N- and C-terminal domains held together by disulfide linkages, most protein activities are performed by C-terminal domain. That is the case of the lysine-rich domain of PTN 111-136 C-terminal residues, which is required for its mitogenic and tumorigenic activities (12). MDK was identified as the ligand of different tyrosine kinase receptors such as Anaplastic Lymphoma Kinase (ALK), as well as the Receptor-like Protein Tyrosine Phosphatase (RPTPs) and the LDL-receptor-Related Protein families (LRPs) (13–15). Through these interactions, MDK is involved in important cellular functions, promoting the growth of tumor cell lines (16) and cell survival by inhibiting apoptosis (17) and was shown to promote tumor angiogenesis (18). In particular, MDK was shown to correlate with tumor progression and invasiveness of neuroblastoma. Indeed, plasma MDK levels were found to be significantly correlated with the status of N-myc amplification, TRKA expression and stage, which are known prognostic factors in neuroblastoma (19). In this study, we assessed the effects of the 81-121 deleted MDK (MDKΔ81-121) lacking its last 40 amino acids, and for the first time, demonstrate that this truncated protein could act as a potent inhibitor of tumor growth and angiogenesis in a neuroblastoma tumor model.
Materials and Methods

Plasmid DNA

The pcDNA3-LacZ control plasmid encoding β-galactosidase was constructed as previously described (10). The pcDNA3-MDKΔ81-121-MycHis plasmid was obtained by cloning the MDKΔ81-121 fragment between the BamHI and the EcoRI sites of the pcDNA3.1(+)myc-HisA plasmid (Invitrogen). All plasmids were purified using endofree Plasmid Maxi Kit (Qiagen), and solubilized in endotoxin-free 0.9% NaCl.

Cell Culture and Conditioned Medium

Human neuroblastoma cell lines KCNR, CLB-PE, LAN-1, GIMen were a kind gift of Dr Janoueix-Lerosey (20). The human IGR-N91 cell line was a kind gift of Dr Bénard and was established from an infiltrated bone marrow collected from a stage 4-neuroblastoma (21). These neuroblastoma cell lines as well as SK-N-AS (ATCC CRL2137), SH-SY5Y (ATCC CRL2266), and BE(2)-M17 (ATCC CRL2267), MRC-5 cell line (ATCC CCL-171) and monkey Cos7 cells (ATCC CRL1651) were grown in DMEM supplemented with 10% fetal bovine serum. Human umbilical Smooth Muscle Cells (SMC) were a kind gift of Dr Uzan and were isolated and characterized as described previously (22). Human Umbilical Vein Endothelial Cells (HUVEC, Lonza CC-2517) and SMC were cultured in their adequate medium from Lonza and promocell respectively (10). Experiments involving ATCC or Lonza cell lines were mostly performed in the same year they were purchased. Authors performed no authentification on the cell lines used in this paper. To obtain Cos7-MDKΔ81-121 and Cos7-LacZ cells, Cos7 cells were transfected with 8µg of either pcDNA3-MDKΔ81-121 or pcDNA3-LacZ plasmids, using jetPEI reagent (Polyplus-Transfection). The medium was changed 4 hours after transfection and stably transfected cells were selected with Geneticin/G-418 (700µg/ml, Invitrogen). After two weeks of culture,
single cells were added to a 96-well cell culture plate for subcloning and expanded in presence of G-418. Conditioned medium was harvested, aliquoted, and stored at -20°C until use.

**Small interfering RNA**

IGR-N91 and SH-SY5Y cells were seeded in 24-well tissue culture dishes, at a density of 5x10^4 cells per well in the appropriate medium. After 24 hours, MDK small interfering RNA (siRNA) (SI02663059, Qiagen) alone or in combination with PTN siRNA (SI00043764, Qiagen) and Scrambled siRNA (control) were added at a final concentration of 10nM. Three days after transfection cells were collected for real-time RT-PCR, or were used to perform proliferation assay. Cell supernatants were harvested and stored at -20°C for an ELISA assay.

**Western Blot Analysis, ELISA and Fluorescence in situ Hybridization**

Samples protein concentration was quantified using Dc Protein Assay (Biorad). PTN expression in neuroblastoma cell pellets was assessed as previously described (10). For MDK detection in cell pellets by Western Blot analysis, proteins were transferred to a nitrocellulose membrane (Pall) that was blocked in PBS 0.2% Tween and 5% powdered milk. Membranes were then probed with rabbit polyclonal anti-MDK antibody (1:5000; American Antigenix). Swain anti-rabbit peroxidase-linked antibody (1:1000; Dako) was used as secondary antibody. ALK Western Blot, was performed as previously described (10). For the RPTPβ/ζ Western Blot analysis, the protein was detected using a primary rat anti-RPTPβ/ζ antibody (1:500, R&D Systems), and a secondary anti-rat antibody. Actin was detected with monoclonal anti-alpha-actin peroxidase conjugate antibody (1:50000). MDK expression in cell supernatants was quantified by an ELISA assay using a Human Midkine Construction kit (America antigenix), according to manufacturer's instructions. Fluorescence in situ
hybridization was performed using the Vysis LSI ALK (2q23) dual color, break-apart rearrangement probe (Abbott Molecular).

**Real-Time Reverse Transcription-PCR**

The theoretical and practical aspects of real-time quantitative reverse transcription-PCR (RT-PCR) using the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems) as well as the RNA extraction method, cDNA synthesis and PCR conditions, were described in detail elsewhere (10). Target gene expression was normalized relative to an endogenous RNA control (TBP gene, which encodes the TATA box-binding protein), as previously described (10).

**Virus Construction and Infection**

AdMDKΔ81-121 is a ΔE1-ΔE3 recombinant adenovirus that expresses the truncated protein MDKΔ81-121 lacking the 40 last amino acids. The MDKΔ81-121 fragment was retrieved from pcDNA3-MDKΔ81-121 by digestion with AflII and AvrII and inserted into the pCA350 shuttle plasmid. The recombinant adenoviral genome (Ad5ΔE1E3) encoding the truncated protein was generated by homologous recombination between plasmids pCA350 and pOSE1700 in competent *E. coli* JM83 (ATCC 35607). After recombination, the adenoviral genome was excised by PacI and the AdMDKΔ81-121 virus was obtained by transfecting 293 cells (CRL-1573, ATCC) using Lipofectamine (Invitrogen). Recombinant adenovirus expressing no transgene (AdCO1) was used as negative control. Recombinant viruses were expanded in 293 cells and purified by two-step CsCl gradient ultracentrifugation. Viral stocks were desalted using G50 columns (Pharmacia), frozen, and preserved at -80°C in PBS 10% glycerol. Viral titer was quantified as plaque-forming units (PFU)/ml following infection of 293 cells and infection was carried out with a multiplicity of infection (MOI) of 100 PFU/cell.
Proliferation Assay

Human neuroblastoma cell lines IGR-N91, SH-SY5Y and HUVEC were seeded in 24-well tissue culture dishes at a density of 5x10^4 cells per well in complete media. After 24 hours, the medium was replaced by 420 µl of conditioned medium Cos7 containing MDKΔ81-121, and 420µl of medium containing 1% or 5% of FBS respectively for neuroblastoma and endothelial cell lines. The culture medium was refreshed every 24 hours over 4 days, and cell survival was then quantified by a cell proliferation assay using 3-(4',5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT, 0.5mg/ml), as previously described (10). IGR-N91, SH-SY5Y, MRC-5 cell lines, HUVEC and SMC were transduced with AdMDKΔ81-121 or AdCO1 used as control and after 4 days cell proliferation was performed as previously described (10).

Co-culture of SMC and HUVEC

A endothelial-smooth muscle cells co-culture experiment was performed as previously described, using the 24-well transwell culture plates (0.4 µm pore size, Corning) (23). Briefly, SMC were seeded in 24-well culture plates at 5x10^4 cell density per well, infected with 200 MOI of AdMDKΔ81-121 and cultured during 24 hours with DMEM supplemented with 5% FBS. Then HUVEC were seeded onto the culture inserts at a density of 2.0x10^5 cells per well and cultured with DMEM supplemented with 5% of FBS. 100 ng/ml of MDK was added or not to the medium. After 48 hours a MTT assay was performed to quantify the endothelial cell proliferation.

Endothelial Tube Formation Assay
Two hundred µl of growth factor-reduced Matrigel (8mg/ml, BD) were added to a 48-well tissue culture dish and was polymerized during one hour at 37°C. HUVEC cells were then seeded at a density of 6x10^4 cells per well in 250µl of EBM-2 1% FBS and 100µl or 250µl of conditioned medium (control or MDKΔ81-121 medium). After 18 hours of incubation under normal culture conditions, the capillary-like structures were stained with Giemsa and the length of networks was quantified under phase-contrast microscopy, using Archimed and Videomat softwares (GT Vision).

**Plasmid Electrotransfer**

Before the electrotransfer procedure, 6-week-old female athymic mice were anesthetized with an i.p injection of 100mg/kg ketamine (Ketalar, Panpharma), and 40mg/kg xylazine (Rompun, Bayer). Forty µg of pcDNA3-MDKΔ81-121 or pcDNA3-LacZ plasmids were resuspended in 30µl sterile 0.9% normal saline and injected in both tibialis cranialis muscles of mice using a Hamilton syringe. The electrotransfer was performed with a Cliniporator system (IGEA), as previously described (10).

**Tumor Cell Inoculation**

IGR-N91 cells were harvested, washed and resuspended in sterile 0.9% normal saline at a concentration of 10x10^6 cells in 100µl and were injected subcutaneously into the athymic mice, 7 days after plasmid electrotransfer. The tumor size was monitored after 41 days by measuring the largest (a) and the longest (b) tumor diameters and the tumor volume was calculated using the following formula: 

\[ \frac{((a+b)/2)^3 \times \pi}{6} \]

Mice were sacrificed using a lethal dose of isofluorane: for each mouse, the electrotransferred muscles and the tumor were collected and stored under appropriate conditions for a further immunohistochemical analysis. Serum was collected at day 41 for the quantification of MDKΔ81-121 by a specific ELISA.

**Immunohistochemistry and Image Analysis**
After mice were sacrificed, muscles and tumors were collected and fixed in FineFix (Milestone). Paraffin sections of IGR-N91 tumor cells were prepared to quantify the number of intratumoral vessels by CD31 immunostaining, as well as Ki67, Caspase 3 and SMA immunostaining, as previously described (4,10). Images of histological slides were recorded with a Nikon SuperCoolscan 8000 ED scanner, and analyzed with the PixCyt® image analysis software. Paraffin sections of electroporated muscle were stained for the detection of β-galactosidase and MDKΔ81-121, as previously described (10).

**Statistical Analysis**

Each experiment was done at least twice. Statistical significance was evaluated using the Student’s *t* test (unilateral and unpaired).
Results

Expression of MDK, PTN and Their Receptors

To analyze the expression of MDK and PTN in neuroblastoma cell lines (KCNR, SK-N-AS, BE(2)-M17, CLB-PE, LAN-1, SH-SY5Y, GlMen, and IGR-N91), we first quantified endogenous MDK and PTN mRNA expression by real-time RT-PCR. We showed that MDK and PTN mRNAs were detected in all of the 8 cell lines and that MDK mRNA level was significantly higher (p=0.039) than PTN mRNA (Fig. 1A). Indeed, in 5 cell lines (KCNR, CLB-PE, LAN-1, SH-SY5Y, and IGR-N91) MDK mRNA was 3.5 to 11-fold higher than PTN mRNA. In order to study MDK protein expression after 48 hours of culture, cell supernatants and cell extracts were analyzed using ELISA and Western Blot, respectively. Secretion of MDK in the culture media was demonstrated for all the cell lines (Fig. 1B). The IGR-N91 cell line was found to produce and secrete the highest level of MDK and was also found to produce a high level of MDK mRNA (Fig. 1A and 1B). Furthermore, immunoreactive MDK protein was also detected at a high level in IGR-N91 cell extracts that also expressed a low level of PTN compared to other cell lines (Fig. 1C and 1D). All these results clearly showed heterogeneous expression of MDK and PTN in all the cell lines tested and that IGR-N91 and SH-SY5Y cell lines appeared to produce and secrete high levels of MDK certainly contributing to their proliferation.

To evaluate the potential contribution of HBGH signaling, we then studied the expression of its receptors (ALK, RPTPs, and LRPs) using qRT-PCR. Table 1 shows that ALK mRNA was present in all neuroblastoma cell lines tested with clear heterogeneity of expression since 5 out of 8 cell lines (KCNR, BE(2)-M17, CLB-PE, LAN-1, SH-SY5Y) expressed higher levels of ALK mRNA. Our results were confirmed by Western Blot analysis showing the presence of two ALK isoforms, a
220 kDa native transmembrane protein and a 140 kDa cleaved soluble ALK (Fig. 2A). We then focused on the ALK gene to verify whether there was a correlation between ALK protein expression and possible somatic amplification of the ALK genomic locus. No gene amplification (<5 copies) was found in IGR-N91 (Fig. 2C) and SH-SY5Y (Fig. 2D). Moreover, the level of ALK expression was not strongly correlated with the N-myc status since SH-SY5Y (not amplified for N-myc) and IGR-N91 (amplified for N-myc) respectively expressed high and low ALK levels. The second well known HBGH receptor, RPTPβ/ζ, was also detected by RT-PCR (Table 1) and Western Blot analysis (Fig. 2B) in all cell lines at the same levels and thus seemed to be highly ubiquitous in all cell lines tested. Concerning other RPTP isoforms and LRP receptors, all the neuroblastoma cell lines exhibited RPTPS, RPTPA, RPTPD and LRP6 mRNA. Some cell lines expressed LRP1B but none of the cells expressed LRP2 (Table 1).

**In vitro Expression and Functional Characterization of MDKΔ81-121**

To determine whether MDK is functionally relevant in neuroblastoma models and thus might represent a tractable therapeutic target, we examined the consequences of disrupting MDK signaling by mRNA knockdown. We transiently transfected siRNAs directed against MDK alone or in combination with PTN-targeted siRNAs into two neuroblastoma cell lines expressing high levels of MDK (IGR-N91 and SH-SY5Y) and screened growth inhibition. We first demonstrated knockdown of the mRNA and protein in these two cell lines. The MDK mRNA level was undetectable in IGR-N91 cell line, and was inhibited by 80% in SH-SY5Y cells (Fig. 3A). MDK-specific siRNA induced a drastic decrease in the secretion of endogenous MDK compared to cells transfected with scrambled control siRNA. The combination of MDK- and PTN-specific siRNAs reduced the MDK secretion to the same level as the cells transfected...
with MDK siRNA alone (Fig. 3B). In order to evaluate the consequence of MDK suppression on cell proliferation, we performed a proliferation assay on the cells transfected with MDK-specific siRNA alone or in combination with PTN siRNA, after 4 days of culture. Both cell lines showed significant inhibition of proliferation to MDK knockdown (Fig. 3C). The proliferation of IGR-N91 cells was inhibited by 34% ($P < 2 \times 10^{-5}$) and of SH-SY5Y by 15% ($P < 0.01$) with MDK siRNA alone. The additive inhibitory effect of PTN siRNA on neuroblastoma cell proliferation compared to MDK siRNA alone was not significant (Fig. 3C).

We then produced Cos7 cells expressing MDKΔ81-121 constitutively. We harvested 26 clones stably transfected with the pcDNA3-MDKΔ81-121-MycHis plasmid or pcDNA3-LacZ as control and assessed the expression of the truncated MDK protein using a specific ELISA assay on cell supernatants. The expression of MDKΔ81-121 was up to 30 ng of MDKΔ81-121/mg of protein in 3 clones (clones 9, 10 and 11), whereas no expression was detected in the supernatant of Cos7 cells transfected with pcDNA3-LacZ (Fig. 3D). As the MDKΔ81-121 is fused to the c-Myc epitope tag, we performed two different Western Blots in order to verify the molecular mass of the secreted MDKΔ81-121 protein. The first was performed towards the c-Myc epitope and showed the presence of the c-Myc tag in the 6 clones tested (Fig. 3E). The second was performed towards the MDK epitope and showed the secreted immunoreactive protein with a molecular mass of 11.2 kDa (Fig. 3F).

To assess the potential paracrine inhibitory effect of MDKΔ81-121 on tumor growth, we performed a cell proliferation assay using 2 neuroblastoma cell lines (IGR-N91, SHSY-5Y) expressing a high level of MDK. Conditioned media from Cos7-MDKΔ81-121 (clone 11) and Cos7-control cells were added to tumor cells in culture over 4 days. As illustrated in Fig. 4A, tumor cell proliferation was significantly inhibited by...
33% for IGR-N91 cells ($P < 3.10^{-7}$), and by 43% for SH-SY5Y cells ($P < 1.10^{-6}$). To confirm these results, we used a recombinant adenovirus carrying a CMV-driven MDKΔ81-121 peptide (AdMDKΔ81-121) and an isogenic adenovirus (AdCO1) as control. The efficacy of adenovirus-mediated gene delivery was first evaluated by quantifying the proportion of cells infected with the AdCMV-GFP reporter virus after 48h by FACS analysis (Fig. 4B). The dose of 100 multiplicity of infection (MOI) enabled us to efficiently transduce all IGR-N91 and SH-SY5Y cell lines as well as human primary endothelial cells (HUVEC). Under these conditions, transduction of IGR-N91 and SH-SY5Y cell lines by AdMDKΔ81-121 resulted in stronger significant inhibition of cell proliferation compared to the use of the Cos7-conditioned medium (Fig. 4C).

**Effect of MDKΔ81-121 on angiogenesis**

To evaluate a possible antiangiogenic role of MDKΔ81-121, we first performed a proliferation assay on HUVEC, under the same conditions as for the tumor cells. We observed 60% inhibition of HUVEC proliferation ($P < 5.10^{-7}$) with the supernatant containing MDKΔ81-121, compared to the control or after their infection with 100 MOI of AdMDKΔ81-121 (Fig. 4D). These results were confirmed by quantitative RT-PCR with complete repression of Ki67 expression in AdMDKΔ81-121 transduced HUVEC compared to control (AdCO1-transduced) cells (Fig. 4E).

As endothelial cell migration is also an important process in angiogenesis we then used an *in vitro* matrigel angiogenesis assay based on the differentiation of endothelial cells and the formation of tube-like structures on an extracellular matrix. HUVEC cells were seeded on matrigel in the presence of 100µl or 250µl of conditioned medium (MDKΔ81-121 or control medium). As shown in Figure 4F, we
observed dose-dependent inhibition of tube-like structure formation with a 59% mean reduction after adding 250µl of MDKΔ81-121 conditioned medium.

To obtain further evidence for antiangiogenic activity of MDKΔ81-121, we studied the Endoplasmic Reticulum (ER) stress-mediated apoptosis in endothelial cells transduced with AdMDKΔ81-121. C/EBP Homologous Protein (CHOP), also known as DNA damage-inducible transcript 3 (DDIT3) is the key proapoptotic effector of ER stress-mediated apoptosis. CHOP expression was significantly upregulated in HUVEC transduced with AdMDKΔ81-121 (70 fold increase, \( P < 0.01 \)) as assessed by quantitative RT-PCR (Table 2). We observed significant activation of a set of genes implicated in Unfolded Protein Response (UPR) pathway, which triggers apoptosis in the case of intensive ER-stress, such as Heat Shock 70kDa Protein 5 (HSPA5), Growth Arrest- And DNA Damage-Inducible Protein (GADD34), Activating Transcription Factor 2 and 3 (ATF2 and ATF3) and the spliced variant of X-Box Binding Protein 1 (XBP1S) in endothelial cells expressing truncated MDKΔ81-121 protein (Table 2).

As endothelial cells boarding blood vessels are surrounded by smooth muscle cells and fibroblasts, we therefore investigated the inhibitory effect of MDKΔ81-121 on SMC (Fig. 5B) and MRC-5 fibroblast (Fig. 5A) using the MTT proliferation assay. Cell proliferation was significantly inhibited when MRC5 (\( P<0.0001 \)) and SMC (\( P=0.005 \)) were infected with AdMDKΔ81-121 with 57% and 38% of inhibition respectively. In contrast when SMC were treated with MDK (100 ng/ml), no increase in cell proliferation was observed (Fig. 5C).

We further studied the effect of AdMDKΔ81-121 on endothelial cells proliferation when co-cultured with SMC during 48 hours in the presence or not of 100 ng/ml of MDK (Fig. 5D). In these experiments when HUVEC were treated only with MDK no
increase in cell proliferation was observed ($P=0.65$). However, when HUVEC were co-cultured with SMC, MDK significantly increased the cell proliferation ($P=0.0002$). Furthermore co-culture of HUVEC with AdMDKΔ81-121-transduced SMC with or without MDK repressed significantly HUVEC proliferation (Fig. 5D).

**Inhibition of Tumor Growth by MDKΔ81-121 *in vivo***

To confirm the antitumor potency of MDKΔ81-121 *in vivo*, we used a prophylactic protocol after muscle electrotransfer of the pcDNA3-MDKΔ81-121-MycHis plasmid. We first electrotransferred 30µg pcDNA3-MDKΔ81-121-MycHis or pcDNA3-LacZ into the *tibialis cranialis* muscles of nude mice (n=10 per group). Seven days later, $10^6$ IGR-N91 cells were inoculated subcutaneously. Serum was collected from mice 41 days after the electrotransfer to quantify the systemic amount of MDKΔ81-121 secreted by the electrotransferred muscle cells. As shown in Figure 6A, IGR-N91 tumors from the MDKΔ81-121-treated group did not grow and after two weeks the tumor size reduced drastically, while the tumors from the control group treated with pcDNA3-LacZ increased in size. This inhibition correlated with the detection of MDKΔ81-121 immunoreactive material in the serum (Fig. 6B) and in the muscles (Fig. 6C). IGR-N91 tumors treated with MDKΔ81-121 appeared much less vascularized than the β-galactosidase-treated control group (Fig. 6A). Intratumoral angiogenesis was thus assessed by CD31 and alpha Smooth Muscle Actin (SMA) immunostaining of tumor sections (Fig. 6F). The results indicated a marked reduction (37%) of intratumoral vascularization within MDKΔ81-121-treated tumors (score of $3.15 \pm 0.15$) as compared with the β-galactosidase-treated group (score of $2.14 \pm 0.05$) (Fig. 6D and 6E). These results correlated with SMA immunostaining showing again the presence of large vessels only in tumors treated with MDKΔ81-121 (Fig.
6F). Tumor proliferation activity was assessed by Ki67 immunostaining, which is widely used in clinical pathology diagnostics. As shown in Fig. 6F the proliferation appeared to be drastically decreased within MDKΔ81-121-treated tumors (50% versus 90% of positive tumor cells) with a higher proportion of apoptotic cells through activation of Caspase 3-mediated apoptosis pathway (35 ± 8 versus 3 ± 2 positive nuclei per field) (Fig. 6F)
Discussion

PTN and MDK are members of a new family of neurotrophic factors known to be expressed in many human tumors (24–27). They participate in tumorigenesis through their action on angiogenesis and cell proliferation (18,28), and their expression is highly correlated with metastasis development (29).

MDK and PTN were found to play an important role in regulating growth and differentiation in neuroblastoma, the third most common childhood solid tumor, which originates from the sympathoadrenal lineage of the neural crest. Both factors were found to be expressed in many primary neuroblastomas, but their patterns of expression were found to be quite different. PTN was found to be highly expressed in favorable neuroblastomas (stages I, II and IV-S), whereas it was expressed at a significantly lower level in advanced tumors (stages III and IV) (30). Furthermore PTN was not expressed in aggressive neuroblastoma with N-myc amplification nor in eleven different neuroblastoma cell lines. In contrast, MDK was highly expressed in almost all primary neuroblastomas and cell lines and showed no correlation with disease stage or N-myc amplification. Unlike PTN, MDK expression was higher in undifferentiated and aggressive neuroblastomas (stage III and IV) and the plasma MDK level was thus found to be a prognostic factor for neuroblastoma patients (31). These observations support the idea that MDK is a better molecular target candidate than PTN for neuroblastoma therapy and may therefore be considered as a new strategy for blocking tumor development and angiogenesis.

We collected 8 neuroblastoma cell lines to explore the MDK/PTN pathway. In contrast to PTN, all the cell lines ubiquitously produced and secreted MDK and expressed all the RPTP-receptor-related proteins. As several studies have suggested PTN and MDK as possible ligands for ALK (13,32), we quantified ALK mRNA,
identified the protein in the 8 different cell lines and demonstrated that ALK was not expressed at the same level in all cell lines. Five out of 8 cell lines exhibited high ALK expression, while 3 expressed only low levels. These results are consistent with previous reports which showed that full-length ALK is detected in almost half of the cell lines derived from neuroblastomas (33,34). We were unable to show that these patterns of expression were associated with ALK amplification and that there was an association between ALK and N-myc amplification. Furthermore, significant amplification of ALK appeared to be rarely observed in human neuroblastoma samples (33) and in neuroblastoma cell lines (20). Recently, somatic and germline activating mutations of the ALK kinase domain of the receptor were documented in human neuroblastoma samples and in neuroblastoma cell lines. These mutations were mainly clustered in two hotspots located in exon 23 (F1174V) or exon 25 (R1275Q). The F1174V mutation was observed more frequently in cell lines than in primary tumors, suggesting that the former may provide a selective \textit{in vitro} growth advantage (20). Interestingly, the knockdown of ALK mRNA by siRNA resulted in profound inhibition of cell proliferation in all cell lines harboring mutant or wild-type ALK (20).

In the present study, we investigated a new strategy evaluating the antitumor and antiangiogenesis effects in a neuroblastoma model of a truncated MDK lacking the COOH-terminal 81 to 121 portion of the molecule (MDK$\Delta$81-121), that might act as a dominant-negative effector for its mitogenic, angiogenic, and tumorigenic activities by interfering with the wild-type native protein.

We decided to use two cell lines (SH-SY5Y and IGR-N91) to perform a further analysis of the effect of MDK$\Delta$81-121 for several reasons. Both cell lines expressed a high level of MDK and a low level of PTN compared with other cell lines, suggesting
the existence of a dominant autocrine MDK signaling loop. In addition, ALK mRNA was highly expressed in SH-SY5Y cells and was 52-fold higher than the mRNA level detected in IGR-N91 cells and both cell lines identically expressed the other tyrosine kinase receptors (RPTPs and LRPs).

Our study was completed with the use of specific MDK siRNA, to down-regulate the level of MDK mRNA in both cell lines. As MDK and PTN share same receptors and have 45% structural homology, we investigated whether knockdown of PTN expression together with MDK could have a synergic effect on cell proliferation. Our results showed that MDK siRNA alone or together with PTN siRNA induced significant specific inhibition of proliferation of both cell lines. That was more pronounced for IGR-N91 cell lines which expressed the highest level of MDK. We thus believe in the existence of a strong autocrine MDK signaling loop in both cell lines. However, extinction of PTN expression together with MDK did not have any additive or synergic inhibitory effect on IGR-N91 and SH-SY5Y proliferation. This observation is consistent with the data regarding the low PTN expression level in these two cell lines (Fig. 1A) and our observation that PTN protein was not detectable in these two cell lines supernatant once assessed by ELISA (data not shown).

We demonstrated that MDK\(\Delta81-121\) is able to significantly inhibit the proliferation of both IGR-N91 and SH-SY5Y cell lines \textit{in vitro}. In this case, it might be probably due to the heterodimerization of MDK\(\Delta81-121\) with endogenous MDK which mainly explains its dominant-negative effect and that MDK\(\Delta81-121\) might be able to dissociate MDK homodimers, thereby might induce the formation of non-functional heterodimers. Thus, the inhibition of MDK in both cell lines exerts several effects on different important cellular systems. In this context, we believe that the inhibition of
MDK by MDKΔ81-121 abolishes the MDK/ALK and/or the MDK/RPTPβ/ζ signaling pathways, as previously suggested with a PTN-derived peptide lacking the C-terminal residues (PTNΔ111-136). PTNΔ111-136 was shown to act as a potent inhibitor of PTN biological activities by the formation of inactive heterodimers (PTN/PTNΔ111-136) (9). In particular, binding studies using a biotinylated peptide showed that PTNΔ111-136 could interfere with PTN binding to DU145 prostatic tumor cells, in an RPTPβ/ζ-dependent fashion (35). Furthermore PTNΔ111-136 was shown to bind to the extracellular domain of ALK and to inhibit PTN-induced mitogenesis (9,12) and thus is also able to interfere with the PTN/ALK pathway. MDK and PTN are members of the same neurotrophic family factors and are both basic, cysteine-rich polypeptides containing identical heparin-binding motifs, which are essential for the dimerization of PTN and MDK. They exhibit about 50% of homology and no sequence homology with other heparin-binding proteins, such as fibroblast growth factors. In this context, the formation of MDKΔ81-121/PTN heterodimers is not excluded and further experiments are warranted to explore this hypothesis.

As angiogenesis is also known to be a key mechanism via which MDK promotes tumorigenesis, we then investigated the effects of MDKΔ81-121 on endothelial cell proliferation and migration. We were able to show that the inhibition of MDK by MDKΔ81-121 has major consequences on different stages of angiogenesis, because MDKΔ81-121 strongly inhibited the proliferation and the migration of endothelial cells in vitro. We also showed that MDKΔ81-121 could have major effects on their structural organization in a three-dimensional model. MDKΔ81-121 is able to reduce the formation of capillary-like structures, a crucial step in tumor neovascularization. The effects of MDKΔ81-121 on HUVEC could be mediated by the inhibition of ALK and/or RPTPβ/ζ tyrosine kinase receptors since HUVEC have been shown to
express MDK and both receptors (36,37) enabling the stimulation of the phosphorylation of ALK (36) and the activation of the Akt and ERK pathways (38). We also showed that MDKΔ81-121 inhibits endothelial cell cycling with a complete extinction of Ki67 expression, while promotes cell apoptosis via activation of a set of genes, including CHOP, implicated in ER stress-mediated apoptosis pathway. These data are consistent with the observations from U87MG glioblastoma cell line and the truncated protein PTNΔ111-136 which enhances apoptosis via CHOP protein (39).

Angiogenesis process involves interactive dialog between endothelial and smooth muscle cells. These interactions were previously studied by Sumi et al. using an artificial blood vessel model (23). They documented that MDK had no effect on the human aortic SMC or endothelial cells when cultured separately and these observations are in concordance with our results showing no effect of MDK on SMC and HUVEC. They also clearly showed that MDK promotes growth and glycosaminoglycan synthesis of HUVEC through its action on smooth muscle cells that are able to produce Interleukin-8. In order to evaluate if MDKΔ81-121 is able to disrupt this interaction we have co-cultured HUVEC and SMC that were transduced or not with an adenovirus expressing MDKΔ81-121. We were able to show that only the co-culture of HUVEC and SMC treated with MDK could trigger the proliferation of HUVEC. This effect was abolished by MDKΔ81-121.

To complete and confirm our in vitro experiments, we investigated the antitumoral efficacy and antiangiogenic properties of MDKΔ81-121 using an in vivo therapeutic approach, namely the intramuscular electrotransfer of a plasmid delivering a secretable form of MDKΔ81-121. A single intramuscular injection of pcDNA3-MDKΔ81-121 but not the control plasmid was shown to inhibit primary tumor growth without apparent toxicity. This antitumor inhibitory effect was tightly correlated with
markedly decreased intratumoral vascularization and with the detection of MDKΔ81-121 immunoreactive material in muscle sections and blood. As MDK is known to both promote proliferation and inhibit caspase-dependent apoptosis (40), MDKΔ81-121 can be considered to induce these two mechanisms, and thereby, to inhibit the proliferation of tumor cells. The MDKΔ81-121-treated tumors apparently failed to establish a vascular network within the tumor mass to support rapid and extensive growth, and this failure translated into >80% inhibition of tumor growth. The treated tumors were shown to be less aggressive and less vascularized and to have a higher apoptotic index. This effect was attributed to the efficient action of MDKΔ81-121 on endothelial cell growth and migration and on smooth muscle cells which participate in the formation to the vascular network.

In conclusion, we showed that specific targeting of MDK by MDKΔ81-121 results in strong inhibition of a neuroblastoma model and inhibition of the angiogenesis process. Nevertheless, the fact that cDNA muscle electrotransfer failed to completely abolish tumorigenesis emphasizes the importance of improving the gene transfer method, so that sustained expression can be achieved for maximum clinical benefits. We also intend to combine PTN inhibitors or tyrosine kinase inhibitors targeting ALK and/or RPTPβ/ζ receptors to enhance MDK inhibitor potency and thus improve the clinical outcome of children with neuroblastoma.
Acknowledgements

We thank Dr Isabelle Janoueix-Lerosey for providing the neuroblastoma cell lines, Dr Jean Benard for IGR-N91 cell line, Dr Georges Uzan for SMCs and Lorna Saint Ange for editing. The authors are grateful to Aurélie Ducès for providing pcDNA3-MDKΔ81-121-MycHis plasmid construct and Delphine Dargere for technical assistant with siRNA experiments. L’Institut National de la Santé et de la Recherche Médicale (INSERM), l’Association pour la Recherche sur le Cancer (ARC), and le Ministère de l’Enseignement Supérieur et de la Recherche are acknowledged for financial support.
References


Tables

Table 1. Real-time RT-PCR analysis of ALK, RPTPs and LRPs mRNA, P53 mutation and N-myc amplification status in 8 different neuroblastoma cell lines. WT; Wild-Type, M; Mutant.

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<th>Cell lines</th>
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<th>N-myc amplification</th>
<th>ALK</th>
<th>RPTPs</th>
<th>RFTPA</th>
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<th>LRP2</th>
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Table 2. Real-time RT-PCR analysis of CHOP pathway-mediated apoptosis in HUVEC cells transduced with AdMDKΔ81-121 or AdCO1.

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<td>8862 ± 941</td>
<td>65387 ± 6013</td>
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<td>HSPA5</td>
<td>33195 ± 2854</td>
<td>237304 ± 24156</td>
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<td>DDIT3 (CHOP)</td>
<td>5 ± 0.28</td>
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<td>307 ± 21</td>
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<td>XBP1L</td>
<td>2454 ± 133</td>
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Figure Legends

Fig. 1. *In vitro* assessment of MDK and PTN expression. (A) PTN and MDK mRNA expression by real-time quantitative RT-PCR in tumor cell line lysates in 8 different neuroblastoma cell lines. (B) Quantitative detection of MDK by ELISA in the supernatants of 8 different neuroblastoma cell lines. (C) Western Blot analysis of MDK in cell pellets from eight different neuroblastoma cell lines compared to positive control protein (MDK). (D) Western Blot analysis of PTN in cell pellets from different neuroblastoma cell lines compared to positive control protein (PTN).

Fig. 2. *In vitro* assessment of MDK receptors expression. (A) Immunoblots of ALK in various neuroblastoma cell lines. (B) Immunoblots of RPTPβ/ζ in various neuroblastoma cell lines. (C and D) Fluorescence *in situ* hybridization was performed on interphase nuclei from IGR-N91 and SH-SY5Y cultured lines. We used 2 probes (Vysis LSI ALK Dual Color, Abbott Molecular) specific for the 2p23 chromosomal region [SHGC-56576 spectrum orange, SHGC-104192 spectrum green], showing two signals for each probe in all the cells analyzed corresponding to the presence of the ALK chromosome region.

Fig. 3. Transfection of IGR-N91 and SH-SY5Y cell lines by small interfering RNAs (siRNAs) directed against MDK, PTN or nonsilencing sequence siRNAs (control) and Cos7 cells by pcDNA3-MDKΔ81-121-*MycHis* or pcDNA3-LacZ plasmids. (A) Real time RT-PCR expression of MDK mRNA, 4 days after the transfection of IGR-N91 and SH-SY5Y cell lines with 10 nmol/L siRNAs targeting MDK alone or in combination with PTN targeted siRNAs and with nonsilencing sequence siRNAs. (B) Quantitative detection of MDK by ELISA in the supernatant of IGR-N91 and SH-
SY5Y cell lines transfected with siRNAs directed against MDK alone or in combination with PTN siRNAs or nonsilencing sequence siRNAs after 4 days of culture. (C) siRNAs directed against MDK induced inhibition of IGR-N91 and SH-SY5Y cell lines proliferation compared to the transfection of nonsilencing siRNAs. PTN targeted siRNA did not induced any significant additive effect on proliferation of neuroblastoma cell lines when transfected in combination with MDK targeted siRNAs.

NS; Not Significant, *P < 0.05; **P < 0.005

(D) Quantification of MDKΔ81-121 in the supernatant of Cos7-transfected cells by ELISA. Cos7 cells were transfected with 8µg of either pcDNA3-MDKΔ81-121 or pcDNA3-LacZ plasmids and subclones were expanded. Supernatants of 26 different Cos7-MDKΔ81-121 subclones were tested by ELISA compared to Cos7-LacZ control cells. (E) Western Blot analysis of the c-Myc epitope in 6 different Cos7-MDKΔ81-121 subclones. (F) MDKΔ81-121 immunoblotting of the culture supernatant of Cos7-transfected cells (subclones 10).

Fig. 4. MDKΔ81-121 and AdMDKΔ81-121 induced the inhibition of cell proliferation. (A) MDKΔ81-121 induced inhibition of IGR-N91 and SH-SY5Y tumor cell proliferation. Both cell lines were cultured with pcDNA3-MDKΔ81-121 Cos7-conditioned medium and cell viability was assessed after 96h by MTT assay. Medium from nontransfected Cos7 cells was used as control. (B) FACS analysis of IGR-N91, SH-SY5Y and HUVEC transduced with 100 MOI of AdCMV-GFP reporter virus. (C) AdMDKΔ81-121 induced inhibition of IGR-N91 and SH-SY5Y tumor cell proliferation. Both cell lines were infected with 100 MOI of AdMDKΔ81-121 and cell viability was assessed after 96h by MTT assay. An adenovirus isogenic control devoid of a transgene (AdCO1) was used as control. (D) MDKΔ81-121 and AdMDKΔ81-121 induced inhibition of HUVEC cell proliferation. (E) qRT-PCR analysis showed
suppression of Ki67 expression in HUVEC transduced with AdMDKΔ81-121. (F) MDKΔ81-121 induced inhibition of tube-like structure formation using an endothelial tube formation assay on matrigel. HUVEC cells were cultured with 100µl or 250µl of conditioned medium (MDKΔ81-121 or control medium) and the capillary-like structures were stained with Giemsa. The length of networks was quantified under phase-contrast microscopy. Each experiment was done at least twice. *P < 0.05; **P < 0.005

Fig. 5. In vitro assessment of AdMDKΔ81-121 effect on HUVEC and SMC apoptosis and proliferation. (A) Proliferation of MRC-5 cells transduced with 50 or 100 MOI AdMDKΔ81-121 was assessed with MTT assay after four days. (B) Cell proliferation of Smooth muscle cells transduced with 200 MOI AdMDKΔ81-121 or AdCO1 as control. (C) Proliferation of SMC treated with MDK (100 ng/ml) was assessed after 96h by MTT assay. (D) Endothelial cell proliferation assay after co-culture with SMC or AdMDKΔ81-121-transduced SMC with or without MDK treatment. Each experiment was done at least twice. NS; Not Significant, *P < 0.05; **P < 0.005

Fig. 6. Tumor growth and inhibition of intratumoral vascularization. (A) Effect of MDKΔ81-121 transgene expression after in vivo muscle pDNA electrotransfer on IGR-N91 tumor growth in nude mice. Animals received a subcutaneous injection of 10x10^6 IGR-N91 cells 7 days after electrotransfer. The mean tumor volume evolution is shown until day 25 in MDKΔ81-121-treated versus β-galactosidase-treated mice (p<0.05). Tumors from MDKΔ81-121-treated and β-galactosidase-treated groups are shown at day 41 after cell inoculation. (B) Quantification of MDKΔ81-121 in serum by ELISA. (C) Detection of β-galactosidase and MDKΔ81-121 in electroporated muscle by immunohistochemistry. (D) The extent of intratumoral vascularization was assessed by CD31 immunostaining and quantified by assessing the mean
immunoreactive score within the whole tumor area at day 41. (E) The extent of vascularization of a representative tumor treated with MDKΔ81-121 and the control (β-galactosidase) is shown 41 days after the subcutaneous injection of cells. (F) Antiproliferative, proapoptotic and antiangiogenic effect of MDKΔ81-121 is demonstrated by immunohistochemistry on tumor slices for Ki67, Caspase 3 and SMA, respectively.
**Figure 1**

A) Graph showing standardized mRNA levels.

B) Bar chart illustrating ng MDK/mg of protein.

C) Western blot analysis of 13 kDa MDK and β-actin.

D) Western blot analysis of 18 kDa PTN and β-actin.
Figure 2

A

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C

IGR-N91

D

SH-SY5Y
Figure 3
Figure 4

A

![Graph showing Absorbance 570nm for IGR-N91 and SH-SY5Y.]

B

![Graph showing Absorbance 570nm for HUVEC.]

C

![Graph showing Absorbance 570nm for IGR-N91 and SH-SY5Y.]

D

![Graph showing Absorbance 570nm for HUVEC.]

E

![Graph showing Standardized Ki67 mRNA level for HUVEC.]

F

![Graph showing Total length of Capillary tubes (µm) for conditioned medium volume (µl).]
Figure 5

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 6
Molecular Cancer Therapeutics

Midkine lacking its last 40 amino acids acts on endothelial and neuroblastoma tumor cells and inhibits tumor development

Noushin Dianat, Barbara Le Viet, Emilie Gobbo, et al.

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