

Therapeutic Angiogenesis Inhibits or Rescues Chemotherapy-induced Peripheral Neuropathy: Taxol- and Thalidomide-induced Injury of Vasa Nervorum is Ameliorated by VEGF

Rudolf Kirchmair^{1,2}, Anne B Tietz¹, Eleftheria Panagiotou¹, Dirk H Walter¹, Marcy Silver¹, Young-Sup Yoon¹, Peter Schratzberger^{1,2}, Alberto Weber¹, Kengo Kusano¹, David H Weinberg³, Allan H Ropper³, Jeffrey M Isner^{1,4} and Douglas W Losordo^{1,4}

¹Department of Cardiovascular Research, St Elizabeth's Medical Center – Tufts University School of Medicine, Boston, Massachusetts, USA; ²Department of Internal Medicine, General Internal Medicine, Medical University Innsbruck, Innsbruck, Austria; ³Department of Neurology, St Elizabeth's Medical Center – Tufts University School of Medicine, Boston, Massachusetts, USA; ⁴Department of Vascular Medicine, St Elizabeth's Medical Center – Tufts University School of Medicine, Boston, Massachusetts, USA

Toxic neuropathy represents an important clinical problem in the use of the chemotherapeutic substances Taxol and thalidomide. Sensory neuropathy has a high incidence, lacks an effective treatment and is the dose-limiting factor for these drugs. The pathogenic basis of these neuropathies is unknown. We investigated the hypothesis that the experimental toxic neuropathies from Taxol and thalidomide results from destruction of vasa nervorum and can be reversed by the administration of an angiogenic cytokine. In animal models of Taxol- and thalidomide-induced neuropathy, nerve blood flow has been attenuated and the number of vasa nervorum has been reduced. Intramuscular gene transfer of naked plasmid DNA encoding VEGF-1 administered in parallel with Taxol injections completely inhibited deterioration of nerve function and diminution of the peripheral nerve vasculature. Gene therapy in animals with established Taxol- or thalidomide-induced neuropathies resulted in recovery of vascularity and improved nerve electrophysiology. These findings implicate microvascular damage as the basis for toxic neuropathy and suggest that angiogenic growth factors may constitute a novel treatment for this disorder.

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INTRODUCTION

Taxol (Paclitaxel) is a potent, widely used chemotherapeutic drug for the treatment of a variety of cancers, especially of advanced ovarian carcinoma and non-small cell lung cancer.

Unfortunately, it produces a predominantly sensory peripheral neuropathy in up to 90% of treated patients.^{1–3} Despite some promising data in animal models with nerve growth factor,⁴ there is currently no effective therapy for this kind of neuropathy and neuropathic toxicity is often the dose limiting complication of Taxol therapy.³ Thalidomide is also used in the treatment of certain malignancies, especially multiple myeloma, and also causes a peripheral neuropathy in up to 30% of patients.^{5–7}

Both drugs are, beside their antimitotic activity, strong antiangiogenic agents.^{8,9} We therefore hypothesized that the neuropathy caused by these substances is due to destruction of the blood supply of the nerve, *i.e.*, the vasa nervorum, and might benefit from gene therapy with the angiogenic cytokine vascular endothelial growth factor (VEGF).^{10–12} This strategy, previously called therapeutic angiogenesis, has been used for the treatment of ischemic heart disease and peripheral artery disease.^{13–21} Recent publications from our laboratory have demonstrated that gene therapy with the endothelial mitogen VEGF restored nerve function in experimental ischemic and diabetic neuropathy.^{22,23} Furthermore, VEGF gene therapy improved nerve function in patients with severe limb ischemia, including in diabetics with neuropathy.²⁴

We report here markedly reduced nerve blood perfusion and density of vasa nervorum in an experimental model of predominantly sensory neuropathy induced by Taxol and thalidomide in the rat. Gene therapy with VEGF restored nerve blood supply and nerve function in these neuropathies. Prophylactic VEGF gene therapy also inhibited pathological changes in Taxol-induced neuropathy. From these observations, we conclude that nerve blood supply is attenuated in toxic neuropathies caused by the antiangiogenic chemotherapeutic substances Taxol and thalidomide. The pathophysiological

Correspondence: Rudolf Kirchmair, Department of Internal Medicine, Medical University of Innsbruck, Anichstr., 35, 6020, Innsbruck, Austria; Feinberg Cardiovascular Research Institute, Northwestern University, 303 E Chicago Avenue, Tally 12-703, Chicago, Illinois 60611, USA.
E-mails: Rudolf.kirchmair@uibk.ac.at; d-losordo@northwestern.edu

importance of this effect is revealed by the fact that angiogenic gene therapy with VEGF reversed or prevented this process.

RESULTS

VEGF inhibits Taxol-induced EC apoptosis

Human umbilical vein endothelial cells (HUVECs) were serum starved for 18 h (2% FBS) and treated with 1 nM Taxol or solvent. This concentration was chosen from preliminary experiments (data not shown) and from the published literature⁸ on the effect of Taxol on endothelial cells (ECs). As shown in **Figure 1**, Taxol significantly decreased cell numbers of cultured HUVECs and increased the percentage of cells with pyknotic nuclei, consistent with EC apoptosis. This apoptotic reaction was completely prevented by addition of VEGF: VEGF at a dose of 100 ng/ml added 1 h before Taxol significantly increased EC number ($*P < 0.01$, $n = 4$) and decreased percentage of cells with pyknotic nuclei ($*P < 0.05$, $n = 4$) compared to cells treated with Taxol alone.

Taxol inhibits PI3-kinase/Akt pathway and MAPK system in ECs. HUVECs were serum starved (1% BSA, no serum) and incubated with 1 μ M Taxol or solvent for 18 h. Subsequently, proteins were extracted and subjected to Western blot analysis for activated (phosphorylated) and total Akt and ERK-2, respectively, and for actin (loading control). Taxol inhibits Akt/PI3-kinase as well as the mitogen-activated protein kinase (MAPK) system in ECs by dephosphorylation of the respective active phosphorylated kinases (**Figure 2a**).

Addition of the PI3 kinase inhibitor LY 294002 (10 μ M) to ECs (**Figure 2b**) treated with Taxol further increased EC

apoptosis, whereas inhibition of the MAPK system by PD 98059 (10 μ M) did not increase apoptotic cells compared to Taxol alone. The importance of the Akt pathway was further demonstrated by transfection of HUVECs with an adenovirus expressing constitutively active myr acutely transforming retrovirus AKT8 in rodent T-cell lymphoma (myrAkt): myrAkt completely inhibited the apoptotic reaction of ECs treated with Taxol (**Figure 2c**).

VEGF expression in sciatic nerves after i.m. plasmid injection

Western blotting of sciatic nerve extracts for VEGF was performed under nonreduced and reduced (addition of β -mercaptoethanol) conditions 2 and 3 days after VEGF plasmid injection. Nerves of saline-injected animals were used as control. Increased VEGF immunoreactivity was observed in the injected side 2 (reduced and nonreduced conditions) and 3 (nonreduced condition) days after i.m. VEGF plasmid injection reflecting higher detection limits for the VEGF monomer (**Figure 3**).

Nerve electrophysiology

Taxol. Mean nerve conduction velocities (NCV) at baseline were: motor nerve conduction velocity (MCV) 52.6 ± 1.2 m/s, $n = 26$, and sensory nerve conduction velocity (SCV) 54.3 ± 1.6 m/s, $n = 26$. Rats received i.m. injections of saline or

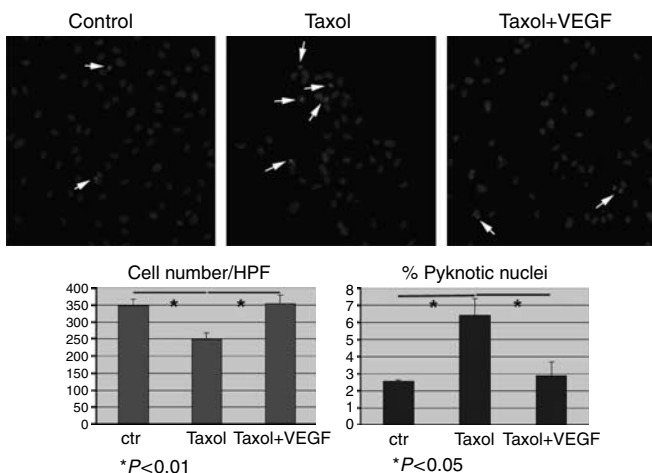


Figure 1 Taxol-induced EC apoptosis is reversed by VEGF. HUVECs were serum-starved for 18 h (starvation medium contained M199 supplemented with 2% FBS and antibiotics) and incubated with 1 nM Taxol alone or in combination with 100 ng/ml VEGF, which was added 1 h before cisplatin. After incubation cells were fixed with 4% paraformaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI). Total cell number per high-powered field and cells with pyknotic, condensed or fragmented nuclei (arrows in upper panels; expressed in percent of total cells) were counted from three different fields per well. Taxol significantly reduced total cell number ($*P < 0.01$, $n = 6$) and increased apoptotic cells ($*P < 0.05$, $n = 6$). Coincubation of Taxol-treated cells with VEGF significantly increased cell number ($*P < 0.01$, $n = 4$) and decreased EC apoptosis ($*P < 0.05$, $n = 4$) compared to cells treated with Taxol alone.

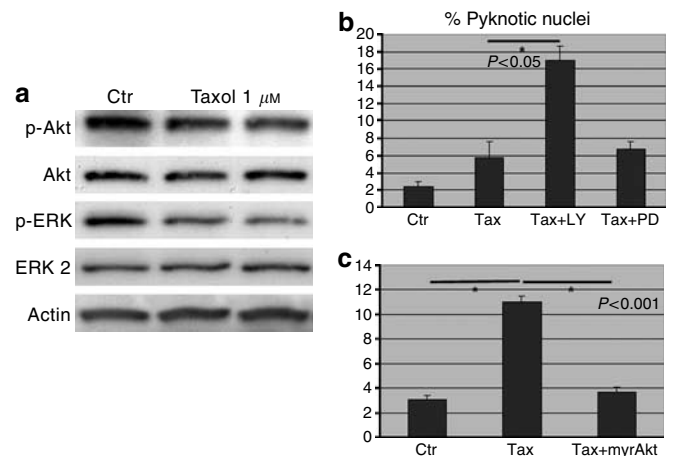


Figure 2 Inhibition of EC PI3 Kinase/Akt Pathway and MAPK system by Taxol. **(a)** HUVECs were serum starved (M199 plus 1% BSA plus antibiotics) and treated with 1 μ M Taxol or 0.01% ethanol (solvent as control) for 18 h. Western blotting against phosphorylated (active) and total Akt and ERK 2, respectively, shows attenuation of active Akt and ERK. **(b)** HUVECs were serum-starved for 18 h (M199 supplemented with 2% FBS and antibiotics) and incubated with 1 nM Taxol alone or in combination with PI3-kinase inhibitor LY 294002 or MAPK inhibitor PD 98059 both at concentrations of 10 μ M. LY and PD were added 1 h before application of Taxol. Incubation with Taxol or in combination with either substance significantly increased EC apoptosis compared to control (not shown). Coincubation of Taxol with LY significantly further increased percentage of cells with pyknotic nuclei compared to Taxol alone, whereas coincubation with PD did not ($*P < 0.05$, $n = 3$). **(c)** A subset of HUVECs were transfected with an adenovirus containing constitutively active Akt (Adeno-myrAkt). Transfected and nontransfected cells were subsequently serum-starved and incubated with saline (control) or 1 nM Taxol for 24 h and afterwards processed by DAPI stain. Overexpression of myr-Akt significantly inhibited Taxol induced EC apoptosis. ($n = 3$, $*P < 0.001$).

VEGF GTx (250 µg VEGF-1 plasmid/limb) before Taxol. At 4 weeks after start of Taxol treatment (**Figure 4a and b**) NCV were significantly reduced in saline injected animals: MCV was 47.2 ± 1 m/s, $n = 18$, SCV was 41.9 ± 1.1 m/s, $n = 18$ ($P < 0.001$ vs baseline for both MCV and SCV). VEGF gene therapy (GTx; **Figure 4a**) before Taxol injections completely prevented deterioration of nerve function: 4 weeks after Taxol MCV was 51.1 ± 0.8 m/s and SCV was 52.1 ± 0.9 m/s ($n = 8$, $P < 0.05$ for MCV and $P < 0.001$ for SCV vs saline injection, P NS vs baseline).

In a second group of animals VEGF GTx or saline injection was performed 4 weeks after Taxol treatment, when neuropathy was already established (**Figure 4b**). NCV measurements 4 and 8

weeks after GTx or saline showed that SCV had completely recovered in VEGF-treated animals; 4 weeks: VEGF 54.4 m/s, saline 44 m/s; 8 weeks: VEGF 53 m/s, saline 45.6 m/s ($P < 0.01$ VEGF vs saline 4 weeks after GTx; $P < 0.05$ VEGF vs saline 8 weeks after GTx; SCV $P < 0.001$ before vs 4 and 8 weeks after VEGF GTx; $n = 8$ for each group). Measurement of MCV revealed accelerated recovery in VEGF-treated rats, although even the control rats exhibited spontaneous recovery (4 weeks: VEGF 53.8 m/s, saline 50.6 m/s; 8 weeks: VEGF 50.8 m/s, saline 51 m/s; $P =$ NS VEGF vs saline, $n = 8$ each group). NCV in control rats were stable over the time period investigated (data not shown).

Thalidomide. Owing to the long study period (8 months) and the known changes of NCV with age and weight of rats, three groups were studied; controls received solvent (hydroxymethyl cellulose), the remaining rats received thalidomide (all 6 months orally). After the treatment period NCV measurements showed development of a sensory neuropathy in thalidomide-treated animals (SCV: controls 53.7 ± 2 m/s, thalidomide 43.7 ± 1.8 m/s, $P < 0.01$; **Figure 4c**). After thalidomide treatment rats were randomly allocated to receive VEGF GTx (250 µg VEGF-1) or saline i.m. SCV improved in VEGF-treated animals and reached levels of control animals (without thalidomide treatment), whereas saline injected rats showed no improvement of SCV (4 weeks after VEGF/saline: controls 53.3 ± 1.5 m/s, VEGF 52.5 ± 3.3 m/s, saline 45.8 ± 1.2 m/s, $P < 0.05$ ctr, VEGF vs saline; 8 weeks after VEGF/saline: controls 52.5 ± 0.6 m/s, VEGF 52.4 ± 1.2 , saline 44 ± 1.8 m/s, $P < 0.001$ ctr, VEGF vs saline; $P < 0.05$ before vs. 4 weeks after VEGF GTx, $P < 0.01$ before

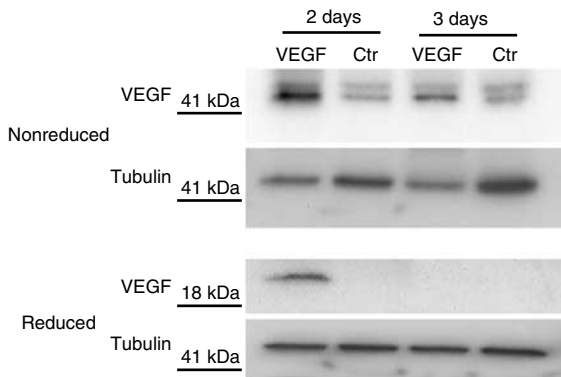


Figure 3 Western Blotting of Sciatic Nerves for VEGF. Extracts of sciatic nerves from VEGF plasmid or saline-injected rats were subjected to VEGF western blotting. Increased VEGF expression was observed 2 and 3 days after VEGF plasmid injection.

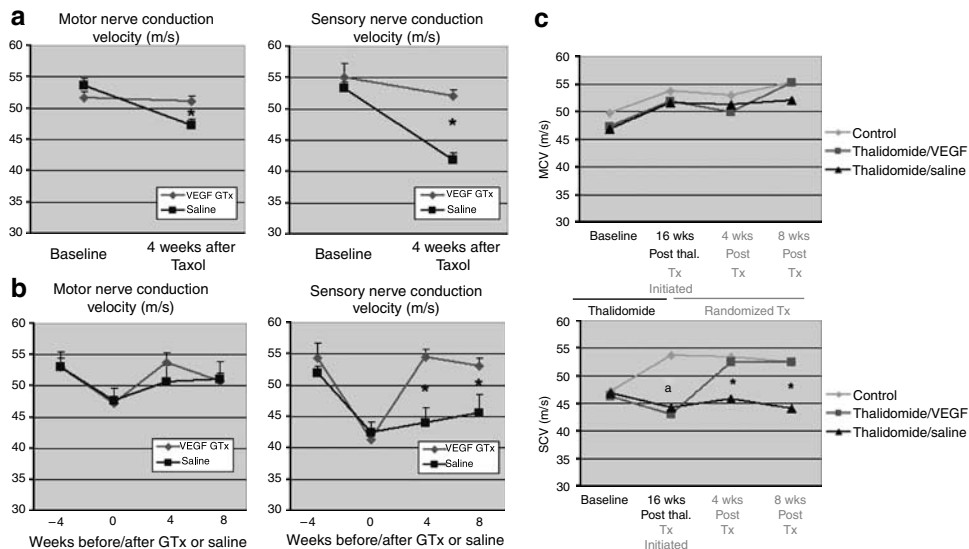


Figure 4 Electrophysiological measurement of nerve conduction velocity. **(a)** Both SCV and to a minor extent MCV decreased significantly after Taxol treatment compared to baseline **a** ($P < 0.001$, $n = 26$). VEGF gene therapy 48 h before each Taxol injection ($n = 8$) completely inhibited deterioration of nerve function (MCV, SCV $P =$ NS vs baseline) yielding a significant difference between saline and VEGF-treated animals ($*P < 0.05$ for MCV, $*P < 0.001$ for SCV). **(b)** VEGF GTx 4 weeks after Taxol injections significantly improved SCV 4 weeks ($*P < 0.01$, $n = 8$) and 8 weeks ($*P < 0.05$, $n = 8$) after VEGF injection compared to saline. MCV improved spontaneously also in saline-treated animals ($P =$ NS VEGF vs saline, $n = 8$). **(c)** At 6 months after thalidomide treatment ($n = 14$) SCV was significantly reduced compared to controls (solvent-treated animals $n = 4$; $*P < 0.001$). VEGF GTx ($n = 7$) significantly improved SCV compared to saline ($n = 7$) 4 weeks ($*P < 0.05$) and 8 weeks ($*P < 0.001$) after VEGF or saline injections. MCV was similar in all three groups, consistent with a predominantly sensory neuropathy.

vs 8 weeks after VEGF GTx, $n = 7$ for each group). MCV showed no difference between controls and thalidomide-treated rats (randomized to VEGF or saline) at any time point.

Laser-Doppler perfusion imaging of sciatic nerve blood-flow

Laser-Doppler perfusion imaging (LDPI; **Figure 5**) of the exposed sciatic nerve showed reduced blood flow in Taxol-treated rats (LDPI units: controls 1181 ± 101.5 , Taxol 528.6 ± 32.7 , $P < 0.01$). VEGF GTx before Taxol injections completely inhibited deterioration of nerve blood flow (1152 ± 147.1 LDPI units, $P < 0.01$ vs Taxol, $P = \text{NS}$ vs ctr). VEGF therapy 4 weeks after Taxol injection also improved nerve perfusion, however, due to spontaneous recovery in saline-injected rats, differences between treatment groups were not statistically significant (VEGF 1093 ± 338 , saline 836 ± 104 LDPI units). Thalidomide treatment and subsequent VEGF or saline injection showed mild reduction of blood flow in saline-injected rats and restoration of perfusion with VEGF treatment (LDPI units: ctr 1329 ± 474 , thalidomide + saline 1062 ± 47 , thalidomide + VEGF 1434 ± 180). However, differences did not reach statistical significance.

In vivo staining of vasa nervorum by BS-1 lectin perfusion

Whole mount staining. Gross observation of sciatic nerve whole mount staining showed marked reduction of vasa nervorum after treatment with Taxol or thalidomide, whereas VEGF treatment, either before Taxol application (**Figure 6a**) or after establishment of the neuropathy by Taxol (not shown) or thalidomide (**Figure 6b**) restored vasa comparable to control nerves.

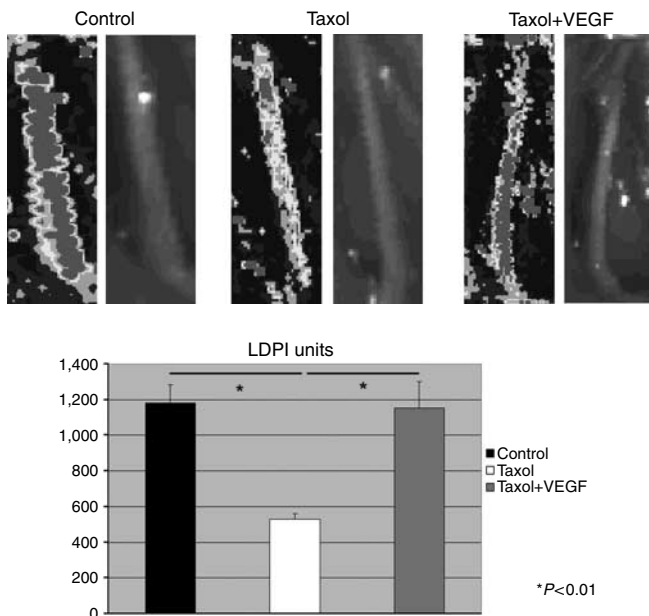


Figure 5 LDPI of sciatic nerve blood flow. LDPI was performed in control rats ($n = 10$) and in rats 4 weeks after taxol/saline ($n = 5$) or taxol + VEGF ($n = 4$) injections. Taxol significantly reduced nerve blood flow (* $P < 0.01$), whereas concomitant VEGF GTx inhibited this reduction. (* $P < 0.001$ vs taxol/saline, $P = \text{NS}$ vs control).

Quantification of vasa nervorum. Endoneurial vasa nervorum were counted on cross-sections of sciatic nerves. Taxol treatment showed marked reduction of vasa (ctr 48.1 ± 3.4 , Taxol 20.1 ± 2.8 , $P < 0.01$). VEGF GTx before Taxol treatment inhibited

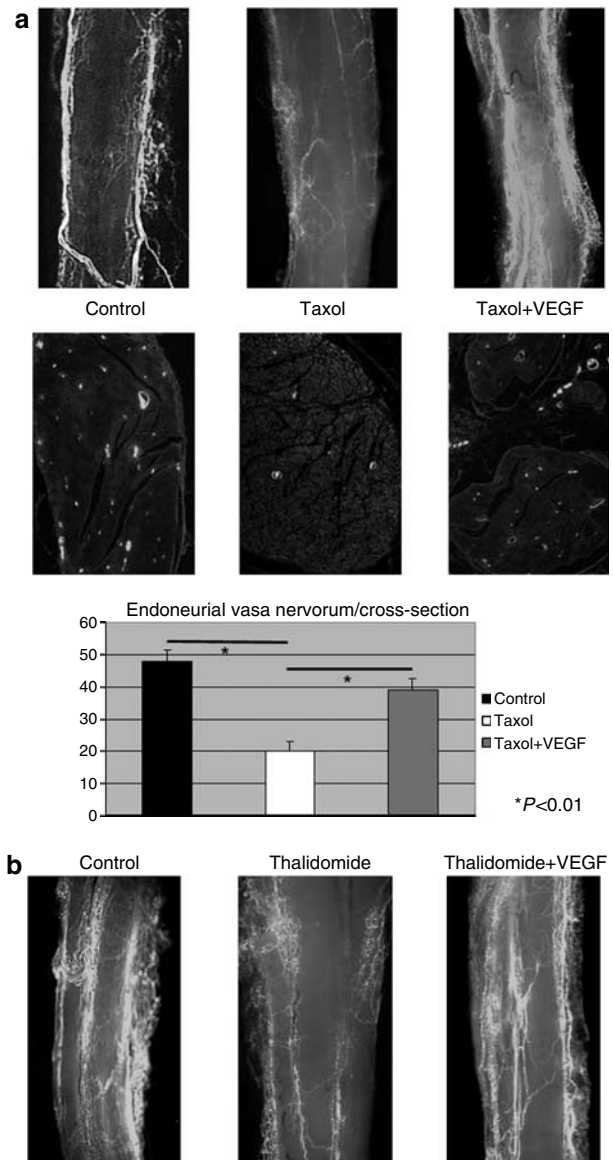


Figure 6 In vivo BS1 lectin staining of sciatic nerve vasa nervorum. (a) Whole mount fluorescent microscopic pictures of sciatic nerves (upper panel) after in vivo BS1 lectin perfusion show marked reduction of vasa nervorum in Taxol-treated rats whereas concomitant VEGF GTx inhibited loss of vasa. Sciatic nerve cross-sections (lower panel) after BS1 lectin perfusion demonstrate abundant endoneurial vasa nervorum in nerves of control animals, whereas treatment with Taxol led to a marked reduction of vasa nervorum. In contrast, VEGF gene therapy before Taxol showed normal density of vasa. Quantification of endoneurial vasa nervorum per cross section revealed significant reduction of vasa in Taxol treated, saline injected rats ($n = 5$) compared to controls ($n = 4$); * $P < 0.01$. VEGF GTx prevented loss of vasa ($n = 4$, $P < 0.01$ vs taxol/saline, $P = \text{NS}$ vs controls). (b) Representative whole mount fluorescent microscopic pictures of sciatic nerves of a control rat and thalidomide treated animals show reduced vasa nervorum after thalidomide treatment, whereas subsequent VEGF GTx restores vasa, yielding a picture comparable to controls.

loss of vasa nervorum (vasa/cross-section 39.0 ± 3.9 , $P < 0.01$ vs Taxol, $P = \text{NS}$ vs controls). VEGF GTx after induction of neuropathy by Taxol or thalidomide also increased vasa compared to saline treatment (Taxol: saline 45.7 ± 3.4 , VEGF 49.4 ± 1.5 ; thalidomide: saline 32.4 ± 13.7 , VEGF 44.7 ± 4.0) but these differences, while consistent with the LDPI measurements and suggestive of a restoration of the vasa nervorum, did not reach statistical significance.

DISCUSSION

Previous studies in our laboratory have demonstrated that gene therapy with the endothelial mitogen VEGF restores nerve function in animal models of ischemic and diabetic neuropathy^{22,23} as well as in patients with neuropathy associated with peripheral vascular disease, including in some diabetics.²⁴ In experimental diabetic neuropathy, the deterioration of nerve function was associated with a decrease in nerve blood supply via vasa nervorum, which was restored after VEGF therapy, thereby demonstrating the regenerative capacity of this component of the peripheral nerve.²³

Peripheral neuropathy induced by chemotherapeutic substances such as Taxol, thalidomide, and cisplatin¹² represents, beside other adverse events of these drugs, a major clinical problem due to the frequency of this toxic process and the lack of therapeutic measures to treat the resultant disability.^{3,5,25} Furthermore, this adverse effect often represents the dose-limiting factor in therapeutic oncologic regimen, where higher doses might be otherwise desirable.

The mechanism of chemotherapy-induced neuropathy is still uncertain. Direct toxic damage to axons and Schwann cells and disturbed cytoplasmic flow are considered to be the main pathogenic factors.³ Spontaneous improvement of nerve function over time, as observed in some animal models,²⁶ suggests involvement of components of the nerve which have regenerative capacity, unlike neurons themselves. However, involvement of the vasa nervorum is a more attractive hypothesis since the majority of substances causing this type of neuropathy, *i.e.*, Taxol, thalidomide, and cisplatin exhibit antiangiogenic properties in addition to their direct effects on tumor cells.^{8,9,27}

Previous reports have demonstrated that Taxol exhibits antiangiogenic properties *in vitro* and *in vivo*.^{8,28} We found that Taxol induces apoptosis of ECs. It has been reported that the PI3-kinase/Akt pathway plays an important role in Taxol-mediated intracellular signal transduction in tumor cells;²⁹ furthermore blockade of this pathway by the PI3-kinase inhibitor LY potentiated the antitumor effects of Taxol *in vitro* and *in vivo*.³⁰ Akt also plays an essential role in EC survival³¹⁻³³ and was inhibited by Taxol in ECs in our experiments. In contrast to tumor cells, where prolonged activation of MAPK system by Taxol has been observed,³⁴ this pathway was inhibited by the drug in ECs. Evidence for the importance of the Akt pathway in Taxol-induced EC apoptosis is provided by the fact that inhibition of this pathway further increased EC apoptosis compared to Taxol alone, whereas inhibition of MAPK, did not do so. Additionally, transfection of ECs by an adenovirus expressing the constitutively active form of Akt (Adeno-myrAkt) completely inhibited Taxol-induced EC apoptosis. VEGF also

inhibited Taxol-induced EC apoptosis *in vitro*, indicating also a possible effect of this substance *in vivo*. However, these studies do not exclude the possibility that other apoptotic pathways (*e.g.*, the Bcl-2 pathway) are involved in Taxol-induced EC apoptosis. One other limitation of this study is that effects observed in HUVECs might not be directly translated to ECs in vasa nervorum.

The most important finding of this study was the marked reduction of peripheral nerve intrinsic blood supply in Taxol-induced neuropathy as shown by laser-Doppler perfusion scanning and BS1 lectin staining. The role of vasa nervorum in this type of neuropathy was further elucidated by the strategy of *i.m.* gene transfer of the endothelial mitogen VEGF-1 in proximity to the sciatic nerve.²³ Western blotting of sciatic nerve extracts revealed increased VEGF expression in the nerve after *i.m.* VEGF plasmid injection, indicating VEGF transgene expression also at the site of the nerve.

This therapy prevented the expected reduction of nerve conduction velocities and of nerve blood supply when administered 2 days before Taxol injections. Furthermore, when VEGF was injected after neuropathy was already established it restored electrophysiologic nerve function.

Thalidomide represents another oncologic drug^{5,6} which is a potent antiangiogenic substance⁹ and causes a peripheral neuropathy.^{5,7} Long-term treatment of rats with thalidomide caused electrophysiological evidence of a sensory neuropathy, which recovered after VEGF gene therapy. Nerve blood supply and vasa nervorum were reduced after treatment and restored after VEGF.

Although recovery of nerve function was not accompanied with a statistically significant increase in blood flow and vasa nervorum in animals, which received VEGF after establishment of Taxol or thalidomide neuropathy the absolute changes observed favor an effect of VEGF on reconstitution of nerve vascularity. The lack of significance might be due to some spontaneous recovery of nerve function and vascularity (Taxol) or just mild damage to nerves and vasculature (thalidomide). Nevertheless, we cannot exclude some direct trophic effects of VEGF on Schwann cells^{22,35} or neuronal cells,^{36,37} however, complete recovery of an established neuropathy would be unlikely if VEGF would just act on neuronal cells because this cell type lacks regenerative capacity.

These data from experimental neuropathies induced by Taxol and thalidomide, as well as results from cisplatin neuropathy¹² support the hypothesis that toxic neuropathies induced by antiangiogenic chemotherapeutic substances are caused, at least in part, by vascular damage to the vasa nervorum and benefit from gene therapy with the angiogenic cytokine VEGF. Although these data suggest a potential therapeutic strategy to preserve nerve function in patients undergoing chemotherapy we are aware of the risks of a therapy with an angiogenic cytokine in tumor patients. The role of angiogenesis in tumor growth³⁸ is well established, based on preclinical data and the success of antiangiogenic therapies in humans.³⁹ Thus, the concern that a strategy involving the promotion of angiogenesis might accelerate cancer growth, is well founded. On the other hand, it is also known that angiogenesis alone is not sufficient to drive

tumorigenesis. In fact, the only evidence that we are aware of in the literature suggesting that overexpression of VEGF could lead to increased tumor growth involved either the stable transfection of a breast cancer cell line resulting in constitutive, unregulated, permanent overexpression of VEGF⁴⁰ or generation of a transgenic mouse with permanent unregulated VEGF overexpression.⁴¹ The low level, local and transient expression of VEGF that results from gene therapy with naked DNA is orders of magnitude less than these strategies. In human studies, a change in circulating VEGF levels was barely detectable only at 7 days after gene transfer.⁴² Moreover, because of our concern about this matter, we tested the ability of our approach of local VEGF gene therapy to increase vascularity or growth of tumors implanted in animal models. We implanted tumors in rats and administered VEGF plasmid repeatedly, starting before tumor implantation and at bi-weekly intervals, and found no impact of the transient expression of VEGF on tumor vascularity or growth compared to controls.¹² Finally, and most compellingly, approximately 1000 patients have now been enrolled in placebo controlled trials of angiogenic therapies for cardiovascular diseases and no evidence has emerged regarding an increase in cancer incidence in treated vs control patients.

Our study was not intended to suggest an immediate translation into clinical use, but rather to illustrate a concept and to identify a possible novel mechanism that could be exploited to avoid an important complication of certain chemotherapeutic agents. Therefore, further experiments are necessary before VEGF gene therapy could be considered as a preventative or therapeutic in humans with cancer.

MATERIALS AND METHODS

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured⁴³ in six- or 24-well plates and allowed to attach overnight. Cells in six-well plates were starved in M199/1% bovine serum albumin (BSA) (Sigma, St Louis, MO), treated with 1 μ M Taxol (Bristol-Myers Squibb, Syracuse, NY) for 18 h and processed for Western blotting. Twenty-four-well plates were treated with different concentrations of Taxol for 18 h in M199 containing 2% FBS, cells were fixed by 4% paraformaldehyde and stained with DAPI (4'-6'-diaminidino phenylidole, Roche, IN, USA; 1 μ g/ml PBS for 20 min). A dose-dependent effect of Taxol on EC proliferation and apoptosis was observed as reported in the literature. Unless stated otherwise 1 nM Taxol was used for further experiments. In some experiments, cells were starved and incubated with 1 nM Taxol with or without 100 ng VEGF (Sigma), 1 μ M LY294002 (Sigma), or 1 μ M PD98059 (Sigma) for 18 h and stained with DAPI.

In another set of experiments, HUVECs were transfected with an adenovirus expressing a constitutively active form of Akt (Adeno-myrAkt),³¹ using a MOI of 50. Cells were afterwards starved for 24 h with or without 1 nM Taxol and subjected to DAPI staining.

Cells were examined by fluorescent microscopy and three randomly selected fields per well were photographed. Total cell number as well as apoptotic cells, characterized by condensation and fragmentation of the nucleus was counted. Experiments were performed in triplicate.

Western blotting. Antibodies were purchased from Cell Signaling (Beverly, MA, USA): Phospho-Akt (Ser 473) and Akt, from Promega (Madison, WI, USA): Anti-ACTIVE MAPK and Anti-ERK 1/2 and from Santa Cruz (Santa Cruz, CA, USA): VEGF, Tubulin and Actin. Cells were

lysed, lysates processed and Western Blotting performed as suggested by the manufacturer. Bands were visualized by enhanced chemiluminescence (ECL) staining for 1 min (Amersham Pharmacia Biotech, Piscataway, NJ).

Animal models. All protocols were approved by the St Elizabeth's Medical Center Institutional Animal Care and Use Committee. In all experiments, investigators performing the follow-up examinations were blinded to the identity of the treatment administered.

Taxol- and thalidomide-induced neuropathy in rats. Male Sprague-Dawley rats (retired breeders, Charles River Laboratories, Wilmington, MA) weighing between 550 and 650 g at the outset were used. Rats were fed standard lab rodent chow and water *ad libitum* and housed individually.

Induction of Taxol-induced neuropathy. Twenty-six rats were treated by intravenous (i.v.) injections of 12 mg/kg Taxol (in cremophor/ethanol/saline 10/10/80% from Bristol-Myers Squibb, Syracuse, NY) as described by other investigators²⁶ on day 0 and day 3 under anesthesia. Age- and weight-matched rats injected with cremophor/ethanol/saline were used as control animals.

Induction of thalidomide-induced neuropathy. Rats ($n=14$) were treated by oral application (gavage) of 100 mg/kg thalidomide in 1% carboxymethylcellulose (CMC) daily from Monday to Friday for 6 months; a similar protocol was previously used to induce neuropathy in rabbits.^{44,45} Controls ($n=4$) received CMC.

VEGF plasmid and gene transfer. *phVEGF₁₆₅*: Gene therapy was accomplished with intramuscular (i.m.) injection of plasmid DNA expressing human VEGF₁₆₅ (*phVEGF₁₆₅*¹⁹). We demonstrated in a previous publication, that i.m. injection of plasmid DNA results in transgene expression in myocytes.⁴⁶ To demonstrate, that VEGF protein indeed is increased in nerves after i.m. VEGF plasmid injection Western blotting for VEGF was performed in sciatic nerves of injected and control sides 2 and 3 days after plasmid injection.

Gene transfer in Taxol-treated rats: Two groups were studied. In the first ($n=16$), VEGF was given 4 weeks after Taxol injections. In the second ($n=8$), VEGF was given concomitantly with the Taxol treatment (48 h before each Taxol injection) in an attempt to study the potential prophylactic effect of gene therapy on the neuropathy. Animals received i.m. injections of 250 μ g *phVEGF₁₆₅* as described.²³ Placebo injected animals received saline.

Gene transfer in thalidomide-treated rats: After the end of thalidomide treatment rats were randomly selected to receive 250 μ g *phVEGF₁₆₅* or saline ($n=7$ each).

Neurophysiological measurements. *Electrophysiology:* Sciatic nerve motor and sensory nerve conduction velocity (NCV) was measured as described previously.²³ NCV was measured bilaterally in all rats at baseline before the first taxol/thalidomide application and 4 weeks after Taxol and 1 week after last thalidomide application, respectively. VEGF gene therapy or placebo injection was performed and NCV measured again at 4 and 8 weeks after gene therapy (GTx).

In vivo assessment of nerve perfusion and vascularity. *Laser-Doppler imaging of vasa nervorum blood flow:* Perfusion of sciatic vasa nervorum was measured with a laser Doppler perfusion imager (LDPI) system (Moor Instruments, Wilmington, DE) as described previously in detail.²³ Briefly, nerves were exposed and blood flow measurements were repeated three times. Low or no perfusion was displayed in dark blue, while the maximum perfusion intensity was displayed in red.

Computer assisted analyses were performed by calculating the average perfusion for each nerve expressed as relative laser-Doppler color units.

Fluorescent imaging of vasa nervorum: Vascularity of sciatic nerves was assessed by *in situ* fluorescent staining using the endothelial cell (EC)-specific marker BS-1 lectin conjugated to FITC (Vector Laboratories, Burlingame, CA) as described previously.²³ Briefly, hindlimbs were perfused with BS-1 lectin (2 mg/rat) via the abdominal aorta. After killing, sciatic nerves were excised and fixed in 1% paraformaldehyde. After fixation, nerves were either whole-mounted for longitudinal analysis, or paraffin-embedded for cross-sectioning. Samples were analyzed using a fluorescence microscope (Nikon Inc., Melville, NY) and a digital camera (Bolton, MA). Fluorescent-labeled endoneurial vasa nervorum were counted per cross-section; typically four to six different sections were counted and averaged.

Statistics. All results are expressed as the mean ± SEM. Statistical comparisons between groups were performed by analysis of variance. $P < 0.05$ were considered to denote statistical significance. In case of non-normal distribution nonparametric tests were used.

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