Short Communication

Chemotherapy-Associated Angiogenesis in Neuroblastoma Tumors

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The influences of cytotoxic drugs on endothelial cells remain incompletely understood. Herein, we examined the effects of chemotherapeutic agents in experimental angiogenesis models and analyzed vessel densities in clinical neuroblastoma tumor samples. Cisplatin (20 to 500 ng/mL), doxorubicin (4 to 100 ng/mL), and vincristine (0.5 to 4 ng/mL), drugs commonly involved in neuroblastoma therapy protocols, induced pro-angiogenic effects in different angiogenesis models. They enhanced endothelial cell tube formation, endothelial cell sprouting from spheroids, formation of tip cells in the sprouting assay, expression of $\alpha v\beta 3$ integrin, and vitronectin binding. All three drugs increased global cellular kinase phosphorylation levels, including the angiogenesis-relevant molecules protein kinase Cβ and Akt. Pharmacological inhibition of protein kinase Cβ or Akt upstream of phosphatidylinositol 3-kinase reduced chemotherapy-induced endothelial cell tube formation. Moreover, the investigated chemotherapeutics dose dependently induced vessel formation in the chick chorioallantoic membrane assay. Tumor samples from seven high-risk patients with neuroblastoma were analyzed for vessel density by IHC. Results revealed that neuroblastoma samples taken after chemotherapy consistently showed an enhanced microvessel density compared with the corresponding samples taken before chemotherapy. In conclusion, our data show that chemotherapy can activate endothelial cells by inducing multiple pro-angiogenic signaling pathways and exert pro-angiogenic effects *in vitro* and *in vivo*. Moreover, we report a previously un-recognized clinical phenomenon that might, in part, be explained by our experimental observations: chemotherapy-associated enhanced vessel formation in tumors from patients with neuroblastoma. *(Am J Pathol 2012, 180: 1370–1377; DOI: 10.1016/j.ajpath.2011.12.011)***

Tumor angiogenesis, the ability of malignant tumors to establish their own blood supply, is regarded as an important event during oncogenesis and cancer disease progression. Moreover, inhibition of tumor angiogenesis has evolved as an anti-cancer treatment strategy. Different anti-angiogenic agents are registered for cancer treatment, and many other angiogenesis inhibitors are experimentally investigated in numerous cancer models.[1]

Many cytotoxic drugs have been shown to not only affect cancer cells but also to interfere with endothelial cell viability and angiogenesis, providing the basis for low-dose metronomic anti-angiogenic chemotherapy.[2] However, chemotherapeutics have also activated different cell types, such as monocytes, macrophages, and natural killer cells.[3–5] Various cytotoxic drugs were demonstrated to induce pro-inflammatory and pro-angiogenic gene expression in cancer and normal epithelial cells.[6–12] Moreover, chemotherapeutics caused pro-angiogenic effects through mobilization of bone marrow–derived circulating endothelial progenitor cells[13,14] or induced a stress response in endothelial cells that resulted in the paracrine protection of lymphoma cells from toxicity.[15] Also, (low-dose) ionizing radiation may exert pro-angiogenic potential through direct activation of endothelial cells.[16,17]

Neuroblastoma is the most frequent extracranial solid tumor of childhood. Approximately half of patients are confronted with high-risk disease associated with overall survival rates <40%, despite intensive multimodal treatment.[18]

Like tumors from numerous cancer entities, the ability of neuroblastoma tumors to establish their own blood supply is generally considered to be crucial for tumor progression and malignancy, and anti-angiogenic treatment regimens are considered for patients with neuroblastoma.[1,19]

Herein, we investigated the influence of the cytotoxic drugs cisplatin, doxorubicin, and vincristine on pro-angiogenic activity of endothelial cells (tube formation assay, spheroid sprouting assay, and compensation assay for analysis of tip and stalk cells) and on vessel formation in the chick chorioallantoic membrane (CAM). Finally, we compared vessel formation in tumor pairs from patients with neuroblastoma, obtained before and after chemotherapy.

Materials and Methods

Drugs

Cisplatin, doxorubicin, and vincristine were obtained from GRY-Pharma GmbH (Kirchzarten, Germany). Enzastaurin was obtained from Selleck Chemicals LLC (distributed by Biozol GmbH, Munich, Germany). LY294002 was obtained from Merck KGaA (Darmstadt, Germany). Luconyl Black was obtained from BASF (Ludwigshafen, Germany).

Cells

The cell lines UKF-NB-2, UKF-NB-3, and UKF-NB-6 were isolated from bone marrow metastases from patients with *MYCN* (alias N-*myc*)–amplified stage 4 neuroblastoma.[20–22] IMR-32 cells were obtained from American Type Culture Collection (Manassass, VA). NLF and IMR-5 cells were kindly provided by Dr. Angelika Eggert (University Children's Hospital Essen, Essen, Germany), and SMS-KAN cells were provided by Dr. C. Patrick Reynolds (Children's Hospital Los Angeles, Los Angeles, CA).

All neuroblastoma cell lines were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin at 37°C. All cell lines are quarterly tested and found free of mycoplasma contamination. Cell line authentication is regularly performed by analysis of short-tandem repeats.

Human umbilical vein endothelial cells (HUVECs) were received from PromoCell GmbH (Heidelberg, Germany) and cultivated as described previously.[23]

Cell Viability Assay

Cell viability was investigated using the MTT dye reduction assay, as described previously.[23]

Endothelial Cell Tube Formation

Endothelial cell tube formation was investigated using HUVECs seeded on extracellular matrix (BD Biosciences, Heidelberg, Germany), as described.[23]

Flow Cytometry

A monoclonal mouse antibody directed against integrin αVβ3 (MAB 1976Z-20; Millipore, Schwalbach, Germany) and a secondary phycoerythrin-conjugated goat anti-mouse antibody (R&D Systems GmbH, Wiesbaden, Germany) were used to detect integrin αVβ3 expression by flow cytometry (FACSCalibur; BD Biosciences). Vascular endothelial growth factor receptor 2 (VEGFR2) was detected using a phycoerythrin-conjugated VEGFR2 antibody (FAB357P; R&D Systems GmbH).

Cellular tyrosine phosphorylation status was determined in cells fixed with 4% formaldehyde and permeabilized with Perm/Wash buffer (BD Biosciences) using a mouse anti-phosphorylated tyrosine antibody (Cell Signaling via New England Biolabs GmbH, Frankfurt/Main, Germany) and a phycoerythrin-conjugated secondary anti-mouse antibody (R&D Systems GmbH).

Western Blot

Cells were lysed in Triton X sample buffer and separated by SDS-PAGE, as described previously.[23] Proteins were detected using specific antibodies against β-actin (Sigma-Aldrich Chemie GmbH, München, Germany), extracellular signal–regulated kinase (ERK) 1/2, the phosphorylated forms of ERK 1/2, MEK 1/2, Akt, the phosphorylated forms of Akt, protein kinase Cβ (PKCβ), the phosphorylated form of PKCβ, glycogen synthase kinase 3β (GSK3β), or the phosphorylated form of GSK3β (each from New England Biolabs, Frankfurt am Main, Germany). They were visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany).

Sprouting and Compensation Assay

HUVECs were treated with doxorubicin (20 ng/mL), vincristine (2 ng/mL), or cisplatin (200 ng/mL) for 24 hours before endothelial cell spheroids containing 400 cells were generated, as described.[24] After 24 hours in the collagen gel, angiogenesis was quantified by measuring the cumulative length of all capillary-like sprouts, the mean length of the sprouts, and the mean sprout number of each spheroid using a computer-assisted microscope.

For the compensation assay,[25] HUVECs were labeled with either cerulean or green fluorescent protein using a lentiviral expression system.[26,27] Afterwards, one group was treated with solvent and the other group was treated with doxorubicin (20 ng/mL), vincristine (2 ng/mL), or cisplatin (200 ng/mL) for 24 hours. Spheroids were generated by mixing control cells and drug-treated cells 1:1 and embedded in a collagen gel. After 24 hours, Z-stacks of the spheroids were generated and the three-dimensional projection was analyzed for the number of sprouted control cells and treated cells and the respective position as a tip cell or a stalk cell. Fluorescences were displayed in arbitrary colors.

At least five spheroids per experimental group and experiment were analyzed.

CAM Assay

The CAM assay was performed using drug-loaded gelatin sponges, as described.[23,28] Sponges were placed onto the CAM at day 8. Vessel formation was determined at day 12.

To better visualize the vascular system of the CAM, 20% Luconyl Black in PBS was injected into a vitelline vein. The pro-angiogenic response was scored from 0 (no vessel formation) to 5 [maximal vessel formation, as induced by basic fibroblast growth factor (bFGF), 10 ng].

Pathological Examinations

All patients were treated by the NB97 study protocol of the German Society of Pediatric Oncology and Hematology. The study was evaluated and approved by the Ethics Committee of the University of Cologne (Cologne, Germany). All patient data used herein were analyzed anonymously.

Formalin-fixed, paraffin-embedded tissue was used for immunohistochemical (IHC) staining of CD31 (monoclonal mouse antibody, clone JC70A; Dako, Hamburg, Germany) and actin (monoclonal mouse antibody, clone 1A4; Dako). Slides (5 μm thick) were deparaffinized. For IHC staining of CD31, antigen retrieval was performed with enzymatic digestion (5 minutes, Proteinase K, Ready-to-use; Dako). For IHC staining of actin, no antigen retrieval was necessary. The slides were incubated with the primary antibody (dilutions: actin, 1:400; CD31, 1:100) for 1 hour. Immunoreactivity was detected with the Dako REAL Detection System AP/RED, Rabbit/Mouse (code K5005; Dako).

Vessel formation was described in vascular hot spots by two parameters, microvessel density (MVD) and microvessel pericyte coverage index (MPI), following described procedures.[29] To determine MVD, CD31-stained tumor microvessels were counted in five different fields of 1 mm². To determine MPI, sequent slides were used. The first one was stained for CD31, indicating endothelial cells; and the second one was stained for α-smooth muscle actin, indicating pericytes. The MPI was expressed as percentage of α-smooth muscle actin–surrounded vessels relative to total vessels.

Statistical Analysis

Two groups were compared by Student's *t*-test; more groups were compared by analysis of variance. A pairwise multiple comparison was performed by the Student-Newman-Keuls test.

Results

Endothelial Cells Are Less Sensitive to Chemotherapeutics than Neuroblastoma Cells

HUVECs showed a lower sensitivity to the anti-cancer drugs doxorubicin, vincristine, or cisplatin, compared with a panel of seven neuroblastoma cell lines, as indicated by MTT assay after 5-day incubation. Concentrations that decreased cell viability by 50% (IC_{50}s) in neuroblastoma cells ranged as follows: doxorubicin, from 4.28 to 39.02 ng/mL; vincristine, from 0.27 to 1.23 ng/mL; and cisplatin, from 82 to 322 ng/mL. In HUVECs, the doxorubicin IC_{50} was 165.14 ng/mL (4.2- to 38.6-fold

decreased sensitivity relative to neuroblastoma cells), the vincristine IC_{50} was 4.54 ng/mL (3.7- to 16.8-fold decreased sensitivity), and the cisplatin IC_{50} was 2903 ng/mL (9.0- to 35.4-fold decreased sensitivity) (see Supplemental Table S1 at *http://ajp.amjpathol.org*).

Influence of Chemotherapeutics on Endothelial Cell Tube Formation, Endothelial Cell Activation, and Endothelial Cell Sprouting in Vitro

Next, we investigated the influence of anti-cancer drugs on endothelial cell tube formation. HUVEC tube formation was increased by doxorubicin concentrations ranging from 4 to 100 ng/mL, cisplatin concentrations ranging from 20 to 500 ng/mL, and vincristine concentrations ranging from 0.5 to 2 ng/mL. Maximal stimulation was achieved by 100 ng/mL cisplatin, 20 ng/mL doxorubicin, and 2 ng/mL vincristine (Figure 1, A–D).

Activated endothelial cells are characterized by increased expression of $\alpha v\beta 3$ integrin (a vitronectin receptor), increased endothelial cell binding to vitronectin, and enhanced VEGFR2 expression.[30] Treatment with doxorubicin, cisplatin, or vincristine enhanced $\alpha v\beta 3$ integrin expression and cellular vitronectin binding (Figure 1E). Cisplatin treatment increased the number of VEGFR2-expressing cells in a similar manner to bFGF (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

Cisplatin, doxorubicin, and vincristine also enhanced bFGF-induced sprouting *in vitro* in a collagen-based spheroid assay (Figure 2A). Doxorubicin and vincristine increased total tube length, mean tube length, and mean tube number in the same concentration found to be most effective in the tube formation assay (ie, 20 ng/mL doxorubicin and 2 ng/mL vincristine). In contrast, treatment of spheroids with cisplatin, 100 ng/mL, did not result in significant effects (data not shown), whereas 200 ng/mL exerted substantial stimulatory effects (Figure 2A). The reason for this discrepancy remains unclear.

Although all three drugs enhanced the numbers of tip cells, varying influences on the numbers of stalk cells were found. Cisplatin or doxorubicin did not influence the quantities of stalk cells, whereas vincristine treatment resulted in reduced stalk cell numbers (Figure 2B).

Influence of Chemotherapeutics on Vessel Formation in the Chick CAM Assay

Up to certain levels, cisplatin (0.8 μg), doxorubicin (0.25 μg), and vincristine (0.05 μg) enhanced vessel formation in the CAM in a concentration-dependent manner, whereas a further increase in the amounts of applicated drugs resulted in anti-angiogenic effects (Figure 3).

Influence of Chemotherapeutics on Signaling Pathways in Endothelial Cells

Irradiation was transiently associated with enhanced global protein phosphorylation in endothelial cells, as indicated by tyrosine phosphorylation levels.[17] Investigation of tyrosine phosphorylation 30 minutes after addition
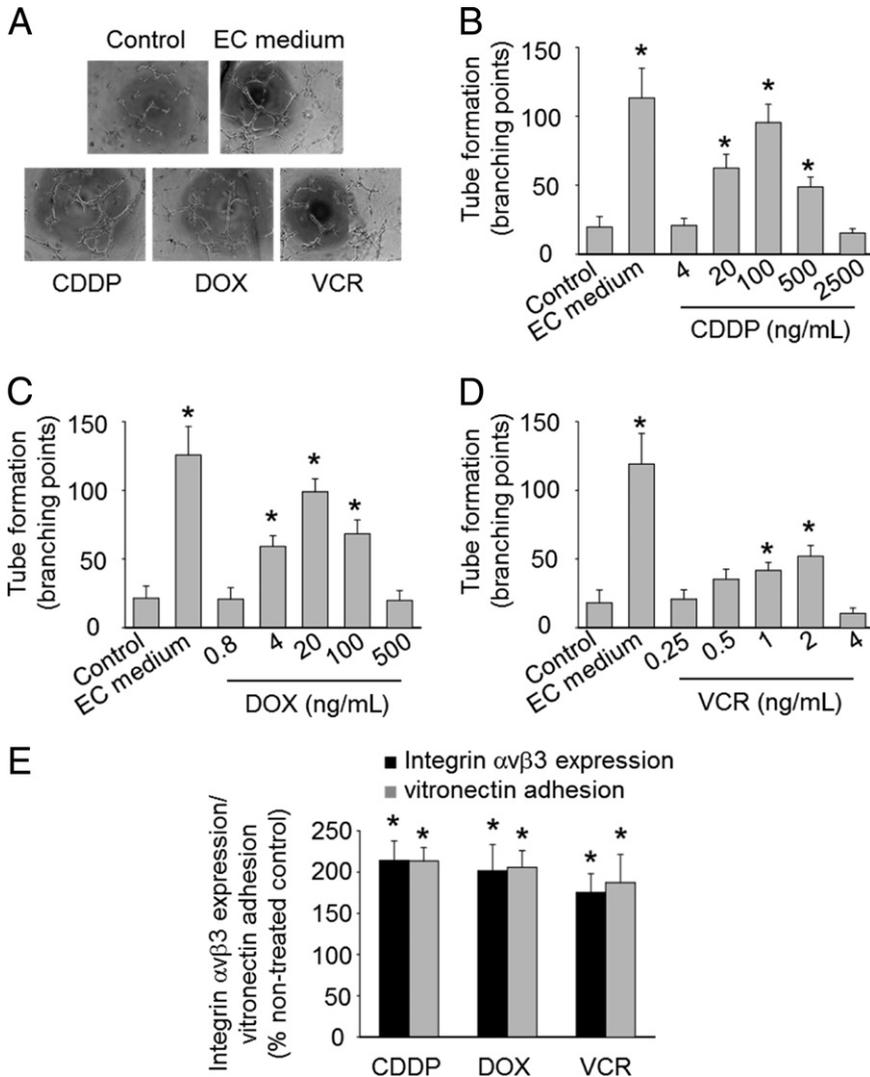
Figure 1. Influence of cytotoxic drugs on endothelial cell tube formation and activation. **A:** Representative pictures of HUVEC tube formation. Cells were serum starved for 24 hours before seeding onto extracellular matrix. Tube formation was investigated 16 hours after cell seeding on extracellular matrix. HUVECs incubated with IMDM supplemented with pooled human serum (10%), fetal calf serum (10%), and bFGF (2.5 ng/mL) (EC medium) served as a positive control. Drug concentrations were as follows: cisplatin (CDDP), 100 ng/mL; doxorubicin (DOX), 20 ng/mL; or vincristine (VCR), 1 ng/mL. **B:** Quantification of tube formation in CDDP-treated HUVECs by determination of the number of branching points. **C:** Quantification of tube formation in DOX-treated HUVECs by determination of the number of branching points. **D:** Quantification of tube formation in VCR-treated HUVECs by determination of the number of branching points. **E:** Relative $\alpha v \beta 3$ integrin expression and relative adhesion to vitronectin of HUVECs treated with CDDP, 100 ng/mL; DOX, 20 ng/mL; or VCR, 2 ng/mL. HUVECs were serum starved for 24 hours and then treated for 16 hours with cytotoxic drugs. $\alpha v \beta 3$ Integrin was measured by flow cytometry. For determination of vitronectin adhesion, cells were seeded into vitronectin-coated wells, allowed to adhere for 1 hour, and washed with PBS. After 2 hours, the metabolic activity of adhered cells was measured by the Alamar Blue assay. Results were expressed relative to nontreated control (ie, 100%). $*P < 0.05$ relative to control.

of cytotoxic drugs revealed clearly enhanced global protein phosphorylation relative to the nontreated control that returned to basal levels after 60 minutes (see Supplemental Figure S2A at *http://ajp.amjpathol.org*).

Moreover, anti-cancer drugs caused phosphorylation of PKCβ and its downstream kinase GSK3β, phosphorylation of the phosphatidylinositol 3-kinase (PI3K) downstream kinase Akt, and weak phosphorylation of the mitogen-activated protein kinases ERK 1/2 (see Supplemental Figure S2B at *http://ajp.amjpathol.org*), all known players in endothelial cell activation during angiogenesis.[30–34]

Inhibition of Chemotherapy-Induced Signaling Prevents Chemotherapy-Induced Angiogenesis

The PKCβ inhibitor, enzastaurin, or the PI3K inhibitor, LY294008, interfered with chemotherapy-induced endothelial tube formation (see Supplemental Figure S2, C and D, at *http://ajp.amjpathol.org*), suggesting that chemotherapy-induced activation of the corresponding signaling pathways contributes to the chemotherapy-induced pro-angiogenic effects.

Increased Vessel Formation in Tumor Samples Taken after Chemotherapy

Finally, neuroblastoma tumor vessel density was compared before and after chemotherapy in seven patients with high-risk neuroblastoma. All patients had *MYCN* amplification and were >1 year at diagnosis. Of the seven patients, six were diagnosed as having stage IV disease, except for one patient, who was diagnosed as having stage II disease (see Supplemental Table S2 at *http://ajp.amjpathol.org*). All patients were treated by the NB97 study protocol of the German Society of Pediatric Oncology and Hematology,[35] with a treatment regimen containing cisplatin, doxorubicin, and vincristine, among other substances. Tumor samples were taken before chemotherapy at diagnosis and after four to seven blocks of chemotherapy.

Neuroblastoma samples taken after chemotherapy were characterized by necrotic areas but consistently showed an enhanced MVD (Figure 4). The MPI did not differ between neuroblastoma samples taken at diagnosis or after chemotherapy (data not shown). Notably,
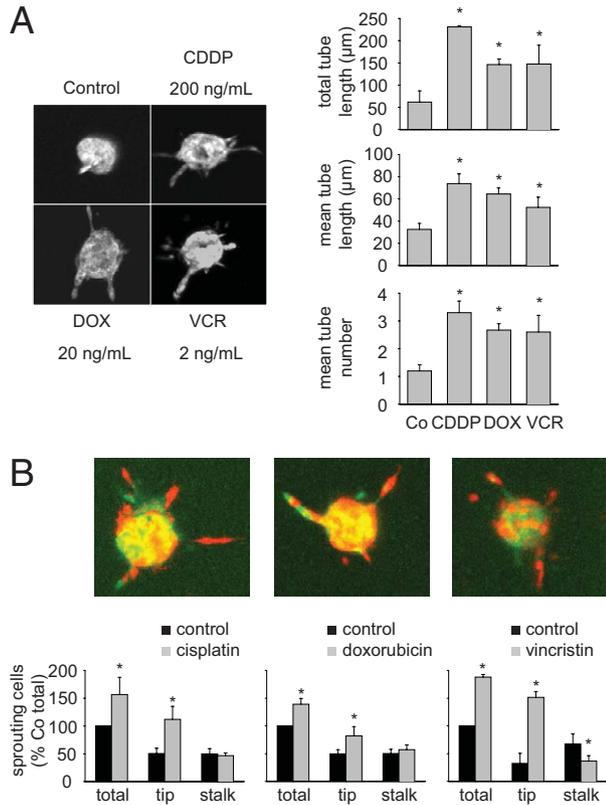
Figure 2. Influence of cytotoxic drugs on endothelial cell sprouting. **A:** Representative photographs and bar graphs demonstrating the effects of cisplatin (CDDP; 200 ng/mL), doxorubicin (DOX; 20 ng/mL), or vincristine (VCR; 2 ng/mL) on endothelial sprouting in the spheroid assay. **B:** Representative photographs and bar graphs showing the effects of CDDP (200 ng/mL), DOX (20 ng/mL), or VCR (2 ng/mL) on the formation of tip and stalk cells in endothelial sprouts. Green fluorescent protein–labeled control cells (green) and cells labeled with cerulean and treated with the respective chemotherapeutic agent (red) were mixed; the resulting spheroids were embedded in a collagen matrix, and endothelial sprouting was observed after 24 hours. *$P < 0.05$ relative to control (Co).

therapy outcome differed between the investigated patients with neuroblastoma. Three patients showed event-free survival during the observation period (4161, 3198, or 3266 days after diagnosis), and four patients experienced recidivism.

Discussion

Although the establishment of its own blood supply is considered to be an important factor during tumor progression, the influence of chemotherapy on endothelial cells and tumor angiogenesis remains to be studied in detail. Herein, we show that certain concentrations of the cytotoxic drugs cisplatin, doxorubicin, or vincristine can directly activate endothelial cells and exert pro-angiogenic effects in different *in vitro* and *in vivo* models. Apparently, the pro-angiogenic effects are the consequence of the stimulation of multiple pro-angiogenic signaling pathways, including PKC and PI3K signaling by cytotoxic drugs. The PKCβ inhibitor, enzastaurin, that is under clinical investigation in phase 2 and 3 studies[36,37] or the PI3K inhibitor, LY294008, interfered with chemo-
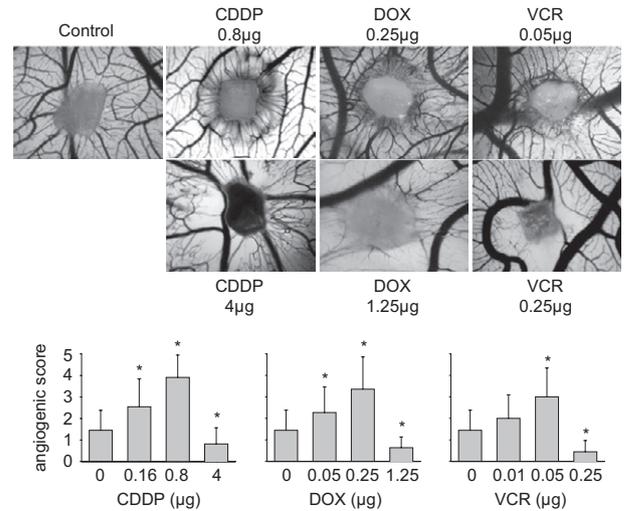

Figure 3. Representative photographs demonstrating the effects of cisplatin (CDDP), doxorubicin (DOX), or vincristine (VCR) on vessel formation in the chick CAM. Bar graphs demonstrate the angiogenic score at each drug concentration. *$P < 0.05$.

therapy-induced endothelial cell tube formation. These results are in accordance with previous findings[38–41] showing that cytotoxic drugs can activate survival pathways in different cell types and that activation of these pathways in endothelial cells is a well-characterized pro-angiogenic event.[31–34] Moreover, chemotherapy-acti-
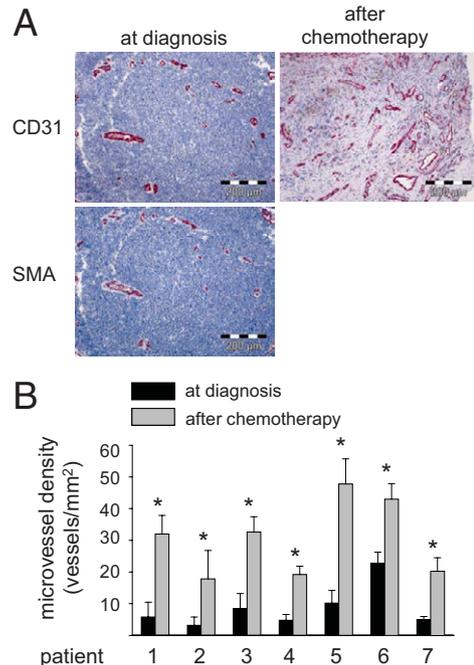

Figure 4. Vessel density in pathological tumor samples. Vessel densities were compared between samples taken from therapy-naïve patients at diagnosis and from subsequent therapy-refractory recidives of the same patients (seven pairs from seven patients). **A:** Representative photomicrographs of one tumor pair stained for endothelial cells (anti-CD31 antibody) or for smooth muscle cells [anti-smooth muscle actin (SMA) antibody]. **B:** Quantification of MVDs at diagnosis or after therapy. *$P < 0.05$ relative to MVD at diagnosis.

vated endothelial cells have previously supported cancer cell survival by paracrine effects.[15]

Endothelial cell treatment with cisplatin, doxorubicin, or vincristine increased the number of tip cells in a sprouting assay. Usually, tip cells are characterized by the expression of VEGFR2 and δ-like ligand 4 (Dll4) and induce Notch signaling in the stalk cells.[42–44] Like the pro-angiogenic growth factor, bFGF, cisplatin increased the number of VEGFR-expressing cells (see Supplemental Figure S1A at *http://ajp.amjpathol.org*). Moreover, cisplatin, like bFGF or VEGF, enhanced Dll4 expression, as determined by quantitative PCR (2.29 ± 0.26-fold relative to control after 24 hours; 10 ng/mL bFGF: 2.18 ± 0.54-fold; 100 ng/mL VEGF: 2.59 ± 0.49-fold), increased the number of Dll4-positive cells, and promoted DNA synthesis in Dll4-negative endothelial cells that are supposed to be the stalk cells (see Supplemental Figure S1, B and C, at *http://ajp.amjpathol.org*). Conflicting results have been published on the effects of Dll4-Notch signaling on endothelial (stalk) cell proliferation, and these effects seem to vary during vessel formation.[44–48] Therefore, further research will be necessary to receive a more detailed picture. Nevertheless, the investigated cisplatin concentration appears to resemble the pro-angiogenic effects of established pro-angiogenic growth factors. Therefore, these data support the potential pro-angiogenic role of cytotoxic drugs in certain concentrations.

A comparison of tumors from seven patients with high-risk neuroblastoma, taken before or after chemotherapy, revealed consistently higher vessel densities in the post-chemotherapy samples than in the corresponding samples taken at diagnosis, although necrotic areas in the post-chemotherapy samples suggested anti-cancer therapy to be effective. Notably, outcomes differed between these patients: three patients showed event-free survival during the observation period (4161, 3198, or 3266 days after diagnosis), and four patients experienced recidivism. These findings demonstrate that high-dose chemotherapy may be associated with enhanced vessel formation in tumors of patients with neuroblastoma.

Based on our findings presented herein, it appears plausible that endothelial cells may be temporally exposed to chemotherapeutic drug concentrations that directly activate endothelial cells during the treatment course. This may contribute to the observed chemotherapy-associated angiogenesis. However, other mechanisms are likely to contribute in the much more complex *in vivo* situation. The patients received granulocyte colony-stimulating factor during therapy[35] that is known to exert pro-angiogenic effects.[49–51] Also, infiltrating immune cells, especially macrophages, may promote tumor vessel formation,[52] although we did not find detectable levels of macrophages (Figure 4A, data not shown). Moreover, chemotherapy-induced mobilization of bone marrow–derived circulating endothelial progenitor cells has been described.[1,13,14]

The therapeutic implications of our observation remain the subject of further investigations. Anti-angiogenic therapy has proved to be clinically effective, especially in combination with maximally tolerated dose chemotherapy in different cancer entities,[1] a phenomenon of which the underlying mechanisms remain only partly understood. Anti-angiogenic therapy–induced vessel normalization, resulting in improved anti-cancer drug delivery into tumors and/or inhibition of mobilization of bone marrow–derived circulating endothelial progenitor cells,[1,13,14,53,54] may be possible mechanisms. Herein, our data suggest that anti-angiogenic therapies may also target vessel formation induced by direct endothelial cell activation by maximally tolerated dose chemotherapy.

In conclusion, our data show that chemotherapy can activate endothelial cells by inducing multiple pro-angiogenic signaling pathways and exert pro-angiogenic effects in different *in vitro* and *in vivo* angiogenesis models. Moreover, we report a previously unrecognized clinical phenomenon that might be, at least in part, explained by our experimental observations: chemotherapy-associated enhanced vessel formation in tumors from patients with neuroblastoma.

Acknowledgments

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