Oncolytic Vaccinia Virus Disrupts Tumor-Associated Vasculature in Humans

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Abstract

Efforts to selectively target and disrupt established tumor vasculature have largely failed to date. We hypothesized that a vaccinia virus engineered to target cells with activation of the ras/MAPK signaling pathway (JX-594) could specifically infect and express transgenes (hGM-CSF, β-galactosidase) in tumor-associated vascular endothelial cells in humans. Efficient replication and transgene expression in normal human endothelial cells in vitro required either VEGF or FGF-2 stimulation. Intravenous infusion in mice resulted in virus replication in tumor-associated endothelial cells, disruption of tumor blood flow, and hypoxia within 48 hours; massive tumor necrosis ensued within 5 days. Normal vessels were not affected. In patients treated with intravenous JX-594 in a phase I clinical trial, we showed dose-dependent endothelial cell infection and transgene expression in tumor biopsies of diverse histologies. Finally, patients with advanced hepatocellular carcinoma, a hypervascular and VEGF-rich tumor type, were treated with JX-594 on phase II clinical trials. JX-594 treatment caused disruption of tumor perfusion as early as 5 days in both VEGF receptor inhibitor-naïve and -refractory patients. Toxicities to normal blood vessels or to wound healing were not evident clinically or on MRI scans. This platform technology opens up the possibility of multifunctional engineered vaccinia products that selectively target and infect tumor-associated endothelial cells, as well as cancer cells, resulting in transgene expression, vasculature disruption, and tumor destruction in humans systemically. Cancer Res; 73(4); 1265–75. ©2012 AACR.

Introduction

Solid tumors must develop a blood supply to grow and metastasize (1). Growth factors including VEGF and fibroblast growth factor (FGF) play critical roles in promoting tumor angiogenesis (2–4). Both angiogenesis and the established tumor vasculature are promising therapeutic targets. VEGF/VEGF receptor (VEGFR)-targeted angiogenesis inhibitors have resulted in transient clinical benefit, although resistance eventually develops through diverse mechanisms including upregulation of alternate proangiogenic pathways (e.g., FGF-2) and “evasive resistance” through development of a more invasive or metastatic tumor phenotype (3, 5). Small-molecule vascular disrupting agents (VDA) are designed to collapse established tumor vasculature, but despite reductions in tumor perfusion, VDAs have not improved survival significantly to date (6, 7). Therefore, novel approaches to targeting tumor angiogenesis and ablating established tumor vasculature are needed, both in antiangiogenic treatment-naïve and -refractory patients. JX-594 is a Wyeth vaccinia vaccine-derived oncolytic virus engineered for viral thymidine kinase gene inactivation, and expression of the human granulocyte-monocyte colony stimulating factor (hGM-CSF) and β-galactosidase (β-gal) transgenes for immune stimulation and replication assessment, respectively (8, 9). In phase I trials, JX-594 was well tolerated and associated with replication in cancer cells, expression of its 2 transgenes, and tumor necrosis after intravenous or intra-tumoral injection (8, 10, 11). Vaccinia has evolved to exploit cellular signal transduction pathway activation for its replication and spread. Specifically, activation of the EGFR pathway has been shown to drive vaccinia replication, as has activation of other pathways (e.g., Abl kinase; refs. 12–15). In addition, JX-594 specifically requires high cellular thymidine kinase levels to replicate. We therefore hypothesized that endothelial cell-specific growth factor signal transduction pathway stimulation would create a cellular milieu supportive of JX-594 replication, including cellular thymidine kinase induction. We hypothesized that JX-594 could infect and disrupt tumor-associated vasculature selectively in humans. First, replication in cancer...
cells is driven by EGFR/Ras pathway signaling and cellular thymidine kinase levels (13, 16–18), and VEGF activates many of the same downstream mediators in endothelial cells (19). Second, we previously made anecdotal observations with oncolytic vaccinia from individual mice and humans that were consistent with this hypothesis (20, 21). Finally, with RNA-based oncolytic virus therapy, decreased tumor perfusion in mice (22, 23) and infection of tumor-associated endothelial cells following anti-VEGFR therapy were reported (24). Nevertheless, definitive clinical and laboratory mechanistic data were lacking.

To determine whether selective tumor vascular infection and disruption could be achieved by JX-594 in patients, we conducted detailed laboratory and clinical investigations. First, we evaluated whether JX-594 could infect and lyse normal human endothelial cells under VEGF and FGF-2 stimulation in vitro. Next, we assessed vascular disruption in a murine tumor model. In humans, we evaluated human tumor biopsies for endothelial cell infection and transgene expression after intravenous administration of JX-594 in a phase I clinical trial, and we prospectively designed an analysis of tumor perfusion over time after JX-594 treatment in phase II trials of patients with advanced hepatocellular carcinoma (HCC), a hypervascular and VEGF-rich tumor type (25). Full safety and toxicity evaluations were conducted, including assessments for toxicity to normal blood vessels and to healing wounds.

Materials and Methods

Virus

JX-594 is a Wyeth vaccinia virus vaccine-derived oncolytic virus with disruption of the viral thymidine kinase gene and expression of the hGM-CSF and β-gal transgenes under control of the synthetic early-late and p7.5 promoters, respectively (8). A version of JX-594 was generated to monitor replication in vitro, JX-594-GFP+/β-gal- expresses GFP instead of β-gal under the synthetic early/late promoter pSE/L in the vaccinia thymidine kinase gene locus. Viruses were propagated in HeLa cells. The vaccinia virus Wyeth strain was obtained from American Type Culture Collection (ATCC; distributed by Cedarlane Laboratories, Burlington, Ontario, Canada) and was propagated on U2OS cells. For mouse tumor model studies, a WR-TK(−) vaccinia was used as a mouse-adapted surrogate of JX-594; WR is a Wyeth strain vaccinia that was isolated through serial passage in mice.

In vitro infection of endothelial cells, Western blotting, and immunofluorescence staining

Human umbilical vein endothelial cell (HUVEC) or human dermal microvascular endothelial cells were subcultured in Endothelial or Microvascular Endothelial Cell Growth Medium-2 (Lonza). Endothelial cells used in experiments were not passaged more than 3 times since resuscitation. For Western blotting, cells were plated in 6-well dishes and stimulated with 50 ng/mL VEGF165 (R&D Systems/Cedarlane) or 50 ng/mL EGF (Sigma). Detergent cell extracts from triplicate wells were pooled and prepared as described (26). Following protein determination by Bradford assay (BioRad Protein Assay Solution), 20 μg of clarified cell extract were electrophoresed using the NuPAGE SDS-PAGE Gel System (Invitrogen). Blots were cut into strips and probed with antibodies specific for tyrosine-1175 phosphorylated VEGFR2 (Cell Signaling NEB), total VEGFR2 (Cell Signaling), thymidine kinase-1 (Abcam), or α-tubulin (Santa Cruz), followed by horseradish peroxidase–conjugated mouse or rabbit secondary antibodies (The Jackson Laboratory, Bar Harbor, Maine). Bands were visualized using Supersignal West Pico Chemiluminescent substrate (ThermoScientific Pierce). To assess the effect of endothelial growth factors on vaccinia replication, cells were plated in MCDB131 medium (Gibco/Invitrogen) in 24-well dishes and pulsed with 30 ng/mL VEGF165 or 5 ng/mL FGF-2 (R&D Systems) in the presence of JX-594-GFP+/β-gal- or wild-type Wyeth control at multiplicity of infection (MOI) 0.01. Fluorescent images were taken 72 hours postinfection with an Axiovert Si10 Fluorescence microscope (Carl Zeiss Ltd). Relative fluorescence units for GFP were obtained using the Synergy Mx microplate reader and Gen5 1.10 software (Biotek). Cells and supernatant were collected 48 to 72 hours postinfection and JX-594-GFP+/β-gal- and Wyeth vaccinia were titered from cell lysates by plaque assay on U2OS. Virus output was normalized to cell numbers following growth factor stimulation. Viable cells were counted by Trypan blue exclusion assay. For in vitro wound healing assays, HUVECs were grown on glass coverslips until confluent, treated with 30 ng/mL VEGF165, scratched with a micropipette tip, and infected with JX-594-GFP+/β-gal- at MOI 0.01 for 24 hours. Coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked using 5% goat serum with 1% BSA in PBS, and incubated with mouse monoclonal anti-CD105 (Dako) or rabbit monoclonal EPR3193 thymidine kinase 1 antibody (Abcam) overnight at 4°C. For detection, coverslips were incubated with Alexa-Fluor 555-conjugated goat anti-mouse or rabbit secondary antibodies (Molecular Probes, Invitrogen) and mounted and sealed with Dako Prolong anti-fade (Dako) with 4’, 6-diamidino-2-phenylindole (DAPI). Images were collected using the Zeiss Imager M1 microscope equipped with AxioCam HRm camera (Carl Zeiss Ltd). For microtubule disruption assays, endothelial cells cultured on glass coverslips were infected with JX-594 (MOI = 0.05) or mock infected. After 36 hours postinfection, cells were fixed as above and incubated with goat anti-tubulin antibody (Abcam) followed by Alexa Fluor 594 donkey anti-goat (Molecular Probes, Invitrogen) and visualized as above.

Mouse treatments

BALB/c mice (female, 8 to 10 weeks) were implanted with 1 × 10^5 4T1 cells (ATCC) subcutaneously on the hind limb. Tumors were allowed to form (7 to 10 days until tumors were approximately 100 mm^3). Mice were then treated intravenously with 1 × 10^5 plaque-forming units (pfu) of WR-TK(−) Vaccinia strain WR with a deletion in the viral thymidine kinase gene; strain WR was used in these experiments as it is better able to replicate in mouse cell lines relative to Wyeth; n = 3 per group, experiment repeated 3 times). All animal work was conducted with the approval from the Institutional Animal Care and Use Committee.
Patients

Infection of tumor-associated vasculature was assessed in patients treated on a phase I dose-escalation trial of i.v. JX-594 (ClinicalTrials.gov number NCT00625456; ref. 11; n = 23). Patients had treatment-refractory, histologically confirmed advanced/metastatic solid tumors. Patients were treated at doses ranging from 1 × 10^5 to 3 × 10^6 pfu per kg; a sixth cohort was added at 1 × 10^9 pfu (fixed dose). Acute effects of JX-594 on tumor perfusion were evaluated in patients with advanced HCC on randomized phase II trials of JX-594 (ClinicalTrials.gov number NCT00554372 and NCT01171651). On one randomized study, patients were treated intratumorally with a single dose of JX-594 at a dose level of 1 × 10^6 or 1 × 10^9 pfu before the evaluation of tumor perfusion on day 5 (dynamic MRI). One additional patient with metastatic rectal adenocarcinoma was treated intratumorally in liver metastases at a dose of 1 × 10^9 pfu. Patients with HCC on a second phase II study were treated with a single intravenous infusion of JX-594 at a dose of 1 × 10^9 pfu before the assessment of effects on tumor perfusion on day 6.

All patients gave written informed consent according to guidelines on good clinical practice. Protocol and consent forms were approved by the United States Food and Drug Administration, Health Canada, the Korea Food and Drug Administration, as well as the Institutional Review and Infection Control Committees at each hospital.

Patient biopsy analysis

Biopsies (excisional, core needle, or fine-needle aspirate) were obtained from all subjects 7 days (±1) after i.v. phase I treatment and formalin-fixed/paraffin-embedded. Hematoxylin and eosin (H&E)-stained sections were evaluated for the presence of tumor tissue, normal tissue, and vascular structures. Additional sections were used to detect vaccinia and β-gal by immunohistochemistry (IHC). IHC for vaccinia was conducted as previously described. For immunohistochemical detection of β-gal, an anti-β-gal polyclonal antibody (Abcam) was used. Negative controls were run without primary antibody, and tumors from mice treated with JX-594 were included as positive controls. The proportion of vessels infected by JX-594 was determined by a Board-certified human pathologist on vaccinia-stained sections; if vessels were too numerous to count, 5 random fields at ×20 magnification were chosen for quantification. Tumor neovasculature was identified by 2 independent pathologists.

Imaging

Dynamic contrast-enhanced MRI imaging was conducted 5 days after intratumoral injection of JX-594 into primary liver tumors or liver metastases or 6 days after intravenous infusion of JX-594; this hyperacute MRI scan was conducted to evaluate early changes in tumor vascularity, and to determine whether early MRI responses correlated with longer term clinical endpoints. Imaging was conducted using an extracellular gadolinium contrast agent and a three-dimensional spoiled gradient echo pulse sequence with fat suppression. Lesion signal intensity measurements were used to calculate changes in contrast enhancement, a reflection of tumor perfusion.
kinase-positive HUVECs at the leading edge of a scratch in the cell monolayer were preferentially infected. Furthermore, infection colocalized with the endothelial cell activation marker CD105 (Fig. 1F). We then sought to investigate in vitro whether JX-594 infection of endothelial cells resulted in microtubule depolymerization and cell rounding; these effects are associated with vascular disruption in vivo. This phenotype was confirmed following HUVEC infection with JX-594-GFP+/−β-gal− (Fig. 1G). In summary, these data showed that JX-594 could selectively infect and express transgenes in VEGF- and FGF-2–activated human endothelial cells of 2 subtypes.

**JX-594 infects and disrupts tumor vasculature in tumor models in vivo**

We subsequently evaluated the ability of a mouse-adapted thymidine kinase gene-deleted vaccinia [WR-TK(−)] to infect and disrupt tumor vasculature after intravenous administration in an immunocompetent BALB/c murine tumor model (4T1 breast cancer, subcutaneous hind limb location). Intravenous WR-TK(−) was previously shown to have significant efficacy in this model, and tumors contained high VEGF concentrations at baseline; VEGF concentrations in tumors were more than 10-fold higher than those in the liver and 100-fold higher than those in the spleen (data not shown). Tumors were harvested 48 hours after infusion of either WR-TK(−) (10^6 pfu) or PBS, and immunofluorescence staining was conducted detecting tumor vasculature (anti-CD31, endothelial cell marker), replicating vaccinia (anti-vaccinia polyclonal), and hypoxia (hypoxyprobe detection). Endothelial cell infection was evident (Fig. 2A). In contrast, cancer cell infection was relatively limited at this early time point suggesting that endothelial cell infection can precede cancer cell infection (cancer cell

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**Figure 1.** JX-594 expresses transgene and replicates in activated human endothelial cells in vitro following VEGF or FGF-2 stimulation. A, HUVECs were pulsed with 0.50 ng/mL EGF or 50 ng/mL VEGF165 for 30 hours and cell lysates were collected and resolved by gel electrophoresis. The blot was cut into strips and probed for pVEGFR2, VEGFR2, TK1, or tubulin as a loading control, as indicated. B, VEGF- and FGF-2-stimulated vaccinia replication: HDMECs were infected with JX-594-GFP+/−β-gal− or wild-type Wyeth control at an MOI of 0.01 for 72 hours after pulsing with either 30 ng/ml VEGF165, 5 ng/ml FGF-2 or neither. Cells and supernatant were collected and infectious virus titer was assayed on U2OS cells. C, HUVECs were pulsed with 0 to 400 ng/mL VEGF165 and infected with JX-594-GFP+/−β-gal− for 48 hours. Mean fluorescence values for GFP (485 and 528 nm) were obtained and plotted as the average of quadruplicates. D, JX-594 transgene expression: HDMECs were pulsed with 0 or 30 ng/mL VEGF165 and infected with JX-594-GFP+/−β-gal− at MOI 0.01. Fluorescent images were taken after 72 hours. E, HUVECs grown in the presence of 25 ng/mL VEGF165 infected with JX-594-GFP+/−β-gal− at an MOI of 0.01 for 24 hours. Fixed cells were stained for TK1 and DAPI counterstain. Images were collected using a ×10 objective. Scale bar, 100 μm. F, HUVEC cells were treated with VEGF165 as above and infected with JX-594-GFP+/−β-gal− for 24 hours. Fixed cells were stained for CD105 (endothelial cell activation marker) and DAPI counterstain. Images were collected using a ×5 objective. Scale bar, 100 μm. G, immunofluorescence staining for tubulin in HUVECs infected with JX-594-GFP+/−β-gal− for 36 hours at an MOI of 0.05. White arrows indicate tubulin depolymerization in JX-594-GFP+/−β-gal− infected cells. Images are representative of 3 independent experiments.
infection was extensive at later time points (data not shown). The density of CD31+ intratumor vessels had decreased significantly, and tumor hypoxia had increased significantly, at this time point in treated animals (Fig. 2B). Vessels in all normal tissues examined (liver, spleen, and lungs) and harvested at the same time point did not show infection as determined by immunofluorescence staining (not shown). Similar infection of tumor-associated vessels was shown by immunohistochemical analysis in a second strain of mice (C57BL/6) after intravenous infusion of WR-TK(−) vaccinia in immunocompetent animals bearing subcutaneous MC38 tumors (Fig. 2E).

We subsequently tested whether blood flow was selectively decreased to solid tumors within 48 hours after intravenous administration in the 4T1 model with contrast-enhanced ultrasound microbubble technology (28). Within 48 hours after intravenous vaccinia injection, the tumor perfusion was reduced by more than 90% from pretreatment (P = 0.0006, Student t test); in contrast, no change was noted in immediately adjacent normal tissue perfusion (P = 0.78; Fig. 2C and D). Therefore, TK(−) vaccinia induced tumor vessel-specific infection, blood flow disruption, and hypoxia within 48 hours in the 4T1 model. Importantly, these effects were evident before widespread tumor cell infection and/or necrosis; these effects, therefore, preceded widespread cancer cell infection and necrosis shown at later time points.

To determine whether normal tissue vasculature would be damaged with high dose i.v. JX-594, rabbits (a species that is more sensitive to Wyeth vaccinia and human GM-CSF than mice or rats) were treated with 3 weekly doses of 107 pfu/kg (the highest dose per kg given to humans intravenously to date, and approximately twice the dose resulting in reproducible tumor infection); the study was conducted under Good Laboratory Practice guidelines by an independent contract toxicology group. Blood analyses showed no change in lab parameters, including those that would be consistent with disseminated vascular damage (changes in platelets, prothrombin time, PT/INR, creatinine), on days 3, 7, 17, 21, and 44. Detailed histopathology of solid organs revealed no vascular pathology in any tissues, including heart, liver, brain, and kidney (Supplementary Table S1).

**JX-594 infects tumor-associated endothelial cells in patients after intravenous infusion**

The distribution of JX-594 infection and transgene expression was evaluated within tumor biopsies collected...
approximately 7 days after treatment from patients treated with a single intravenous infusion of JX-594 on a phase I dose-escalation trial (n = 18 patient tumor biopsy samples had evaluable vascular structures); we previously described dose-dependent delivery, replication, and transgene expression in cancer cells in these biopsy samples (11). Infection of tumor-associated vascular endothelial cells was shown in dose-related fashion: 0 of 10 low-dose (≥1 × 10^7–3 × 10^8 pfu) versus 5 of 8 high-dose (≥1 × 10^9 pfu) patients (n = 18 biopsies contained vascular structures). Positive immunohistochemical staining of endothelial cells was shown in patient tumor biopsies of diverse histologies including colorectal carcinoma, ovarian carcinoma (high-grade serous), and leiomyosarcoma (Fig. 3A, D–F). Endothelial cells in negative control sections (no primary antibody; Fig. 3B and G), as well as control tumors from the same patients collected before JX-594 treatment, were

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**Figure 3.** Patient tumor biopsies show dose-dependent JX-594 infection of tumor-associated endothelial cells after intravenous infusion. Patients in high dose cohorts on a phase I dose-escalation trial of i.v. JX-594 received 1 to 3 × 10^9 pfu, and 7 days later, tumor biopsies were conducted and samples were assessed for tumor-associated endothelial cell infection by IHC. A, JX-594 infection of tumor-associated endothelial cells (black arrows) in metastatic ovarian cancer biopsy (lymph node) visualized by immunohistochemical staining with polyclonal antivaccinia antibody (scale bar, 50 μm). B, negative control: corresponding tissue to A stained with secondary antibody only (scale bar, 50 μm). C, negative control: tumor biopsy from patient in A, B, D, E collected before JX-594 treatment stained with polyclonal anti-vaccinia antibody (scale bar, 50 μm). D, Lower power magnification of sample in A. Black arrows indicate infected vessels (scale bar, 100 μm). E, high magnification of JX-594 infection of additional tumor-associated vasculature in ovarian tumor (black arrows indicate infected vessels; scale bar, 50 μm). F, JX-594 infection of tumor-associated vessels in tumor biopsy of patient with metastatic leiomyosarcoma visualized by immunohistochemical staining with polyclonal antivaccinia antibody (scale bar, 50 μm). G, negative control: corresponding serial section to F stained with secondary antibody only (scale bar, 50 μm). H, immunohistochemical detection of β-gal in vessel of patient with ovarian cancer in A. Black arrow indicates vessel. I, serial section shows colocalized vaccinia staining (polyclonal antivaccinia antibody) in vessel positive for β-gal (linear adjustments to brightness and contrast made in H and I; scale bars, 100 μm). J, patients in escalating dose cohorts on a phase I dose-escalation trial of intravenous JX-594 received approximately 3 × 10^7 to 3 × 10^9 pfu and 7 days later, tumor biopsies were collected and assessed for tumor-associated endothelial cell infection in visible vessels. Percentage of vessels positive for vaccinia IHC in tumor by patient and dose cohort. Number of vessels counted per patient biopsy indicated (all vessels in specimen or vessels in 5 random fields at ×20 magnification). Asterisks indicate patients who have received prior antiangiogenic therapy.
negative for endothelial cell staining (Fig. 3C). To confirm that immunohistochemical staining for vaccinia proteins was associated with productive infection of tumor-associated vasculature, sections were stained for the presence of the JX-594 marker transgene β-galactosidase (β-gal IHC); β-gal is not present in the product, therefore high-level expression requires JX-594 replication. Endothelial cells that were positive for vaccinia antigen staining by IHC also exhibited positivity for the β-gal transgene (Fig. 3H and I).

Finally, we quantified the total number of evaluable vascular structures, and the number staining positively for JX-594 replication, in all evaluable patient biopsies (n = 18). For all patients treated in lower dose cohorts (n = 10), no positive-staining vessels were identified in any biopsy (range = 6–25/sample). Of note, tumor cells were also negative at these dose levels. In contrast, at doses more than approximately 3 × 10⁸ pfu, reproducible infection of blood vessels (as well as tumor cells) was observed (5/7 patients). The percentage of vascular structures staining positively per patient tumor varied from approximately 50% to 100% (Fig. 3J; range = 8 to 60 per sample). Importantly, extensive vessel infection was observed in 2 patients who had tumor progression on prior antiangiogenic therapy; these patients had non-small cell lung cancer (previous bevacizumab, anti-VEGF antibody) and leiomyosarcoma (previous α-IFN, a known antiangiogenic; ref. 29).

JX-594 triggers acute tumor vascular disruption and decreased perfusion in patients with hypervascular liver tumors

Following the demonstration of tumor vascular endothelial cell infection after i.v. JX-594 in patients, we sought to evaluate acute changes in tumor perfusion within the same time period. Serial dynamic MRI scans were obtained (strict SOP and quality control) and read by expert central radiologists blinded to patient characteristics. Patients on a phase II trial of JX-594 had liver-based tumors of either HCC (n = 15) or colorectal carcinoma (n = 1); histology; of note, intratumoral injection resulted in intravenous dissemination acutely at high concentrations (data not shown). Tumor perfusion decreased significantly over time in both injected and noninjected tumors. In tumors directly injected with JX-594, (33% showed 15% or more decrease in perfusion at 5 days; range = −18% to −66%). For example, a patient with a single 10 cm HCC tumor received 1 × 10⁸ pfu JX-594, and by day 5, the tumor perfusion was markedly decreased in large areas of the tumor mass, as evidenced by decreased contrast enhancement (Fig. 4A). Computer segmentation analysis was used to separate necrotic (yellow) from viable (blue) tumor (Fig. 4B); this analysis was also displayed in a three-dimensional tumor model (Supplementary Video S1 and S2). To evaluate whether vascular shutdown was the result of the intratumoral injection...
procedure itself, perfusion of noninjected tumors was also monitored after intravenous dissemination following intratumoral injection. Similar vascular disruption was observed in the tumors of these patients. For example, vascular disruption was shown in 2 noninjected hypervascular infiltrative HCC tumors separated from the injected tumor by normal liver tissue in one patient (Fig. 4C; blue ovals). In addition, small hypervascular tumors that were distant from the injected tumor site, and therefore were not injected, underwent acute vascular disruption with acute swelling (likely edema) by day 5 (Fig. 4D, Supplementary Fig. S4). Tumor vascular disruption was also observed in a patient with metastatic colorectal cancer after intratumoral injection of $1 \times 10^9$ pfu (Fig. 4E).

This patient also had a noninjected hemangioma with atypical features and an abnormal and hyperproliferative vascular tumor without associated malignant cancer cells (30), in another liver lobe that underwent acute vascular disruption (Fig. 4F); perfusion effects on typical nonmalignant hemangiomas were not assessed (not shown).

Finally, acute effects on tumor perfusion were also shown 6 days after i.v. JX-594 infusion in patients with advanced HCC who had failed prior treatment with sorafenib, a small-molecule VEGFR inhibitor (Fig. 4G and H). Following intravenous infusion, tumors became edematous, centrally hypointense, and thus more apparent.

**Acute vascular disruption is maintained over at least eight weeks**

In addition to acute tumor perfusion measurements, the durability of tumor responses was assessed by dynamic MRI imaging at week 8 in the phase II trials of patients with HCC. In patients exhibiting acute reductions in contrast enhancement at day 5 ($n = 5$), responses were maintained at week 8 (Fig. 5A and B). This indicates that early acute reductions in tumor perfusion can be durable and may predict for subsequent durable necrotic responses.

**No clinical signs of damage to normal vasculature or to wound healing were observed**

JX-594-related adverse events are typically flu-like symptoms lasting up to 24 hours (10, 11). For this study, to assess whether toxicity to normal vasculature occurred, we specifically assessed cardiovascular and coagulation end points over time following intravenous JX-594 administration on a phase I trial ($n = 23$); these included blood pressure, creatinine, prothrombin time (PT/INR), and platelet count. No significant changes were shown at doses associated with intravenous delivery and vascular disruption in tumors. Likewise, patients with liver cancer with documented acute (day 5) perfusion reduction in their tumor(s) following JX-594 treatment in a phase II trial had similar assessments; creatinine and transaminase concentrations, coagulation parameters, and changes in blood pressure were closely monitored for signs of damage to normal vasculature in patients exhibiting acute reduction in tumor perfusion ($n = 5$); no clinically significant changes were detected (Fig. 5C and D). Finally, we closely observed normal wound healing sites following JX-594 injection as endothelial cells are proliferating under growth factor stimulation in this setting. Sites of large bore needle insertion (18 gauge, used for percutaneous intratumoral injections into liver tumors in phase II trials; $n = 33$ patients) induced a puncture wound in the skin and liver capsule; insertion site puncture wounds healed normally clinically with no bleeding, ulceration, or infection at injection sites reported as adverse events (Fig. 5E).

**Discussion**

We report here for the first time in humans that a biologic agent can be used to target and infect tumor-associated endothelial cells, resulting in transgene expression, vascular disruption, and subsequent tumor lysis. JX-594, an engineered transgene-expressing vaccinia virus oncolytic, achieved these effects in patients with diverse solid tumor histologies after either intravenous or intratumoral administration. In vitro studies confirmed that FGF-2, a mediator of VEGFR inhibitor resistance, stimulated JX-594 activation in endothelial cells. In an animal tumor model, tumor endothelial cell infection preceded widespread cancer cell infection and was associated with a significant reduction in perfusion and increased hypoxia. These acute effects on tumor vasculature in humans and animals were frequently observed following durable tumor necrosis. Toxicities related to adverse effects on normal blood vessels were not observed in humans or in a GLP toxicology study. Specifically, hypertension (toxicity commonly observed upon treatment with antiangiogenic agents; ref. 31) was not commonly observed upon treatment with JX-594. This platform opens up the possibility of treating highly vascular solid tumors with JX-594 and similar poxviruses, including hypervascular cancers such as hepatocellular and renal cell carcinomas. These findings describe an important mechanism by which JX-594 achieves efficient intravenous delivery and tumor infection; extravasation of this relatively large poxvirus through endothelial pores, a potentially limiting step, may not be necessary to achieve tumor infection.

We identified mechanisms by which JX-594 was able to specifically target and infect tumor-associated endothelial cells, but other mechanisms may also be operative. Our in vitro studies using primary human endothelial cells showed that VEGF and FGF-2 were able to stimulate JX-594 replication in these cells. Vile and colleagues have shown that oncolytic reovirus and VSV replication can also be induced in endothelial cells through modulation of the VEGFR (24). Pulses of VEGF or chemotherapy-induced damage of blood vessels increased the penetration of oncolytic reovirus into murine tumors (24). However, in these studies, there was no evidence that reovirus could inherently infect intact tumor neovasculature without exogenous VEGF treatment; the authors suggested that increased vascular permeability might allow tumor cell infection, rather than infection of the tumor vasculature itself (24).

For JX-594, EGFR activation itself may play a role in vivo, as this pathway can be activated in tumor-associated endothelial cells in both VEGF inhibitor-naive (32) and -refractory settings (33). Finally, efficient JX-594 replication was shown to be dependent on high cellular thymidine kinase concentrations due to a deletion of the viral thymidine kinase gene (34). Thymidine kinase has been shown to be a key enzyme in the modulation of
DNA synthesis in proliferating endothelial cells (35); elevated thymidine kinase may therefore contribute to the enhanced replication of JX-594 in activated endothelial cells. Of note, vaccinia vaccine replication in humans at sites of scarification (localized skin wounding) or eczema may involve proliferating endothelial cells. It is intriguing that JX-594 was able to cause a 100% decrease in enhancement.

Figure 5. Acute vascular disruption with JX-594 is followed by durable, and in some cases progressive, tumor vascular disruption and necrosis over time. A, waterfall plot showing percent decrease in tumor enhancement (perfusion) from baseline over time (day 5, gray bar; Week 8, black bar) for all patients whose tumors showed significant vascular disruption on day 5 (< = 15% reduced enhancement; n = 5). B, dynamic MRI images at baseline, day 5, and week 14 following intratumoral injection of JX-594 in HCC tumor. C, change in critical blood lab values and blood pressure over time in patients showing infection of tumor vasculature after intravenous JX-594 infusion (n = 4). D, change in critical blood lab values over time in patients showing tumor vascular disruption after intratumoral JX-594 injection (n = 5). E, wound healing over time following percutaneous insertion of large bore injection needle into liver for 1 x 10^9 pfu JX-594 injection.
vascular disruption in an atypical hemangioma, an abnormal
growth of vessels in which overproduction of VEGF is impli-
cated (36); this clinical data, together with preclinical tumor
model data, suggests that tumor cell proximity may not be
necessary. Endothelial cells associated with a hemangioma
reportedly secreted high levels of proangiogenic factors,
including VEGF, when compared with normal endothelial cells
from the same patient (37). Finally, we detected no change in
normal liver perfusion or vascularity at similar time points by
dynamic MRI.

Further research is necessary to confirm clinical safety in
the setting of wound healing, and to elucidate additional
mechanisms of vaccinia infection of endothelial cells and
associated vascular disruption, as well as methods to aug-
ment this feature of JX-594. We are currently exploring
several hypotheses to explain the tumor vascular disruption
observed. First, many tumors reportedly exist in a hyperco-
agulable state (38), with tumor cells producing procoagulant
factors (39). Proinflammatory cytokines (40) that are acti-
ated by vaccinia, as well as other viral infections (e.g., HSV;
ref. 41), have been shown to induce tissue factor on endo-
thelial cells. Therefore, vaccinia replication and cytolysis
within the vascular endothelium presumably could result in
tissue factor upregulation and subsequent blood clotting
within infected vessels. In previous mouse studies, we showed
that oncolytic VSV specifically induced clot formation
within small intratumoral blood vessels (23), as well as
neutrophil infiltration and proinflammatory gene expression
(22). Inflammation-mediated blockage of blood flow to sites
of infection is a known physiologic response to infection
(42). In addition, both vascular disrupting agents (such as
combretastatin and vinblastine) and vaccinia disrupt the
normal cellular cytoskeleton leading to endothelial cell
rounding and loss of normal cell–cell contacts; this phe-
nomenon is believed to potentiate vascular collapse (6, 7).

In vitro findings with JX-594 are consistent with this
hypothesis.

While JX-594 has been generally well tolerated in more 160
treated patients to date, more safety data is needed in relation
to potential normal vessel and wound healing toxicities. For
example, the safety of JX-594 should be assessed in patients
with tumors lining the gastrointestinal or aerodigestive tracts;
whereas gastrointestinal bleeding can occur with bevacizumab
(43), this complication has not been reported with JX-594 to
date. In addition, further safety data are needed in the context
of normal wound healing, including in the postoperative
period. Importantly, impaired healing has not been reported
to date at the site of core needle or excisional biopsies taken
within 7 to 10 days after JX-594 treatment, or at large bore (18
gauge) needle insertion sites as described here. The risk of
these theoretical complications will be assessed as more
patients are treated over time.

These results also have implications for combination
therapy with JX-594 and approved anticancer agents. For
example, it may be important to avoid concomitant admin-
istration of VEGFR and/or FGFR-2 inhibitors if the vascular
targeting effect of JX-594 is dependent on signaling from this
pathway. Sequential therapy, in contrast, may be highly
beneficial given the potential for combined antivascular and
antiangiogenic effects or sensitization to antiangiogenic
treatment due to growth factor rebound. Clinically, pro-
found tumor vascular ablation and subsequent durable
tumor necrosis and long-term survival have been induced
by JX-594 followed by small-molecule inhibitors of VEGFR in
hypervascular HCC and renal cell cancers (both VEGF-rich;
ref. 44). Of note, this potential synergy was reported even in
patients who had failed previous therapy with sorafenib.
Randomized trials will be required to determine whether JX-
594 followed by angiogenesis inhibitors is superior to either
agent alone.

Future derivatives of JX-594 may be engineered to express
multiple therapeutic proteins from tumor-associated blood
vessels, including those with antitumor, antiangiogenic, and/or
antiangiogenic effects. This opens up the possibility of novel
multifunctional products derived from JX-594 that can express
high levels of multiple complementary therapeutic proteins in
metastatic solid tumors and tumor vasculature in a highly
selective fashion.

Disclosure of Potential Conflicts of Interest

C.J. Breitbach is employed (other than primary affiliation; e.g., consulting) as
director of clinical research and has ownership interest (including patents) in
Jennexer. S.H. Thorne has ownership interest (including patents) and is a
consultant/advisory board member of Jennexer Inc. R. Patt is a consultant/advisory
board member of RadMD. A. Moon is employed (other than primary affiliation;
e.g., consulting) as VP product development and has ownership interest (including patents) in
Jennexer Inc. J. Burke is employed (other than primary affiliation; e.g., consulting) as VP Clinical Research and has ownership interest
(interesting owning patents), and is a consultant/advisory board member of Jennexer Biotherapeu-
tics.

J. C. Bell has a commercial research grant and ownership interest (including patents) from Jennexer Inc.

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