Clinical Studies

Oncolytic Vaccinia Virus Disrupts Tumor-Associated Vasculature in Humans

Caroline J. Breitbach, Rozanne Arulanandam, Naomi De Silva, Steve H. Thorne, Richard Patt, Manijeh Daneshmand, Anne Moon, Carolina Ilkow, James Burke, Tae-Ho Hwang, Jeong Heo, Mong Cho, Hannah Chen, Fernando A. Angarita, Christina Addison, J. Andrea McCart, John C. Bell, and David H. Kirn

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 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 vivo. 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 HEK293 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 S100 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 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-Fluor555-conjugated goat antimouse 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 × 10⁶ 4T1 cells (ATCC) subcutaneously on the hind limb. Tumors were allowed to form (7 to 10 days until tumors were approximately 100 mm³). Mice were then treated intravenously with 1 × 10⁶ 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 $1 \times 10^5$ to $3 \times 10^9$ pfu per kg; a sixth cohort was added at $1 \times 10^9$ 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 $1 \times 10^8$ or $1 \times 10^9$ 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 $1 \times 10^9$ pfu. Patients with HCC on a second phase II study were treated with a single intravenous infusion of JX-594 at a dose of $1 \times 10^9$ 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 neovascularation 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.

Clinical/laboratory analysis

Serum chemistry, including creatinine and alanine aminotransferase levels, were evaluated by routine laboratory testing at screening, predose day 1, 15, and 22 after JX-594 injection. Similarly, coagulation parameters, including prothrombin time (PT), activated partial thromboplastin time (aPTT), and international normalized ratio (INR) were evaluated at these time points. Blood pressure measurements were conducted at screening, predose day 1, and day 15.

Statistical analyses

All statistical analyses were conducted using the GraphPad Prism 5.0 software (GraphPad Software). The unpaired t test was used to assess differences in virus output in human dermal microvascular endothelial cells (HDMEC) in the presence/absence of growth factors.

Description of methods for the rabbit GLP toxicity study is available in Supplementary Information.

Results

JX-594 infects VEGF- and FGF-2-activated human endothelial cells in vitro

The ability of JX-594 to infect and express transgenes in normal human endothelial cells (HUVECs and HDMECs) was assessed in the presence and absence of known endothelial cell growth factors; a GFP-expressing version of JX-594 was used (JX-594-GFP+/β-gal-). We first confirmed that our endothelial cells expressed VEGFR, and that stimulation with VEGF resulted in VEGFR phosphorylation and downstream cellular thymidine kinase expression (Fig. 1A). Next, VEGF stimulation resulted in a significant increase (10-fold) in JX-594 output in HDMECs (72 hours postinfection; P < 0.0001; Fig. 1B). In addition, stimulation with FGF-2, an endothelial cell growth factor implicated in resistance to VEGFR-targeted therapy (27) and also resulted in stimulation of JX-594 replication in normal endothelial cells (P = 0.002; Fig. 1B). Virus production on a plaque-forming unit per cell basis was also significantly increased (approximately 15-fold with VEGF; Supplementary Fig. S1). Similar results were obtained with HUVECs. The dependence of JX-594 on VEGF or FGF-2 for replication was related to its viral thymidine kinase deletion, as wild-type vaccinia (TK+) replicated efficiently in their absence (Fig. 1B). Increasing VEGF concentrations correlated with increasing JX-594-GFP+/β-gal- transgene expression following infection (Fig. 1c and 1d). Of note, chronic exposure of HDMECs or HUVECs to VEGF during growth, passage, and infection resulted in equivalent stimulation of thymidine kinase levels, JX-594 transgene expression, and replication as compared with acute VEGF exposure (Supplementary Fig. S2). Finally, we confirmed that the anti-VEGF antibody bevacizumab (Avastin) could block the VEGF stimulatory effect, by reducing thymidine kinase levels, and that VEGF did not sensitize control normal VEGR(−) cells to JX-594 replication (GM38 fibroblasts, Supplementary Fig. S3). We next investigated the ability of JX-594 to selectively infect rapidly proliferating and cellular thymidine kinase-overexpressing endothelial cells in the context of an in vitro wound healing assay. As shown in Fig. IE, when stimulated with VEGF165, the thymidine...
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(−) (10⁶ 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
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 from patients who had undergone intravenous infusion of the virus. The data showed that JX-594 preferentially infects tumor-associated endothelial cells, as evidenced by the presence of viral antigen expression in endothelial cells within the tumor tissue. This suggests a potential mechanism by which JX-594 can induce vascular disruption and tumor necrosis.

The study also highlighted the importance of monitoring tumor perfusion changes as a predictor of therapeutic efficacy. Box-whisker plots were used to compare the relative density of CD31+ vessels in treatment groups versus control groups. A significant decrease in perfusion was observed in treated tumors, indicating successful targeting of the endothelial cells.

Additionally, the study demonstrated the safety profile of JX-594 in humans, with no adverse effects noted in the toxicology group. Laboratory parameters, including platelet count, prothrombin time, and creatinine levels, remained within normal limits.

In conclusion, the study provides strong evidence for the potential of JX-594 as a therapeutic agent for the treatment of cancer, particularly in human trials. Further research is needed to optimize the treatment regimen and to confirm the long-term efficacy and safety of JX-594 in a larger patient population.
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 \(( \geq 1 \times 10^7 \text{ pfu} )\) versus 5 of 8 high-dose \(( \geq 1 \times 10^9 \text{ pfu} )\) 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

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 \( \times 10^7 \text{ pfu} \), 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 \( \mu \text{m} \)). B, negative control: corresponding tissue to A stained with secondary antibody only (scale bar, 50 \( \mu \text{m} \)). 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 \( \mu \text{m} \)). D, Lower power magnification of sample in A. Black arrows indicate infected vessels (scale bar, 100 \( \mu \text{m} \)). E, high magnification of JX-594 infection of additional tumor-associated vasculature in ovarian tumor (black arrows indicate infected vessels; scale bar, 50 \( \mu \text{m} \)). F, JX-594 infection of tumor-associated vessels in tumor biopsy of patient with metastatic leiomyosarcoma visualized by immunohistochemical staining with polyclonal vaccinia antibody (scale bar, 50 \( \mu \text{m} \)). G, negative control: corresponding serial section to F stained with secondary antibody only (scale bar, 50 \( \mu \text{m} \)). Linear adjustments to brightness and contrast made in F and G. H, immunohistochemical detection of \( \beta \)-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 \( \beta \)-gal (linear adjustments to brightness and contrast made in H and I; scale bars, 100 \( \mu \text{m} \)). J, patients in escalating dose cohorts on a phase I dose-escalation trial of intravenous JX-594 received approximately \( 3 \times 10^7 \) to \( 3 \times 10^9 \text{ pfu} \) 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 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 × 10^8 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).

**Figure 4.** Intravenous or intratumoral injection of JX-594 results in acute tumor vascular disruption in both injected and noninjected tumors of patients with advanced HCC and colorectal cancer. A, baseline and posttreatment day 5 dynamic MRI images of 10 cm maximum diameter HCC tumor directly injected with JX-594. B, segmentation analysis of images in A separating the poorly perfused (yellow) from well-perfused (blue) tumor. C, baseline and day 5 dynamic MRI images of JX-594 directly injected tumor (red circle) and noninjected tumors (blue circles) in a patient with advanced HCC. D, noninjected subclinical HCC tumor (<1 cm maximum diameter) at baseline and on day 5 after JX-594 treatment shows vascular disruption and edema in tumor. E, baseline and day 5 dynamic MRI images of metastatic colorectal cancer tumor directly injected with JX-594. F, baseline and day 5 dynamic MRI images of noninjected atypical hemangioma in patient with metastatic colorectal cancer as in C. F, noninjected subclinical HCC tumor (<1 cm maximum diameter) at baseline and on day 5 after JX-594 treatment show vascular disruption and edema in tumor. G and H, baseline and day 6 dynamic MRI images of sorafenib-refractory HCC patients following i.v. JX-594.

**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 × 10^9 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 cardiovasculature 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 followed by 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

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 implicated (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 augment 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 hypercoagulable state (38), with tumor cells producing procoagulant factors (39). Proinflammatory cytokines (40) that are activated by vaccinia, as well as other viral infections (e.g., HSV; ref. 41), have been shown to induce tissue factor on endothelial 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 phenomenon 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 antitumor agents. For example, it may be important to avoid concomitant administration of VEGFR and/or FGF-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, profound 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, antivascular, 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.
References

Oncolytic Vaccinia Virus Disrupts Tumor-Associated Vasculature in Humans

Caroline J. Breitbach, Rozanne Arulanandam, Naomi De Silva, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-2687

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/12/31/0008-5472.CAN-12-2687.DC1

Cited articles
This article cites 43 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/4/1265.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/73/4/1265.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.