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Potential mechanisms of vascular thrombosis after microwave ablation in an in-vivo liver

J Chiang^a, K Nickel^b, RJ Kimple^b, and CL Brace^a

^aDepartment of Radiology, University of Wisconsin: 1111 Highland Ave., Madison WI, 53705, USA

^bDepartment of Radiation Oncology, University of Wisconsin: 1111 Highland Ave., Madison WI, 53705, USA

Abstract

Purpose—The purpose of this study was to evaluate potential biological and thermal mechanisms for the observed differences in thrombosis rates between hepatic vessels during microwave ablation procedures.

Materials and Methods—Microwave ablation antennas were placed in single liver lobes of two in-vivo porcine (n=3 in each animal; n =6 total) liver models in the proximity of a large (>5mm) portal vein and hepatic veins. Each ablation was performed with 100 W for 5 minutes. Conventional ultrasound imaging and intravascular temperature probes were used to evaluate vessel patency and temperature changes during the ablation procedure. Vascular endothelium was harvested 1 hour after ablation and used to characterize genes and proteins associated with thrombosis in portal and hepatic veins.

Results—Targeted portal veins and hepatic veins did not show a significant difference between average vessel size (8.7 ± 2.4 mm vs 9.7 ± 3.4 mm; $p=0.44$). Portal veins within the microwave ablation zone thrombosed at a significantly higher rate compared to hepatic veins (54.5% vs 0.0%, $p=0.0046$). There was a negligible change in intravascular temperature in both portal and hepatic veins during the ablation procedure (0.2 ± 0.4 vs 0.6 ± 0.9 °C, $p=0.46$). Portal veins demonstrated significantly higher gene expression compared to hepatic veins in terms of fold-differences in thrombomodulin (2.9 ± 2.0 ; $p=0.0001$), von Willebrand Factor (7.6 ± 1.5 ; $p=0.0001$), endothelial protein C receptor (3.50 ± 0.49 ; $p=0.0011$) and plasminogen activator inhibitor (SERPINE1: 1.46 ± 0.05 ; $p=0.0014$). Western blot analysis showed significantly higher expression of von Willebrand Factor (2.32 ± 0.92 ; $p=0.031$) in portal veins compared to hepatic veins.

Please address editorial correspondence to: Jason Chiang, MD, PhD cjchiang@wisc.edu, Wisconsin Institutes for Medical Research 1310-O, 1111 Highland Ave. Madison, WI 53705 USA.

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Disclosures

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Conclusions—Large portal veins thrombose more frequently than hepatic veins during microwave ablation procedures. Biological differences in thrombogenicity, rather than heat transfer, between portal veins and hepatic veins may contribute to their different rates of thrombosis.

Keywords

thermal; ablation; thrombosis; heat-sink effect

Introduction

Thermal ablation is an accepted alternative to surgical resection in the treatment of liver cancer due to its association with less bleeding, quicker recovery and fewer comorbidities compared to surgical resection (1). The underlying goal of a thermal ablation is to coagulate the entire tumor volume plus a circumferential boundary of at least 5 mm (2–4). One limitation of thermal ablation treatments is that they are less effective in the presence of nearby vasculature which draw heat away from the ablation zone (5). This “heat-sink” effect can lead to incomplete tumor ablation and increased the rate of local tumor progression (4,6,7).

High-powered microwave ablation systems have been utilized to overcome this heat-sink effect by using an electric field to heat through a variety of tissue conditions, including desiccated and charred states (8). The quick and efficient energy delivery mechanism leads to larger ablation zones, even in well-perfused tissue (9). However, there is a lack of information regarding how individual vessels respond to the high temperatures associated with microwave energy. Current literature documenting incidence of thrombosis during ablation is found mostly within case reports and small retrospective studies (4,10–13). Damage of small hepatic vessels is common but unlikely to have clinical consequence; thrombosis of larger vessels appears more rare but could cause more serious complications in patients with cirrhosis or compromised liver function (14). In the absence of established clinical studies characterizing rates of thrombosis in large vessels, physicians may avoid aggressive treatment of perivascular tumors, leading to higher rates of local tumor progression (7). Further insight into intravascular thrombosis in a controlled, in-vivo setting can shed insight into this phenomenon and may help physicians maximize the efficacy of the ablation treatment while minimizing the risk for clinically important vascular damage.

Early in vivo ablation studies demonstrated that vessels smaller than 3 mm in size are more likely to thrombose (8,9,15). More recent studies have identified greater thrombosis rates in smaller portal veins compared to hepatic veins in both porcine and human livers (7,16). The purpose of this study was to evaluate potential mechanisms for the observed differences in thrombosis rates by quantifying thrombosis-related gene and protein expression in normal vessels as well as differences in heat transfer during the ablation procedure.

Materials and Methods

In Vivo Study

All studies were performed with approval from the Institutional Animal Care and Use Committee (IACUC) and complied with National Research Council Guidelines (17). Female domestic swines (n=2, 50 kg; Arlington Farms, Arlington, WI) were sedated with intramuscular tiletamine hydrochloride-zolazepam hydrochloride (7 mg/kg, Telazol, Fort Dodge, IA) and xylazine hydrochloride (2.2 mg/kg, Xyla-Ject, Phoenix Pharmaceutical, St Joseph, MO). Anesthesia was maintained with inhaled 1.0–2.0% isoflurane (Halocarbon Laboratories, River Edge, NJ). An ear vein was cannulated with a 20-gauge angiocatheter for administration of IV fluids.

Individual microwave antennas (Neuwave Medical Inc, Madison, WI) were inserted to create a single microwave ablation in each individual liver lobe (n=3 for each animal, for a total of n=6) to within 20 mm of larger portal and hepatic veins (>5 mm diameter) under ultrasound guidance (Siemens Antares, Siemens Medical Solutions Inc, Issaquah, WA). Each microwave antenna was strategically placed to target multiple portal and hepatic veins simultaneously. Pre-ablation Doppler ultrasound imaging was performed to measure the peak blood flow velocity in each vessel. Fiber-optic temperature probes (MTG-170 Opsens, Quebec, Quebec) were then inserted under ultrasound guidance into the nearest portal or hepatic vein. Temperature probe tips were identified to guide their placement onto the same imaging plane as the microwave ablation antenna heating ceramic (Figure 1A). Ablation zones were then created by applying 100 W for 5 minutes, with the fiber-optic temperature probes recording temperatures every second. Post-ablation ultrasound imaging was performed to confirm the placement of the fiber-optic temperature probe and evaluate vascular damage such as vessel stricture or thrombus formation (Figure 1B).

After the ablation procedure, animals were sacrificed by an IV injection of Beuthanasia-D (390 mg/mL pentobarbital sodium and 50 mg/mL phenytoin sodium at 0.2 mL/kg; Schering-Plough, Kenilworth, NJ). The liver was then removed en-bloc and sectioned along the axis of each antenna to expose the transverse cross section of the nearest vessels and confirm the location of the temperature probe (Figure 1C). Normal sections of the portal and hepatic veins, identified outside of the ablation zone, were excised using sterile technique and frozen for qPCR and Western blot analysis.

Gene Analysis

Endothelial cells were detached by dissection of the inner lining of the vessel from portal (n=3) and hepatic veins (n=3). Removal was performed within 1-hour of animal sacrifice to isolate primary cell samples. Endothelial samples were snap frozen in liquid nitrogen and stored at –80 °C. Total RNA was isolated from the primary portal and hepatic veins using the RNAeasy Plus Mini Kit (Qiagen, Venlo, Netherlands). Quantitative real-time PCR (qRT-PCR) was performed after construction of a cDNA library using 1 µg of RNA and the iScript Reverse Transcription Supremix kit (Bio-Rad, Hercules, CA, USA).

Seven primer sets (Integrated DNA Technologies, Coralville, IA, Table 1) associated with pro and anti-coagulation proteins and an endogenous control glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) were quantified with qRT-PCR using a Bio-Rad CFX96 thermocycler (BioRad, Hercules, CA). Amplification of complementary DNA (cDNA) was performed with primers and the FastStart Master SYBR Green reaction mix (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instruction manual. qRT-PCR was performed under cycling conditions of 95 °C for 10 minutes, followed by 30 cycles of denaturation at 95 °C for 10 seconds, annealing at 55 °C for 10 seconds and extension at 72 °C for 20 seconds. Primer sets were validated and quantified according to the PCR platform instructions. Measurements of mean cycle threshold value from triplicates (from individual samples n=3, for a total of 9 measurements) were used to calculate the expression of each gene using the 2^{-Ct} formula, normalized to GAPDH in each vessel group as an internal control.

Protein Analysis

Changes in gene expression identified by qRT-PCR were confirmed by performing Western blotting on the cellular lysates with replicates. Briefly, endothelial tissue from portal veins (n=3) and hepatic veins (n=3) were homogenized in cell lysis buffer, and equal amounts of protein separated by 3–8% NuPAGE Tris-Acetate precast gels (Invitrogen, Carlsbad, CA). Proteins were then transferred to Immobilon-FL PVDF (Millipore, Billerica, MA) at 30V overnight in the cold room. Membranes were probed with anti-von Willebrand Factor (vWF) antibody and anti-endothelial protein C receptor (EPRC) antibody (Catalog #ab6994 and #ab78280, respectively, Abcam, Cambridge, MA) with GAPDH (Cell Signaling, Catalog # 2118, Beverly, MA) used as a loading control. Reliable porcine antibodies for proteins associated with PLAT and THBD genes were not commercially available at the time of the study. Primary antibodies were detected with infrared dye IR Dye 800CW or 680 RD-conjugated IgG (LI-COR Biosciences, Lincoln, NE). Immunoblots were imaged and quantified with the Odyssey Fc Dual-Mode Imaging System (LI-COR Biosciences).

Statistics

For the in vivo studies, mean vessel size, peak blood flow velocity, vessel-antenna spacing and peak intravascular temperature elevation were compared between portal and hepatic veins using Mann-Whitney U test. Mean up-regulation of differential expression of mRNA, as well as protein expression in portal and hepatic veins were also compared with the Mann-Whitney U test. Odds ratio comparisons for thrombosis events between vessel types were compared with the Fisher's Exact test. P-values less than 0.05 were considered to be significant.

Results

In Vivo Study

Targeted portal veins and hepatic veins did not show a significant difference between average vessel size (8.7 ± 2.4 mm vs 9.7 ± 3.4 mm; $p=0.44$), peak blood flow velocity (14.9 ± 4.9 mm vs 22.3 ± 11.7 mm; $p=0.07$) and vessel-antenna spacing (10.1 ± 9.9 mm vs 10.1 ± 2.6 mm; $p=0.99$). Thrombus formation using ultrasound and Doppler imaging was detected in significantly more portal veins compared to hepatic veins (54.5% vs 0.0% respectively, $p=0.0046$) (Figure 2A and 2B).

No significant difference in peak temperature elevation was observed between blood in the lumen of the portal vein or hepatic vein during the ablation period (0.2 ± 0.4 vs 0.6 ± 0.9 °C, $p=0.46$) (Figure 2C). The presence of thrombus had no effect in intravascular temperature elevation. However, a single temperature measurement at the vessel wall showed temperatures exceeding 100 °C during the ablation period. No acute thrombus formation was identified in that particular sample.

Gene Analysis Study

Compared to hepatic veins, portal veins exhibited significantly higher gene expression of tissue plasminogen activator (fold-difference $\pm$ standard deviation — PLAT: 3.0 ± 0.5 ; $p=0.004$), thrombomodulin (THBD: 2.9 ± 2.0 ; $p=0.0001$), von Willebrand Factor (vWF: 7.6 ± 1.5 ; $p=0.0001$), endothelial protein C receptor (PROCR: 3.50 ± 0.49 ; $p=0.0011$) and plasminogen activator inhibitor (SERPINE1: 1.46 ± 0.05 ; $p=0.0014$) (Figure 3A). The remaining genes were not considered to be significantly elevated in portal veins compared to hepatic veins (PLAU: 1.14 ± 0.07 ; $p=0.7468$ and TF: 0.839 ± 0.13 ; $p=0.4739$).

Protein Results

Total protein from both hepatic and portal veins were isolated to confirm differences in protein expression derived from differences in gene expression. Portal veins were found to have significantly higher expression of vWF compared to hepatic veins (Normalized signal: 2.32 ± 0.92 vs 1.00 ± 0.21 , respectively; $p=0.031$) (Figure 3B and 3C). While there was higher expression of EPCR (antibody to PROC) in portal veins compared to hepatic veins, the difference was not significant (Normalized signal: 0.13 ± 0.05 vs 0.08 ± 0.02 ; $p=0.69$). Unfortunately, no validated porcine antibody for the THBD or TF protein was available so these targets were excluded from this analysis.

Discussion (800)

This study showed that significantly higher rates of thrombus formation were found in portal veins compared to hepatic veins of near-equivalent vessel size, velocity and vessel-antenna spacing. Intravascular measurements during ablation showed an absence of temperature elevation within the lumen of the vessel, even in the presence of thrombus formation. Subsequent analysis of gene expression differences between portal and hepatic veins demonstrated significantly greater upregulation of genes coding for PLAT, vWF, EPCR, SERPINE1 and THBD in portal veins compared to hepatic veins. Confirmatory Western blots only demonstrated significantly greater levels of vWF expression in portal veins compared to hepatic veins. This difference in clotting protein expression, combined with the lack of intravascular temperature change during ablation, suggests that portal veins are more susceptible to thrombotic events at baseline compared to hepatic veins.

Predicting which vessels will thrombose or remain patent remains an important component of ablation treatment planning, particularly to minimize the risk of complications while maximizing treatment efficacy (4). Early in vivo microwave ablation studies have shown that vessel diameters less than 3 mm is a strong predictor of vessel thrombosis (18). This conclusion was supported by more recent in vivo studies, but with the caveat that vessel type

plays a more important role than size, velocity or even vessel-antenna spacing (16). Similar discrepancies in thrombotic rates between larger portal and hepatic veins were identified in this in-vivo study.

The mechanism for thrombus formation in portal veins compared to hepatic veins is presently unclear. Mechanically, the flow pattern of portal veins and hepatic veins differ drastically as hepatic veins drain through an increasingly lower-resistance path to the inferior vena cava, while the portal veins flow into the higher resistance hepatic sinuses. In addition, hepatic veins have a highly-pulsatile flow that corresponds to the cardiac cycle while portal veins are associated with a more damped, continuous flow (19–21). The higher peak flow rate of hepatic veins compared to portal veins in this study, while not statistically significant, may have contributed to a larger heat-sink effect and protected the veins from thrombosis (5). Another contributor to the differences in thrombus formation rates is the fact that thermal damage can activate platelets and the coagulation process (22). The results showing portal veins with higher expression of vWF, a key protein in platelet aggregation and subsequent thrombus growth, supports this biologic mechanism (23). The discrepancy in flow pattern between vessel types, coupled with the underlying differences in coagulation protein and gene expression serve as potential contributors to vessel thrombosis patterns seen in this in vivo study.

There are limitations of this study that need to be considered. Each ablation on a single liver lobe was evaluated as an independent event within the same animal. There are systemic effects as part of the reactive tissue response of the liver subsequent to liver ablations, although no particular porcine marker has been tied to heat-induced thrombus formation (24–26). Additional studies that measure circulating inflammatory markers or coagulation profiles may help clarify whether this is a relevant concern. With respect to the gene and protein analysis, only normal portal and hepatic veins were investigated. As a result, this study highlighted only the intrinsic differences between untreated portal and hepatic vascular endothelia. Post-ablation vessel damage effects on thrombus-related gene expression beyond 1-hour after ablation were not evaluated. Future studies should include post-ablation analysis with time-dependent changes to coagulation-related genes and proteins to fully capture the effect of microwave ablations on hepatic vessels.

Conclusion

This study demonstrated that portal veins thrombose significantly more often compared to hepatic veins during microwave ablation in an in vivo porcine liver model. The lack of temperature changes in either vessel type ruled out thermal coagulation of the flowing blood as a mechanism for thrombosis. However, significant differences in the upregulation of genes related to clot formation were identified in portal veins compared to hepatic veins. Additional studies looking at the effects of these specific proteins is warranted to fully explore the higher rates of portal veins compared to hepatic veins during microwave ablations.

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Figure 1.

Microwave ablation antenna being placed next to a large portal vein. A) The tip of the temperature probe (arrowhead) appears hyper-echoic under ultrasound imaging, and used to guide the placement within the vessel. B) Immediate post-ablation imaging of the portal vein showing a echogenic mass within the vessel. The ablation zone next to the vessel appears hypoechoic, reflecting the increased tissue density within the ablation zone. C) Post-ablation gross histology confirming the location of the ablation zone next to the portal vein and thrombus formation.

Hepatic Vein Occlusion Rates

Portal Vein Occlusion Rates

Figure 2.

Vessel thrombosis versus patency as a function of vessel size and blood flow velocity. A) Hepatic veins of sizes 5–18 mm had no thrombotic events (0/11). B) Portal veins thrombosed at a rate of 54.5% (6/11) for sizes 4–14 mm. C) Sample temperature probe readout during ablation showing vessel insertion and subsequent intravascular temperature measurement. There was not an appreciable rise in blood vessel temperature in either vessel type.

Figure 3.

Gene and protein analysis of the primary portal vein and hepatic vein endothelial cells from in-vivo porcine liver. A) qRT-PCR results (expressed as mean $\pm$ SD) showing significant increase in PLAT (Tissue Plasminogen Activator), THBD (Thrombomodulin), vWF (von Willebrand Factor), PROCR (Endothelial Protein C Receptor) and SERPINE1 (Plasminogen Activator Inhibitor) in terms of fold-difference between portal veins and hepatic veins (reference). Asterisk denotes significance. B) Follow-up Western blotting results of staining with von Willebrand Factor and Endothelial Protein C Receptor antibodies on primary endothelial tissue of the liver C) Normalized signal showing significantly higher expression of vWF ($p=0.031$) but not EPRC (antibody for PROCR) ($p=0.69$) in portal veins compared to hepatic veins.

Table 1

Genes associated with coagulation within endothelial tissue

Gene	Forward Primer/Reverse Primer
PLAU	5'-GATCCCTCTGACTACCTCTATCC-3'
	5'-ACTTCGCTGCCGTAGTAATG-3'
SERPINE1	5'-CAGGGTTTCATGCCCTACTT-3'
	5'-GTGTCTCTTCACCCAGTCATTT-3'
THBD	5'-AAGTGGACAGAGGACCATTTTC-3'
	5'-CCAGAGACCACCAACCAATAA-3'
TF	5'-CACTGTTTCAGATAAGCCCTAGAC-3'
	5'-CCTTCACGTTCTCATGATCTC-3'
vWF	5'-CTCGAACCCAAGAAGAGAATGG-3'
	5'-GGATCACTTCCTCCACAAACTC-3'
PLAT	5'-ACATGTCTGCAGGCTATCTATTT-3'
	5'-GGTGGCTCTGGCATCTATTT-3'
PROCR	5'-GAAACACATCACCACGCATAAC-3'
	5'-CCACTCCTGCGATGATGAAA-3

Gene Abbreviations: PLAU – Urokinase-type Plasminogen Activator; SERPINE1 – Serpine Peptidase Inhibitor (Plasminogen Activator Inhibitor); THBD – Thrombomodulin; TF – Tissue Factor, Thromboplastin, vWF – von Willebrand Factor, PLAT – Tissue Plasminogen Activator, PROCR – Endothelial Protein C Receptor