RAPID COMMUNICATION

Assessment of hepatic VX₂ tumors with combined percutaneous transhepatic lymphosonography and contrast-enhanced ultrasonographic imaging

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Abstract

AIM: To evaluate the feasibility and efficacy of percutaneous transhepatic lymphosonography (PTL) as a novel method for the detection of tumor lymphangiogenesis in hepatic VX₂ of rabbits and to evaluate combined PTL and routine contrast-enhanced ultrasonographic imaging for the diagnosis of liver cancer.

METHODS: Ten rabbits with VX₂ tumors were included in this study. SonoVue (0.1 ml/kg) was injected into each rabbit via an ear vein for contrast-enhanced ultrasonographic imaging, and 0.5 ml SonoVue was injected into the normal liver parenchyma near the VX₂ tumor for PTL. Images and/or movie clips were stored for further analysis.

RESULTS: Ultrasonographic imaging showed VX₂ tumors ranging 5-19 mm in the liver of rabbits. The VX₂ tumor was hyperechoic and hypoechoic to liver parenchyma at the early and later phase, respectively. The hepatic lymph vessels were visualized immediately after injection of contrast medium and continuously visualized with SonoVue® during PTL. The boundaries of VX₂ tumors were hyperechoic to liver parenchyma and the tumors. There was a significant difference in the values for the boundaries of VX₂ tumors after injection compared with the liver normal parenchyma and the tumor parenchyma during PTL.

CONCLUSION: PTL is a novel method for the detection of tumor lymphangiogenesis in hepatic VX₂ of rabbits. Combined PTL and contrast-enhanced ultrasonographic imaging can improve the diagnosis of liver cancer.

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Key words: Percutaneous transhepatic lymphosonography; Ultrasound; Contrast-enhanced ultrasonographic imaging; Ultrasound contrast media; VX₂ tumor

INTRODUCTION

The liver is the largest organ in the abdominal cavity and the main region of primary tumor and distant metastasis of malignant tumors. Detection of tumor nodules in the liver is of major importance for formulating therapeutic strategies and predicting the prognosis in malignant tumors.

Non-ionizing radiation, portable and noninvasive real-time imaging, ultrasonography (US) are the most commonly used imaging techniques. Introduction of microbubbles as contrast agents for ultrasound has improved the image quality and diagnostic value. Contrast-enhanced ultrasonographic imaging enables noninvasive measurements of microvascular perfusion in the heart, brain, kidney, skeletal muscle, skin grafts and solid tumors and provides functional images of angiogenesis in animals and humans.

At present, contrast-enhanced ultrasonographic imaging research has mainly focused on angiogenic blood vessels, blood vessel function and efficacy of...
angiogenesis inhibitors. Recently, lymphangiogenesis has become a new research frontier\cite{10}. Tumor lymphangiogenesis is the process of forming new lymph vessels in tumors and closely related to tumor development and progression. It is necessary to find noninvasive methods for evaluating lymphangiogenesis in situ. However, little is known about the contrast-enhanced ultrasonographic imaging used to detect tumor lymphangiogenesis. Recently, lymphosonography after interstitial injection of microbubble-based contrast agents can trace the lymphatic channels from the injection site up to the draining sentinel lymph nodes\cite{11-13}. However, no report is available on lymphosonography for tumor lymphangiogenesis.

The aim of the present study was to evaluate the feasibility and efficacy of PTL with a small volume of SonoVue\textsuperscript{®} as a novel method for the detection of tumor lymphangiogenesis of hepatic VX\textsubscript{2} in rabbits and to evaluate the combined PTL and contrast-enhanced ultrasonographic imaging in the diagnosis of liver cancer.

**MATERIALS AND METHODS**

**Animal model**

Ten male health New Zealand rabbits, weighing 2.5-3.0 kg, were included in this study and housed in an approved facility with free access to water and standard diet throughout the study. The study, approved by the Institutional Review Board for Animal Research, was performed following the Guidelines for the Care and Use of Laboratory Animals\cite{14}.

An undifferentiated VX\textsubscript{2} carcinoma growing rapidly in rabbits served as the experimental tumor. Two VX\textsubscript{2} tumors were implanted into the right and left lobes of liver, respectively. In brief, rabbits were anesthetized with ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (5 mg/kg) intramuscularly. The rabbits were intermittently given small supplementary doses of sodium chloride (5 mg/kg) intramuscularly. The rabbits were in ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (40 mg/kg) for routinely contrast-enhanced ultrasonographic imaging.

The VX\textsubscript{2} tumor in liver of rabbits ranging 5-19 mm was stabilized in a phospholipid shell, 1-10 μm in diameter, averaging about 2.5 μm. The SonoVue\textsuperscript{®} preparation was reconstituted just before administration by adding 5 mL sterile saline to the freeze-dried powder, so that sulfur hexafluoride had a concentration of 45 μg/mL in the suspension.

**SonoVue\textsuperscript{®} injection**

SonoVue (0.1 mL/kg) was injected via an ear vein as a rapidly injected bolus, followed by a 1.5 mL saline flush for routinely contrast-enhanced ultrasonographic imaging.

SonoVue (0.5 mL) was injected into the normal liver parenchyma near the VX\textsubscript{2} tumors as a rapidly injected bolus using a tuberculin syringe and a 26-gauge needle for PTL. The absorption of the contrast agent and its flow were observed in lymphatic channels of the VX\textsubscript{2} tumors.

**Statistical analysis**

For quantitative analysis, videodensities of the appropriate regions of interest (ROI), including perineoplastic liver parenchyma, boundaries of the tumor and tumor parenchyma were recorded during PTL. Respective evaluations were made for PTL. Data analysis was carried out using SPSS 16 statistical software. All videodensity data were expressed as mean ± SD. Parameters were tested using paired t test. Statistical analysis was performed using one-way analysis of variance and Dunnett’s multiple comparison tests. P < 0.05 was considered statistically significant.

**RESULTS**

The VX\textsubscript{2} tumor in liver of rabbits ranging 5-19 mm was found to be a low echoic mass. However, because the VX\textsubscript{2} tumor was almost isoechoic with the normal tissue and boundaries of the masses were unclear, detection and delineation of the lesion were difficult before SonoVue\textsuperscript{®} injection (Figure 1A).

Since the typical enhancement pattern of VX\textsubscript{2} tumor detected by routine contrast-enhanced ultrasonographic imaging was hyperechoic and hypoechoic to liver...
parenchyma during the early and later phase, respectively, a much more rapid wash-in and -out of ultrasonographic contrast agent was observed compared to the normal liver parenchyma (Figure 1B and C).

The enhancement pattern of VX2 tumors detected by PTL was significantly different from the typical enhancement pattern of VX2 tumors detected by routine contrast-enhanced ultrasonographic imaging. The hepatic lymph vessels were visualized immediately and continuously during PTL. SonoVue® was deposited in the parenchyma relatively quickly in winding channels. At the same time, the boundaries of VX2 tumors were hyperechoic to liver parenchyma and the tumors. The hyperechoic boundaries clearly delineated VX2 tumors compared with the normal liver and tumor parenchyma (Figure 2A-C). The difference in the videodensitometric measurements of the boundaries of VX2 tumors was significantly higher than the baseline (Figure 3). Conversely, videodensity in the normal liver and tumor parenchyma had no signal enhancement compared with the baseline (Figure 3). There was a significant difference in the boundaries of VX2 tumors compared with the baseline as well as the normal liver and tumor parenchyma (Figure 3).

DISCUSSION

Ultrasound is an important and useful imaging method for the detection of tumors. Ultrasound contrast agents containing encapsulated microbubbles are mainly used to increase the diagnostic imaging of tumors. McCarville et al.[18] showed that gray-scale US measurements of microbubble contrast agent flow can be used to detect the functional consequences of antiangiogenic therapy for tumors and to assess angiogenesis inhibitors that act through different mechanisms.[19–21]
Recently, lymphangiogenesis has become a new research frontier\(^{[25]}\). The important functions of the lymphatic system are to remove damaged cells from the body and to prevent the spread of infection and cancer for the maintenance of normal tissue fluid balance and immune surveillance. In spite of its important functions in physiological and pathological conditions, including tumor metastasis, lymphoedema and inflammation, lymphatic vessels have not received as much attention as blood vessels, and the mechanisms regulating their development and growth have been poorly understood\(^{[34]}\). Lymphangiogenesis is associated with increased tumor cells in lymphatics and lymph nodes, served as an independent prognostic factor and a potential target in the development of new therapies for hilar cholangiocarcinoma\(^{[29]}\). At present, neovessel formation, including lymphangiogenesis, represents the key event in tumor progression. Inhibition of metastatic spread may be achieved by restriction of lymphatic vessel growth with novel therapeutic strategies for anti-lymphangiogenic therapies\(^{[34]}\).

Currently, histologic determination of the mean intratumoral or peritumoral lymphatic vessels is the most commonly used method for assessing lymphangiogenesis. However, obtaining tissue for histologic evaluation may require an invasive procedure that cannot be normally accepted by patients. Furthermore, determination of the lymphatic microvessel density does not provide an accurate assessment of the functionality of tumor lymphatic vessels because many poorly functioning or collapsed lymphatic vessels have endothelial cells that are stained and counted. Therefore, the lymphatic microvessel density \textit{in vivo} may be a potentially useful marker for assessing lymphangiogenesis in tumors at diagnosis, and accurately reflects the effectiveness of antitumor therapy.

Ultrasound lymphography with subcutaneous injection of ultrasound contrast material enables direct visualization of the lymphatic drainage pathways and sentinel lymph nodes of breast diseases, melanoma, etc\(^{[11-13]}\).

In the present study, the traditional percutaneous hepatic injection method was used to deliver SonoVue® microbubbles into the liver under US guidance to investigate tumor lymphangiogenesis. To the best of our knowledge, lymphsonography for the detection of tumor lymphangiogenesis has not been reported before. Hepatic lymph vessels were visualized immediately after injection of contrast agent and opacified with SonoVue® during PTL, whereas liver parenchyma was not enhanced by SonoVue®. SonoVue® was deposited in the parenchyma relatively quickly in winding lymph vessels. At the same time, the boundaries of VX\(_2\) tumors were hyperechoic to liver parenchyma and the tumors, indicating that hyperechoic boundaries clearly delineate the peritumoral lymphatic vessels of VX\(_2\) tumors. Compared with the hyperechoic boundaries of VX\(_2\) tumors, the videodensity in the tumor parenchyma had no signal enhancement compared with the baseline. This is consistent with the findings in a previous study\(^{[27]}\). It was reported that three-dimensional changes of lymphatic architecture in rabbit VX\(_2\) tongue cancer, dynamics of its adjacent lymphatic architecture, especially the increased number of capillaries in preexisting lymphatic vessels outside the tumor margin, are associated with lymph node metastasis\(^{[28,29]}\). The morphological features of lymphatic vessels during PTL may be important predictive markers for evaluating lymphatic metastasis and prognosis of tumors. The lymphatic drainage paths and lymphatic distribution pattern in hepatic tissue have been found to be very constant, showing that angiogenesis is a critical factor for tumor growth and metastasis\(^{[23]}\). In this study, the typical enhancement pattern of VX\(_2\) tumors detected by routine contrast-enhanced ultrasonographic imaging was hyperechoic and hypoechoic to the liver parenchyma at the early and later phases, respectively, confirming that routine contrast-enhanced ultrasonographic imaging can assess tumor vascularity and reveal the microvascular perfusion and function\(^{[23,30,31]}\).

The specific mechanism by which the contrast agents used in this study enter the lymphatic system is unclear. SonoVue® microbubbles have a mean diameter of 2.5 μm with 99% smaller than 11 μm, allowing a free passage of capillaries, but keeping within the vascular lumen. This means that SonoVue® microbubbles in the hepatic inter-space cannot come into blood vessels. Although the optimal particle diameter for lymphatic uptake is 10-50 nm, particles up to hundreds of nanometers in diameter appear to be able to cross the lymphatic endothelium\(^{[32,34]}\). Due to the flexibility of microbubbles, phospholipidic shell and poor solubility and diffusivity of SF\(_6\), SonoVue® is highly resistant to pressure. This means the microbubbles may more easily distort and traverse lymphatic wall fenestrations into lymph capillaries.

Due to the different membranes, 99% of Sonazoid and Optison are phagocytosed by Kupffer cells, whereas only 7.3% of SonoVue® is phagocytosed by Kupffer cells\(^{[38]}\). This means that the SonVue® microbubbles are
not easily phagocytosed by macrophages. Tracing the SonVue® microbubble flowing in the lymph vessels can improve the pathologic staging of the disease and its treatment.

At the same time, microbubbles are used not only for contrast enhancement of ultrasound images and improvement of diagnosis, but also for delivery of drugs and genes. The ability to localize lymphatic vessels in tumors may be of value for a new route to the administration of drugs, gene and immunotherapy, etc. Drugs/genes containing vesicles may be injected simultaneously with microbubbles or microbubbles in combination with microbubble-forming vesicle aggregates. Using microbubbles oscillation and cavitation under US guidance might assist in delivering drugs/genes from vesicles to the interstitial tissue, which may be an effective treatment for some diseases.

Since few studies about hepatic lymphography are available at present, it is difficult to find microbubbles in lymphatic vessels. Due to this reason, the study only limited to the ultrasound characteristic aspects of PTL, which were not compared with the histopathologically aspects of rabbit VX2 tumors.

In conclusion, PTL with a small volume of SonVue microbubbles is a novel method for the detection of tumor lymphangiogenesis of hepatic VX2 tumors in rabbits. Combined PTL and contrast-enhanced ultrasonographic imaging can improve the diagnosis of liver cancer. Additional research is needed to determine the potential advantages of PTL and to determine if PTL can be used in clinical practice.

Comments

Background

Ultrasoundography (US) is one of the most commonly used imaging techniques. Lymphangiogenesis has become a new research frontier. Tumor lymphangiogenesis is the process of generating new lymph vessels within and surrounding tumors, which is closely related to tumor development and progression. It is necessary to develop noninvasive methods for evaluating lymphangiogenesis in situ. However, to the best of our knowledge, lymphosonography showing tumor lymphangiogenesis with percutaneous hepatic injection of ultrasound contrast material has not been reported before.

Research frontiers

This study investigated tumor angiogenesis and lymphangiogenesis with combined percutaneous transhepatic lymphosonography (PTL) and contrast-enhanced ultrasonographic imaging for hepatic VX2 in rabbit liver.

Innovations and breakthroughs

Contrast-enhanced ultrasonographic imaging enables noninvasive measurements of microvascular perfusion in the heart, brain, kidney, skeletal muscle, skin grafts and solid tumors in animals and humans. It was recently reported that lymphosonography after interstitial injection of microbubble-based contrast agents can trace lymphatic channels from the injection site up to the draining sentinel lymph nodes. This is the first study to evaluate the feasibility and efficacy of PTL with a small volume of SonVue® as a novel method for the detection of tumor lymphangiogenesis of hepatic VX2 in rabbits and to evaluate the role of combined PTL and contrast-enhanced ultrasonographic imaging in improving the diagnosis of liver cancer.

Applications

PTL with a small volume of SonVue microbubbles is a novel method for the detection of tumor lymph angiogenesis of hepatic VX2 in rabbits. Combined PTL and contrast-enhanced ultrasonographic imaging can improve the diagnosis of liver cancer. Additional research is needed to determine the potential advantages of PTL and to determine if PTL can be used in clinical practice.

REFERENCES

18. McCarrville MB, Streck CJ, Dickson PV, Li CS, Nathwani AC, Davidoff AM. Angiogenesis inhibitors in a murine neuroblastoma model: quantitative assessment of
intratumoral blood flow with contrast-enhanced gray-scale US. Radiology 2006; 240: 73-81


28 Seki S, Fujimura A. Three-dimensional changes in lymphatic architecture around VX2 tongue cancer--dynamic changes after administration of antiangiogenic agent. Lymphology 2003; 36: 199-208

29 Seki S, Fujimura A. Three-dimensional changes in lymphatic architecture around VX2 tongue cancer--dynamics of growth of cancer. Lymphology 2003; 36: 128-139


38 Feinstein SB. The powerful microbubble: from bench to bedside, from intravascular indicator to therapeutic delivery system, and beyond. Am J Physical Heart Circ Physiol 2004; 287: H4450-H4457


40 Borden MA, Caskey CF, Little E, Gillies RJ, Ferrara KW. DNA and polysyline adsorption and multilayer construction onto cationic lipid-coated microbubbles. Langmair 2007; 23: 9401-9408

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