High-Resolution Ultrasound Perfusion Imaging of Therapeutic Angiogenesis

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OBJECTIVES The purpose of this study was to test the feasibility of contrast pulse sequence (CPS) ultrasound imaging for high-resolution perfusion imaging after gene transfer (GT) for therapeutic angiogenesis.

BACKGROUND Imaging modalities capable of accurate and feasible perfusion measurement are essential for the preclinical and clinical development of therapeutic angiogenesis. However, current methods suffer from compromises between spatial and temporal resolution and sensitivity. Contrast pulse sequence ultrasound is a recently developed real-time perfusion imaging method that generates high-contrast agent-to-tissue specificity and spatial resolution.

METHODS Contrast pulse sequence ultrasound was used to noninvasively assess parameters of blood flow 6 days after adenoviral vascular endothelial growth factor (AdVEGF) GT in rabbit and mouse hind limbs with bolus intravenous injection of a microbubble contrast medium. Blood volume, mean transit time, perfusion, and time to the arrival of the contrast bolus were calculated with the gamma variate function. Contrast-enhanced power Doppler ultrasound (CEU), dynamic contrast-enhanced (DCE) magnetic resonance imaging (MRI), and histological capillary measurements were used as reference methods.

RESULTS Blood volume and perfusion increased over 40- and 20-fold, respectively, 6 days after AdVEGF GT in rabbit skeletal muscles. Perfusion values measured with CPS correlated well with those obtained with CEU (r = 0.975) and DCE-MRI (r = 0.854). However, CPS provided superior spatial and temporal resolution showing blood flow in vessels of only 10 to 20 μm in diameter. Contrast pulse sequence ultrasound was also feasible for imaging of therapeutic angiogenesis in mouse hind limbs both at the arterial and capillary levels. The CPS ultrasound revealed that AdVEGF mainly induces angiogenesis in adipose tissue rather than in the skeletal muscle of mouse hind limbs.

CONCLUSIONS Contrast pulse sequence ultrasound is an efficient and accurate noninvasive real-time perfusion imaging modality in small laboratory animals and also offers a means for the assessment of muscle perfusion in future clinical trials of therapeutic angiogenesis. (J Am Coll Cardiol Img 2008;1:83–91) © 2008 by the American College of Cardiology Foundation
Gene therapy for therapeutic angiogenesis is a potential new approach to alleviate tissue ischemia, but so far, promising animal experiments have not resulted in clinical success (1,2). Novel imaging methods capable of measuring blood flow noninvasively and reliably at the capillary level are clearly needed for the development of better angiogenic gene therapy approaches. The current imaging techniques, such as contrast-enhanced power Doppler ultrasound (CEU) and harmonic ultrasound imaging, laser Doppler and optical imaging methods, magnetic resonance imaging (MRI), computed tomography (CT), single-photon emission computed tomography (SPECT), and positron emission tomography (PET), are hampered by compromises between the spatial and temporal resolution, penetration, and sensitivity; might be unsuitable for small animals; or might not be used for imaging of angiogenic and leaky vessels, owing to small-sized contrast media particles (3). Moreover, MRI and PET in particular are less available and require a high level of expertise.

Contrast pulse sequencing (CPS) is a recently introduced contrast-enhanced ultrasound imaging technology that uses nonlinear fundamental frequencies, resulting in improved spatial resolution and higher sensitivity to microbubble contrast media (4). Furthermore, CPS provides better tissue penetration, less attenuation, and more improved tissue subtraction than previous ultrasound perfusion imaging techniques (4). In this study, we used CPS imaging to assess angiogenesis and subsequent perfusion increases in rabbit and mouse skeletal muscle after adenoviral (Ad) gene transfer (GT) of vascular endothelial growth factor (VEGF). Our findings show that CPS is feasible and accurate in small laboratory animals, reaching the level of microcirculation, and is attractive also for clinical studies of therapeutic angiogenesis.

METHODS

GT. Adenoviral VEGF165 (AdVEGF-A, n = 9) or adenoviral beta-galactosidase (AdLacZ, n = 10) intramuscular GT was performed in the right semimembranosus muscle of New Zealand white rabbit hind limbs (5 × 0.1 ml, titer 10^{11} viral particles [vp]/ml) as previously described (5,6). In C57Bl/6Ja mice, AdVEGF (n = 6) or AdLacZ (n = 6) GT was done in the adductor compartment of the thigh and in the posterior calf (both 50 μl, titer 10^{11} vp/ml). All animal experiments were approved by the Experimental Animal Committee, University of Kuopio.

High-resolution ultrasound perfusion imaging. The Acuson Sequoia 512 system and 15L8 transducer (Siemens, Malvern, Pennsylvania) were used for ultrasound imaging. Power Doppler CEU with the bolus injection technique has been previously validated in skeletal muscle perfusion measurement both in animals and humans (6–8). First, CEU (power Doppler mode at 8.5 MHz, mechanical index = 0.60, dynamic range = 10 dB, power = −18 dB, Doppler gain = 40) was performed in rabbits with a 0.3-ml bolus administration of Sonovue (Bracco, Milan, Italy) via the ear vein as previously described in detail (6). There was no blooming of contrast medium with the mechanical index used. In mice, power Doppler imaging was performed without contrast administration (power Doppler at 14 MHz, dynamic range = 20 dB, power = −5 dB, Doppler gain = 50). Cadence CPS imaging was performed in rabbits after an intravenous (IV) bolus injection of 0.5 ml Sonovue at 8.0 MHz (dynamic range = 75 dB, power = −16 dB, CPS gain = −10, T1/0/1/4, Δ4, mechanical index = 0.31). In mice, 50 μl of Sonovue was injected as an IV bolus via a cannula placed in the external jugular vein, and CPS was performed at 14 MHz (dynamic range = 80 dB, power = −8 dB, CPS gain = −15, S1/0/2/4, Δ4, mechanical index = 0.25). The contralateral intact limb was used as an internal control to exclude the effect of cardiac output.

The power Doppler and CPS signal intensities (dB) of video clips were quantified with Datapro (version 2.13, Noesis, Courtaboeuf, France), and signal intensity–time curves were generated. After a bolus injection of a contrast medium, the gamma-variate function (Fig. 1) models the first-pass kinetics of the contrast medium in the region of interest (ROI), which allows the calculation of blood flow (and perfusion) via the determination of blood volume (area under the curve) and the mean transit time (MTT) (8–10). The peak signal intensity of the curve is the maximal contrast enhancement in the ROI representing a simpler estimate of blood flow/perfusion (6,11,12). The time from bolus injection to the arrival of contrast agent in ROI can also be determined, which reflects peripheral resistance (6). PowerShowCase (version 4.95, Trillium
Technology, Ann Arbor, Michigan), Camtasia Studio (version 2.0, TechSmith, Okemos, Michigan), and WinAVI Video Converter (version 7.7, ZJPEG Media Digital Technology) (13) were used for the processing of ultrasound video files for publication in the Windows Media Video 9 format (Microsoft, Redmond, Washington).

Perfusion and blood volume measurement with MRI. The MRI of rabbit thighs was carried out in a 4.7-T magnet (Magnex, Abington, United Kingdom) interfaced to a Varian UnityINOVA (Varian, Palo Alto, California) console with actively shielded gradients and with an in-house-built surface coil (diameter 38 mm) as previously described (6). Briefly, dynamic contrast-enhanced (DCE)-MRI of perfusion with a FLASH pulse sequence (repetition time $T_R$ = 1100 ms, echo time $T_E$ = 5 ms, field of view $= 6 \times 6$ cm$^2$, resolution $= 64 \times 64$, slice thickness $= 5$ mm, number of averages $= 1$, acquisition time $0.58$ s/image) was done after an IV 0.7-ml bolus injection of superparamagnetic iron oxide particles (Resovist, Schering, Berlin, Germany; mean size 62 nm). The signal intensity–time curves were derived from the DCE-MRI data, and perfusion ratios were calculated as previously reported (6). To obtain high-quality images of blood volume, T$_2^*$-weighted gradient echo images (repetition time $= 2$ s, echo time $= 18$ ms, field of view $= 6 \times 6$ cm$^2$, resolution $= 256 \times 128$, slice thickness $= 2.5$ mm, and number of averages $= 2$) were acquired before and 6 min after the bolus contrast agent injection to determine the steady-state distribution of the IV contrast agent. Blood volume-MRI maps ($\Delta R_2^*$) were reconstructed as described (6).

Immunohistochemistry. After MRI, the rabbits were euthanized, perfusion-fixed, and the muscle samples were processed as previously described (5,6). Endothelium was immunostained with a monoclonal antibody against CD31 (dilution 1:50, DAKO, Glostrup, Denmark) in rabbits and with biotinylated Griffonia Simplicifolia lectin I (1:100, Vector, Burlingame, California) in mice with the avidin-biotin-HRP system (Vector) and 3±5-diaminobenzidine (DAB, Zymed, San Francisco, California) color substrate on 7-$\mu$m-thick paraffin-embedded sections (5,6). Photographs and measurements of histological sections were taken with an Olympus AX70 microscope (Olympus Optical, Tokyo, Japan) with analySIS imaging software (Soft Imaging System, Muenster, Germany) and were further processed for publication with Adobe Photoshop CS (Adobe, San Jose, California). The capillary mean area ($\mu$m$^2$, measured along the outer surface) and total capillary vessel area of muscle area (i.e., fractional capillary area, %) were measured from 10 fields of CD31 immunostained sections of rabbit semimembranosus muscles at 200× magnification. All measurements were performed in a blinded manner.

Statistical analyses. Results are expressed as means ± SEM. Statistical significance was evaluated with analysis of variance followed by independent samples $t$ test or the Kruskal-Wallis test, followed by Mann-Whitney $U$ test where appropriate.
tion analyses were performed by the Pearson and Spearman rho tests. A p value <0.05 was considered statistically significant.

RESULTS

CPS perfusion ultrasound imaging provides high spatial and temporal resolution. The vascular effects of AdVEGF GT in rabbit semimembranosus muscles are shown by 3 independent imaging methods: blood volume-MRI, CEU, and CPS ultrasound (Fig. 2). Blood volume-MRI maps (ΔR2*) of AdVEGF and control AdLacZ-injected muscles show strongly increased blood volume in the AdVEGF transduced muscle and effusate surrounding the muscle owing to increased vascular permeability (Figs. 2A and 2B). The CEU imaging is capable of detecting only the large (>100 μm) vessels, whereas microcirculation is invisible (Figs. 2C and 2D). On the contrary, CPS ultrasound with IV-injected contrast microbubbles has a high spatial resolution allowing the detection of perfusion throughout the AdVEGF transduced muscle (Figs. 2E and 2F). Importantly, CPS efficiently subtracts echoes from the tissue and has a high frame rate (Figs. 2E and 2F, Online Video 1). In the CPS “mix” mode, blood flow and tissue can be imaged simultaneously, but the quantification of the microbubble signal is more difficult (Online Video 2). Comparison of CPS imaging with histological capillary staining of the same muscle revealed that CPS is able to detect moving microbubbles in enlarged capillaries that are only 10 to 20 μm in diameter (Fig. 3, Online Video 3).

AdVEGF increases blood volume over 40-fold and perfusion over 20-fold in rabbit skeletal muscle. Blood volume was increased as high as 41- and 51-fold as measured with blood volume-MRI and -CPS methods, respectively, 6 days after AdVEGF GT (Fig. 4A). The perfusion ratio between the transduced and contralateral intact limbs was also found to be very similar, elevated 20- to 24-fold, regardless of the method used (Fig. 4B). The correlations between the different methods were very high (Table 1, Fig. 4B). The shapes of the curves reflect different vessel types in the vascular bed (14): the presence of large arteries and enlarged capillaries without a normal capillary bed produces a steep curve with a high peak intensity (AdVEGF), whereas a normal vascular network with normal-sized capillaries yields a flat curve (AdLacZ) (6).

Microvessel enlargement in AdVEGF-treated muscles decreased peripheral resistance (time to arrival) significantly as compared with AdLacZ control muscles (Fig. 4C). In contrast, MTT was longer in AdVEGF-treated muscles (Fig. 4C). Figures 4D to 4F show histological parameters of capillaries in transduced muscles. As observed previously (6), AdVEGF strongly increases capillary mean size, although capillary density is not changed.
6 days after GT (Figs. 4D and 4E). The capillary enlargement results in a 9.2% total capillary area of muscle area in AdVEGF transduced muscles (0.3% in AdLacZ muscles) (Fig. 4F). As expected, the perfusion increase and blood volume had a positive correlation with mean capillary size and total capillary area (Pearson test), whereas the arrival time had an inverse correlation with capillary size. Capillary density did not correlate with any of the parameters (OnlineTable 1).

CPS perfusion imaging in mouse hind limbs after angiogenic gene therapy. We also tested the feasibility of CPS ultrasound imaging in the assessment of perfusion in mice hind limbs, because there is a shortage of methods capable of measuring real-time perfusion at the capillary level in mice. It was found that CPS has an excellent resolution also in mouse hind limbs (Figs. 5A to 5D, Online Video 4). In fact, because a higher frequency (14 MHz) can be used in mice than in rabbits (8.5 MHz), owing to a smaller ROI and a higher amount of contrast agent used in proportion to the body size, the spatial resolution is even better than in rabbits. Interestingly, CPS in mouse hind limbs revealed that intramuscularly injected AdVEGF induces angiogenesis mainly in the surrounding adipose tissue rather than in the targeted skeletal muscle itself (Figs. 5B, 5D, and 6, Online Video 4). This finding was confirmed by histological staining of capillaries (Figs. 5E to 5H).

DISCUSSION

Imaging of growing vasculature and quantitative measurement of perfusion are very important in the assessment of clinical efficacy of angiogenic therapies. Strong vascular growth during therapeutic angiogenesis by overexpression of VEGF family members involving abundant capillary enlargement leading to capillary arterialization (6) and increased vascular permeability interferes with many current vascular imaging and perfusion measurement techniques. We found in this study that real-time CPS ultrasound imaging is a feasible and accurate perfusion measurement method after GT for therapeutic vascular growth in peripheral muscles of small and large experimental animals.

None of the current noninvasive perfusion measurement techniques, such as DCE-MRI, CT, SPECT, PET, laser Doppler, optical imaging methods, and previous ultrasound techniques, can provide simultaneous capillary level imaging at a
Contrast-enhanced steady-state MRI offers relatively good sensitivity and spatial resolution in vascular imaging, the possibility to perform whole organ scans, as well as functional imaging (3,6). However, in DCE-MRI, much of the spatial resolution must be sacrificed to get a sufficient frame rate for bolus tracking (6). Although highly sensitive, SPECT and PET both suffer from poor spatial resolution (3). Micro-CT is suitable for very accurate (up to 16 μm) postmortem anatomical vascular imaging, but the protocols for dynamic studies in vivo are less well established, in addition to well-known drawbacks of ionizing radiation and the low sensitivity of CT to contrast agents. Moreover, MRI and PET devices are not widely accessible to experimental researchers. Laser Doppler and optical imaging methods suffer from low resolution and poor tissue penetration (3).

In this study, we found that CPS perfusion measurement correlates well with that of DCE-MRI in rabbit skeletal muscle after angiogenic therapy. It was also feasible in mouse hind limbs where perfusion has been generally assessed by laser Doppler, which, however, penetrates only <1 mm in the skin. Importantly, CPS is technically less demanding, and the imaging protocol is much quicker than, for example, that of DCE-MRI. Of utmost importance is that large (>1 μm) microbubbles remain in circulation despite vessel leaki-
Here we used CPS at 8 and 14 MHz in rabbits and mice, respectively. A lower frequency in rabbits allows a better tissue penetration and a lower amount of contrast agent, owing to improved sensitivity, whereas in mice a higher frequency enables the visibility of the smallest vessels.

We found that the perfusion value calculated with the peak signal intensity after a bolus administration of the contrast agent correlated well with the perfusion value obtained by the gamma-variate function, which represents the standard analysis method after a bolus injection (10). Our data are supported by previous reports using both ultrasound and DCE-MRI showing a correlation between the peak enhancement and mathematical models (11,12). This finding is important, because the simple determination of the peak signal intensity from the data allows much quicker data analysis. The use of a continuous infusion of the contrast agent with the destruction-replenishment technique is widely used, especially in the myocardium, and has also been used in rat hind limbs (15). However, it is less practical in the skeletal muscle of big mammals, because of the low basal blood flow.

![Figure 5](image)

**Figure 5.** CPS Allows High-Resolution Perfusion Imaging in Mouse Hind Limbs After Angiogenic Gene Transfer

(A to D) Normal power Doppler without contrast enhancement (left) and 14 MHz CPS imaging (right) 6 days after AdLacZ or AdVEGF gene transfer in mouse hind limbs. (A and B) AdVEGF induces angiogenesis and perfusion increases almost exclusively in the subcutis (brackets), probably owing to the low tropism of adenovirus in mouse skeletal muscle. The femoral artery is indicated by arrowheads. (C and D) Also in the calf, angiogenesis mostly occurs in the adipose tissue (brackets). Here, arrowheads denote the popliteal artery. Lectin capillary stainings of skeletal muscle (E and F) and subcutis (G and H) nearby. Although there are a few slightly enlarged capillaries in skeletal muscle (F, arrows), angiogenesis mostly takes place in the adipose tissue. Note red blood cells inside enlarged blood vessels (asterisks), indicating that the vessels are perfused. Scale bar = 100 μm. See also Online Video 4. Abbreviations as in Figures 1 and 2.

**Figure 6.** Quantitative Measurement of Perfusion in Mouse Hind Limbs After AdVEGF Gene Therapy

The AdVEGF (n = 6) increases perfusion by 2.7- and 2.5-fold in the thigh and calf muscles, respectively, 6 days after gene transfer. *p < 0.05 and **p < 0.01 versus AdLacZ (n = 6). Analyzed by analysis of variance followed by independent samples t test. Q1 = 25th percentile; Q2 = 50th percentile (median); Q3 = 75th percentile; other abbreviations as in Figure 1.
that would require very high amounts of infused contrast medium.

The findings herein show that AdVEGF elevates skeletal muscle perfusion as much as 20- to 24-fold 6 days after GT. It is obvious that this kind of acute and transient (<14 days) supraphysiological perfusion increase might not be therapeutically optimal, but instead, vectors like adeno-associated viruses are needed to promote more persistent angiogenesis without significant tissue edema as a side effect (1). In mice, the perfusion increase was much more modest as compared with rabbits, which is explained by the fact that angiogenesis occurred mainly in the adipose tissue and not in the injected skeletal muscle itself. The reason for this seems to be the poor transduction efficiency of mouse skeletal myocytes by adenovirus (16). The short arrival times (i.e., decreased peripheral resistance) and the steep shape of CPS signal intensity–time curve indicate the presence of large arteries and enlarged capillaries and the lack of a normal small-sized capillary bed after AdVEGF treatment (6,14). The delay in MTT in AdVEGF limbs is likely due to the recruitment of resting capillaries and strong capillary enlargement leading to increased blood volume in AdVEGF-treated limbs (17). It is noteworthy that capillary density did not change by VEGF overexpression over 6 days, nor did it correlate with any of the blood flow parameters.

Contrast-enhanced power Doppler ultrasound imaging has already been established in the assessment of skeletal muscle perfusion both in healthy volunteers and patients with peripheral arterial disease (18). We conclude that CPS ultrasound seems to be the next advance in the field of angiogenesis imaging and one of the most promising means for noninvasive follow-up of patients with peripheral arterial disease in clinical trials of therapeutic vascular growth.

**Study limitations.** In the past, there has been less experience on the analysis of blood flow with the bolus injection technique, whereas the corresponding algorithms are already well established in the method with a continuous contrast agent infusion and the analysis of destruction-replenishment kinetics. Thus, it is necessary to compare these 2 methods in the same experimental setting in the future. The usefulness of the CPS imaging settings used in this study could be limited in clinical use, because high frequencies feasible in smaller animals compromise the penetration of the beam in human skeletal muscle.

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**REFERENCES**


**APPENDIX**

For accompanying videos to Figures 2, 3, and 5, and a table depicting the correlation of parameters of blood flow versus histological analyses of vasculature, please see the online version of this article.