Therapeutic angiogenesis with placental growth factor improves exercise tolerance of ischaemic rabbit hindlimbs

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Aims We investigated the effects of angiogenic gene therapy with adenoviral placental growth factor131 (AdPlGF) on aerobic capacity and exercise tolerance in a rabbit hindlimb ischaemia model. We also assessed whether strong angiogenic changes such as capillary arterialization and formation of artery-venous shunts compromise oxygen transport to target tissues resulting in suboptimal therapeutic efficacy.

Methods and results Hindlimb ischaemia was surgically induced in New Zealand White rabbits (n = 20) that a day later received intramuscular (i.m.) AdPlGF or AdLacZ (3 x 10^11vp) gene transfer (GT). Corresponding GTs were also done in healthy non-ischaemic rabbits (n = 10). Muscle energy metabolism and skeletal muscle perfusion were studied non-invasively before GT and at 6 and 28 days using 31P-magnetic resonance spectroscopy and contrast pulse sequence ultrasound, respectively. Oedema was quantified using modified Miles assay at sacrifice. AdPlGF increased perfusion 7.8-fold and improved aerobic capacity of ischaemic limbs 45% compared with AdLacZ controls (P, 0.05) at 6 days. In non-ischaemic limbs, strong angiogenic response to GT, including capillary arterialization and acute oedema, did not impair muscle energy metabolism.

Conclusion This study shows that proangiogenic gene therapy can significantly improve performance of ischaemic limbs and supports the concept of therapeutic angiogenesis for the treatment of patients with ischaemia.

KEYWORDS Gene therapy; Energy metabolism; Magnetic resonance spectroscopy; Vascular endothelial growth factors; Angiogenesis

Introduction

Adenoviral gene therapy with members of the vascular endothelial growth factor (VEGF) family has been shown to induce capillary arterialization and increase muscle perfusion in preclinical studies.1,2 In peripheral arterial disease (PAD), clinical gene therapy trials endpoints such as increased exercise tolerance have been negative although improved vascularity has been reported.3–6 The assessment of improved exercise tolerance in preclinical studies has also been largely neglected, especially in large animal models due to lack of methods suitable for objective evaluation. Thus, a question has been raised whether aberrant forms of neovascularization, such as capillary arterialization or artery-venous shunts, and muscle oedema induced by strong angiogenic growth factors in fact hinder transport of oxygen and nutrients to target tissues and result in suboptimal therapeutic efficacy. Additionally, the functionality of vessels induced by gene transfer (GT) of a single growth factor or growth factor isoform has been questioned.7–9

Magnetic resonance spectroscopy (MRS) is a non-invasive technique for studying energy metabolism, of skeletal muscle in vivo, and can be used to identify tissue ischaemia in patients with PAD.10 Energy for muscle contraction is released via conversion of adenosine tri-phosphate (ATP) into adenosine di-phosphate (ADP). During exercise, phosphocreatine (PCr) restores ATP reserves and inorganic phosphate (Pi) accumulates.11 Thus, the energy level of the muscle can be monitored using phosphorus (31P) MRS by calculating the ratio of PCr and Pi (PCr/Pi).11 In ischaemic muscles, blood flow is the main limiting factor of aerobic energy production and exercise tolerance. Thus, in ischaemic muscles 31P-MRS can provide objective means for evaluating exercise tolerance.12

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In this study, we investigated using $^{31}$P-MRS whether muscle energy metabolism and exercise tolerance of ischaemic rabbit hindlimbs could be improved by angiogenic gene therapy with adenoviral placental growth factor (AdPlGF). We also assessed whether capillary arterialization and muscle oedema can compromise energy metabolism of normoxic muscles. This manuscript not only reports promising results but also addresses the importance of linking physiological findings with actual changes at the histological level.

**Methods**

**Ischaemia operation and gene transfer**

This model of acute ischaemia induced by a surgical operation, although not completely resembling chronic consequences of atherosclerotic occlusions in humans, is a feasible preclinical approach to study therapeutic angiogenesis as means for revascularization of ischaemic tissues. The time line of the study is shown in Figure 1A. A day before GT, acute ischaemia was induced in the right hindlimbs of a subgroup of New Zealand White rabbits as previously described. Briefly, the superficial part of the femoral artery was ligated under ketamine (Ketalar, Pfizer 0.3 mL/kg) and medetomidine (Domitor, Orion, 0.3 mL/kg) anaesthesia. The re-entry branches for the collaterals growing from the lateral circumflex and deep femoral arteries were ligated. In this model, the calf region is ischaemic while the thigh remains normoperfused (Figure 1B-C). In a sham operation, all other procedures were similar to the actual operation except that ligatures were placed loosely around vessels, not blocking blood flow.

At GT, the rabbits (mean weight 2.5 kg, n = 28) received $3 \times 10^{10}$ adenoviral particles [vp]/mL encoding mPlGF-2$^{12}$ or β-galactosidase (LacZ) under the control of the cytomegalovirus (CMV) promoter. Human clinical grade, first generation, serotype 5, replication-deficient adenoviruses were used. Intramuscular (i.m.) GT was performed in the semimembranosus (8 injections), abductor cruris cranialis (8 inj.), quadriceps femoris (5 inj.), tibialis anterior (3 inj.), and gastrocnemius (6 inj.) muscles using a 1 mL syringe and a 25-gauge needle (each injection 0.1 mL) under ketamine-medetomidine anaesthesia. All animal experiments were approved by the Experimental Animal Committee of University of Kuopio and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**$^{31}$P-magnetic resonance spectroscopy**

Before the gene transfer, and 6 and 28 days after GT, the degree of ischaemia and the aerobic capacity of calf muscles were evaluated using $^{31}$P-MRS. $^{31}$P-MRS was performed with a Varian UNITY INOVA (Varian) imaging console interfaced to a 4.7 T horizontal magnet (Magnex Scientific) with actively shielded gradients (Magnex Scientific). An in-house built linear surface RF-coil consisting of two separate loops tuned to $^1$H and $^{31}$P frequencies with diameters of 38 and 25 mm, respectively, were used for reception. Region of interest under the surface coil was shimmed using $^1$H signal and $^{31}$P data were acquired after ~70° hard pulse excitation using a repetition time of 1.5 s, spectral bandwidth of 10 kHz covered by 6496 data points and a number of averages of 16. JMRui 2.1 software (http://www.mrui.uab.es/mrui) was used for line shape fitting analyses of the spectra after pre-processing the data by discarding the two first data points, using a DC correction of 500 and 15 Hz line broadening.

Small needle electrodes (27G) were placed on both sides of the sciatric nerve on the lateral side of the thigh. During the whole duration of the experiment (20 min) $^{31}$P-MRS spectra were collected each taking 30 s. First, resting spectra were collected for 2 min. Thereafter, the sciatric nerve was electrically stimulated for 6 min at 3 Hz using a voltage of 75–125 V (14A11 Electromyograph, Disa) to induce maximal contractions in the calf muscles. Then, the stimulation was ceased and muscle recovery was followed for 12 min. The ratio between the peak areas of PCr and Pi, i.e. PCr/(PCr$+\text{Pi}$) was calculated and used as a measure of aerobic capacity and fatigue. Example spectra are shown in Figure 3 (see also Supplementary videos S1 and S2 for changes during exercise in intact and ischaemic muscles). The average PCr/(PCr+Pi) ratio during the last 2 min of exercise (at 6–8 min, hereafter referred to as aerobic capacity) and at the end of the recovery period (at 18–20 min, recovery rate) were compared between the groups. Additionally, muscle pH was derived from the chemical shift difference (displacement of the peak on the x-axis) between PCr and Pi peaks.

**Ultrasound imaging of muscle perfusion with contrast pulse sequence technique**

Perfusion in the transduced and contralateral intact, thigh, and calf muscles was measured with Acuson Sequoia 512 and 15L8 transducer (Siemens) using novel Cadence contrast pulse sequence (CPS) application as previously described. Video clips were captured starting at the injection of a 0.5 mL bolus of a second generation contrast agent (SonoVue, Bracco) via the ear vein. CPS signal intensities of the video clips were quantified with Datapro 2.13 (Noesis) and the perfusion ratios between peak intensities in transduced and contralateral muscles were calculated.
Modified Miles assay for plasma protein extravasation

Modified Miles assay with Evans Blue dye was used for the quantification of extravasated plasma proteins at sacrifice as previously described.13 The results are expressed as ratios between transduced and control muscles.

Histology and blood vessel measurements

Avidin-biotin-HRP system (Vector Laboratories) with the 3′-5′-diaminobenzidine (DAB, Zymed) colour substrate was used for immunohistochemistry. The endothelium was immunostained using a mouse monoclonal antibody against CD31 (DAKO, dilution 1:50). Photographs were taken with an Olympus AX70 microscope (Olympus Optical) and analySIS software (Soft Imaging System).1

Mean capillary area (μm²) was measured from 10 fields representing maximal angiogenic effects in CD31 immunostained sections of the skeletal muscle at ×200 magnification from areas covered entirely by skeletal myocytes.2 All measurements were performed in a blinded manner and means of the measurements are reported.

Preparation of video files

The video files were prepared using Windows movie maker (Microsoft) and saved as Windows media video files (WMV, Microsoft). In the case the video files cannot be played, the latest Microsoft Media Player (or equivalent) may have to be downloaded at http://www.microsoft.com/windows/windowsmedia/download/default.aspx. In the case the video files cannot be played, the latest Microsoft Media Player (or equivalent) may have to be downloaded at http://www.microsoft.com/windows/windowsmedia/download/default.aspx. In the case the video files cannot be played, the latest Microsoft Media Player (or equivalent) may have to be downloaded at http://www.microsoft.com/windows/windowsmedia/download/default.aspx. In the case the video files cannot be played, the latest Microsoft Media Player (or equivalent) may have to be downloaded at http://www.microsoft.com/windows/windowsmedia/download/default.aspx.

Statistical analyses

Results are expressed as mean ± SEM. Non-parametric statistical analyses were used as measured values were not always normally distributed. Statistical significances were first evaluated using Kruskal-Wallis test, followed by Mann-Whitney U-test comparing individual groups where necessary. Correlation analysis was performed using a non-parametric Spearman’s Rho correlation test. P < 0.05 was considered statistically significant.

Results

Adenoviral placental growth factor induces angiogenesis and increases perfusion in both normoxic and ischaemic muscles

One day after ischaemia operation, perfusion was normal in the thigh muscles of the operated limbs whereas it was significantly decreased in the calf muscles of both the AdPlGF and the AdLacZ groups (P < 0.05, Figure 2A). The sham operation did not affect muscle perfusion. Six days after GT, perfusion in the AdLacZ-transduced thigh muscles of the operated limbs was at the baseline level (P = NS, Figure 2A and B), see also example ultrasound video in Supplementary video S3). In the ischaemic AdLacZ-transduced calf muscles perfusion was still decreased as it was in the post-operation measurements at day 1 (P = NS, Figure 2A and C, see also Supplementary video S4). In contrast, AdPlGF was found to increase perfusion 15.8- and 7.8-fold in normoxic thigh and ischaemic calf muscles of the operated limbs, respectively (P = 0.01 vs. AdLacZ, Figure 2A, D and E, see also Supplementary videos S5 and S6). Twenty-eight days after GT, no differences in perfusion were observed in any group compared with the intacts (P = NS).

Histological changes in capillary size correlated with the perfusion increases (Spearman’s Rho correlation 0.738, P < 0.05). In the AdLacZ-transduced thigh muscles of the operated limbs, capillary size and histology were similar to that in the intact muscles 6 days after GT (Figure 2F). In the ischaemic calf muscles transduced with AdLacZ, modest capillary enlargement was detected at 6 days (Figure 2G). Also, tissue necrosis, regenerating muscle fibres, and accumulation of inflammatory cells in the ischaemic AdLacZ muscles were observed. AdPlGF GT induced abundant enlargement of capillary vessels in the normoxic thigh muscles 6 days after GT (Figure 2H, arrow). The AdPlGF-transduced calf muscles had almost no ischaemic changes such as necrosis. Capillaries in the AdPlGF-transduced ischaemic calf muscles were strongly enlarged and even the formation of large vessel lacunae was observed (Figure 2I). Twenty-eight days after GT, capillary size in all groups was similar to that in the intact muscles (Figure 2J–M). Growth of branching collateral vasculature was detected in the thigh muscles (arrows, Figure 2J and L). Some inflammatory cells and regenerating muscle fibres were still detected in the AdLacZ-transduced calf muscles (arrowheads, Figure 2K). In contrast, the AdPlGF-transduced calf muscles had normal histology (Figure 2M).

31P-MRS objectively assesses muscle energy metabolism and exercise tolerance

The resting 31P-MRS spectra of normoxic and ischaemic muscles at day 1 were very similar to each other (Figure 3A). They displayed a small Pi peak, a large PCR peak, and three small peaks for ATP. During exercise the differences between the spectra of normoxic and ischaemic animals were visible (Figure 3B–C). In the normoxic muscles, the PCr peak decreased and Pi peak grew as PCr was used for ATP production. At the end of exercise, the PCr and Pi peaks were roughly similar in size (Figure 3B). In the ischaemic muscles, the PCr peak decreased more rapidly and at the end of exercise the Pi peak was higher than the PCr peak (Figure 3C). Very little PCr was left for ATP production indicating that oxidative phosphorylation, restoring PCr, was defected and energy was produced mostly via anaerobic pathways. During the 12 min recovery period, the normoxic muscles restored their PCr level almost back to normal (Figure 3D), whereas the ischaemic muscles had still decreases PCr levels at the end of the recovery period (Figure 3E). See also Supplementary videos S1 and S2 for all the changes in the spectra during exercise and recovery.

Adenoviral placental growth factor improves aerobic capacity of ischaemic rabbit hindlimbs

Decreased aerobic capacity (36%) and recovery rate (67%), as assessed by 31P-MRS, were observed during electrically stimulated exercise in the calf muscles of the ischaemic limbs compared with the intact limbs 1 day after the ischaemia operation (P < 0.01, Figure 4A). The sham operation did not have an effect on the aerobic capacity of the calf muscles (P = NS, Figure 4A). AdPlGF improved aerobic capacity of the ischaemic limbs 45% compared with the AdLacZ controls 6 days after GT (P < 0.05, Figure 4B). In fact, the aerobic capacity of the AdPlGF-treated limbs was surprisingly similar to that of the intact controls (P = NS, Figure 4A), whereas the AdLacZ-treated limbs still had decreased aerobic capacity. However, there was no difference in the recovery rates between the AdLacZ and the AdPlGF groups (Figure 4B). Twenty-eight days after GT, aerobic capacity had recovered.
completely also in the AdLacZ animals, due to the formation of endogenous collateral arteries and thus no difference in the aerobic capacity could be detected between the AdPlGF, the AdLacZ, and the intact limbs (Figure 4C).

**Formation of metabolic acidosis during exercise is reduced after adenoviral placental growth factor gene transfer**

Maximal exercise with electrical stimulation induced acidosis in the calf as muscle pH dropped from 7.2 to 6.6 and 6.3 in the intact and ischemic muscles, respectively, a day after surgery ($P < 0.01$, Figure 5A). During the 12 min recovery period, acidosis almost completely recovered in the intact limbs whereas pH of the ischemic limbs did not, indicating a significantly impaired perfusion in the calf of the operated limbs. Degree of metabolic acidosis during exercise and recovery was reduced in the AdPlGF-transduced limbs compared with the intacts or the AdLacZ-transduced limbs 6 days after gene therapy ($P < 0.05$, Figure 5B). Whereas the intact and the AdLacZ limbs developed a dramatic decrease in muscle pH during exercise, the AdPlGF-transduced limbs had almost normal pH during the whole duration of the test (Figure 5B). The sham-operated muscles showed acidosis similar to that in the intact muscles. A significant increase in acidosis compared to the intacts was still visible in the ischemic AdLacZ legs at 6 days. Twenty-eight days after GT the AdPlGF animals still exhibited improved tolerance to acidosis during exercise compared with the AdLacZ controls ($P < 0.05$, Figure 5C).
Aerobic capacity of non-ischaemic limbs is not altered by adenoviral placental growth factor

To study whether capillary arterialization and muscle oedema can hinder the aerobic capacity of normal muscles, we utilized $^{31}$P-MRS after AdPlGF GT in intact hindlimbs. AdPlGF GT in the intact thigh and calf muscles induced very high increases in perfusion ($P < 0.05$, Figure 6A) and capillary enlargement ($P < 0.05$, Figure 6B), that were comparable to those in the ischaemic animals 6 days after GT. Also in the intact animals, increases in perfusion correlated with capillary enlargement (Spearman’s Rho correlation coefficient 0.719, $P < 0.01$). Significant formation of tissue oedema was also detected after the AdPlGF GT ($P < 0.05$, Figure 6C). However, despite strong capillary growth, increased perfusion or muscle oedema, the aerobic capacity of non-ischaemic limbs was not altered by AdPlGF nor by AdLacZ ($P = NS$, Figure 6D). Instead, the pH of the AdPlGF-transduced muscles was higher than in the AdLacZ or intact controls during exercise and recovery 6 days after GT ($P > 0.05$, Figure 6E). Additional testing of systemic amounts of lactate and blood pH, using blood gas analysis, before and immediately after exercise also indicated reduced anaerobic glycolysis in the AdPlGF-transduced muscles (data not shown). The AdLacZ GT had no effect on muscle pH ($P = NS$) as determined using $^{31}$P-MRS (Figure 6E).

Discussion

Intramuscular delivery of powerful vector growth factor constructs, such as adenoviruses encoding members of the VEGF growth factor family, can induce growth of the whole vascular tree. Intramuscular delivery of adenoviral growth factor can result in the growth of arterioles and capillaries. Capillary arterialization, a process by which capillaries become functionally similar to arterioles, has been shown to increase microvascular flow and decrease muscle oxygen consumption. However, the impact of capillary arterialization on muscle function remains unclear. In this study, we investigated the effect of adenoviral placental growth factor (AdPlGF) on the aerobic capacity of non-ischaemic limbs.

Our results show that AdPlGF GT induced increases in perfusion and capillary enlargement, similar to those seen in ischaemic animals 6 days after GT. However, the aerobic capacity of non-ischaemic limbs was not altered by AdPlGF nor by AdLacZ. Instead, the pH of the AdPlGF-transduced muscles was higher than in the AdLacZ or intact controls during exercise and recovery 6 days after GT. Additional testing of systemic amounts of lactate and blood pH, using blood gas analysis, before and immediately after exercise also indicated reduced anaerobic glycolysis in the AdPlGF-transduced muscles. The AdLacZ GT had no effect on muscle pH as determined using $^{31}$P-MRS.

In conclusion, our findings suggest that capillary arterialization can improve aerobic capacity and reduce anaerobic glycolysis in non-ischaemic limbs. This is important for understanding the impact of capillary arterialization on muscle function and may have implications for the development of gene therapy for muscle diseases.
induced by GTs of a single growth factor or a growth factor isoform has been questioned.7–9 In this study, we show that the AdPlGF GT improved exercise tolerance of acutely ischaemic rabbit hindlimbs 45% compared with the AdLacZ controls. Additionally, we show that in a normoxic rabbit hindlimb angiogenesis accompanied by capillary arterialization and muscle oedema did not impair aerobic capacity which is very important considering clinical angiogenesis gene therapy trials.

Decreased exercise tolerance and metabolic acidosis under exercise leading to pain and claudication are characteristics of lower limb ischaemia in humans. Previously, very little functional data on animal models concerning therapeutic angiogenesis of lower limb ischaemia have been available. Measurement of capillary density, mean capillary area, or blood flow have often been used as the main endpoints, but the actual effects on tissue energy metabolism are rarely described. In this study, using $^{31}$P-MRS we found decreased aerobic capacity, lengthened recovery after exercise, and more severe acidosis in acutely ischaemic rabbit hindlimbs compared with the intact limbs. $^{31}$P-MRS is a highly feasible, non-invasive method for monitoring bioenergetics of muscles quantitatively. In ischaemic muscles, blood flow is the main limiting factor of exercise tolerance and thus the aerobic capacity can be used to evaluate exercise tolerance. $^{12}$ $^{31}$P-MRS parameters have been previously found to correlated with both runoff resistance and ankle-brachial index in patients with PAD. $^{12}$ Where as treadmill tests are hard to put into practise with large animals and are also strongly influenced by the co-operation of the animal, $^{31}$P-MRS allows objective information on the energy state and function of the muscles. Importantly, $^{31}$P-MRS can also be used in clinical trials to objectively monitor patients after angiogenic gene therapy. $^{10,12}$

PlGF is a member of the VEGF family. It induces angiogenesis through binding to VEGFR-1 and has been reported to be as efficient as VEGF-A. $^{17,18}$ In this study, we report that gene therapy with AdPlGF induced abundant angiogenesis and increased perfusion of acutely ischaemic limbs that resulted in improved aerobic capacity and hence exercise tolerance, and alleviated acidosis of the treated limbs. Similar results on improved aerobic capacity of ischaemic tissues have been reported previously after conventional revascularization therapies such as vascular surgery and percutaneous transluminal angioplasty in patients with PAD. $^{19}$ Additionally, decreased metabolic acidosis found after AdPlGF can be not only due to a shift from anaerobic glycolysis to oxidative phosphorylation in producing energy but also...
improved exportation of metabolic waste products such as lactate and protons from the muscle cells. Despite the improvements in the aerobic capacity, the recovery rate in the AdPlGF-transduced limbs was not improved. However, complete restoration of the recovery rates was not observed with conventional revascularization techniques either, possibly indicating tissue damage as a result of ischaemia. Twenty-eight days after GT, no difference in the aerobic capacity or the recovery rate between the AdPlGF, AdLacZ, or normal muscles was observed due to normalization of blood flow due to endogenous growth of collateral arteries. Also, since the adenoviral transduction is transient no increases in perfusion could be detected in the AdPlGF-transfected animals 28 days after GT.

AdPlGF could not improve the aerobic capacity of normal muscles nor could it increase the aerobic capacity of ischaemic muscles above the level of the intact limbs. These findings are congruent with the knowledge of blood flow not being the limiting factor of aerobic capacity or exercise tolerance in normoxic muscles. This can be partially explained by the fact that blood flow in a normal muscle can be increased manifold during exercise. Additionally, although oxygen supply to the muscle would be improved after an angiogenic GT, the rate of mitochondrial energy synthesis is likely not affected. Furthermore, angiogenic vessels that are maximally enlarged, may not have as strong dilatory potential during exercise as normal vessels and thus flow differences between transduced and normal muscles equalize during exercise as previously observed in the pig myocardium. In other words, AdPlGF in healthy muscles keeps capillaries enlarged and perfusion maximally increased even at rest. This will not improve the aerobic capacity but instead might result in an improved acid-base balance of the muscle as metabolic waste products such as lactate and carbon dioxide are efficiently removed from the tissue. Importantly, capillary enlargement leading to capillary arterialization, formation of artery-venous shunts, oedema or a possible 'stealing effect' on tissue perfusion of strong vascular growth in some parts of the limb induced by AdPlGF in normal muscles did not impair the normal muscle energy metabolism.

In summary, we show that capillary arterialization and muscle oedema induced by angiogenic gene therapy do not impair the energy metabolism of normal muscles. In acutely ischaemic muscles proangiogenic gene therapy even with a single growth factor isoform encoded by a powerful vector significantly improves the aerobic capacity, contributes to improved exercise tolerance, and decreases formation of metabolic acidosis. Thus, this study verifies the functionality of vessels grown with gene therapy and supports the concept of therapeutic angiogenesis for the treatment of patients with impaired exercise tolerance due to ischaemia.

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