Thrombin effectuates therapeutic arteriogenesis in the rabbit hindlimb ischemia model: A quantitative analysis by computerized in vivo imaging

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Abstract

We report on an experimental mammalian controlled study that documents arteriogenic capacity of thrombin and utilizes computerized algorithms to quantify the newly formed vessels. Hindlimb ischemia was surgically invoked in 10 New Zealand white rabbits. After quiescence of endogenous angiogenesis heterologous bovine thrombin was intramuscularly injected (1500 units) in one hindlimb per rabbit (Group T). Contralateral limbs were infused with normal saline (Group C). Digital subtraction angiography (DSA) of both limbs was performed after thrombin infusion by selective cannulation of the abdominal aorta and digital images were post-processed with computerized algorithms in order to enhance newly formed vessels. Total vessel area and total vessel length were quantified. In vivo functional evaluation included measurements of blood flow volume at the level of the external iliac artery by Doppler ultrasonography both at baseline and at 20 days after thrombin infusion. Total vessel area and length (in pixels) were $14,713 \pm 1023$ and $5466 \pm 1327$ in group T versus $12,015 \pm 2557$ and $4598 \pm 1269$ in group C ($p = 0.0062$ and 0.1526, respectively). Blood flow volumes (ml/min) at baseline and at 20 days after thrombin infusion were $25.87 \pm 11.09$ and $38.06 \pm 11.72$ in group T versus $26.57 \pm 11.19$ and $20.35 \pm 7.20$ in group C ($p = 0.8898$ and 0.0007, respectively). Intramuscular thrombin effectuates an arteriogenic response in the rabbit hindlimb ischemia model. Computerized algorithms may enable accurate quantification of the neovascularization outcome.

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1. Introduction

Therapeutic angiogenesis constitutes the frontier of modern cardiovascular research in the battle against coronary and peripheral arterial obstructive disease. Thrombin is a serinoprotease with potent angiogenic properties independent of fibrin formation [1–5]. We report on an in vivo experimental study investigating the angiogenic and arteriogenic capacity of thrombin in a well-established hindlimb ischemia model.

2. Methods

2.1. Study design

The well-known New Zealand White rabbit model was utilized. Hindlimb ischemia was bilaterally induced in the rabbits’ hindlimbs [6,7]. Specific doses of thrombin were intramuscularly injected in one ischemic limb, while an equal volume of normal saline was infused in the respective
contralateral limb in order to serve as a control group. Functional evaluation of the evolving revascularization process was performed non-invasively by color-Doppler ultrasonography at regular time intervals. Quantitative morphological assessment of the angiogenic and arteriogenic outcome was finally performed by digital subtraction angiography (DSA). The Hospital’s local Scientific and Ethical Committee approved the study.

2.2. Hindlimb ischemia model

Study population comprised 10 rabbits in total. The femoral artery was completely excised from its origin from the external iliac artery down to its distal bifurcation into the saphenous and popliteal arteries in both hindlimbs per rabbit. Experienced veterinary staff closely monitored all rabbits during the immediate postoperative period and analgesics were regularly administered.

2.3. Thrombin protocol

The thrombin infusion protocol consisted of random intramuscular injections of heterologous bovine thrombin (Thrombin-JMI, King Pharmaceuticals, Inc. Middleton, Wisconsin) in one ischemic hindlimb per rabbit. A dilution of thrombin to a concentration of 1000 units/ml was prepared. The protocol involved 3 hindlimb injections of 0.5 ml thrombin dilution or normal saline in the medial thigh of the ischemic hindlimbs. The injections were made along the course of the excised femoral artery and medially to the bone at approximately equal intervals of 1–2 cm. Injections were performed at the 20th post-surgical day [6]. Hence, one ischemic hindlimb of each rabbit received 1.5 ml of dilution containing 1500 thrombin units in total, whereas the respective contralateral limbs received 1.5 ml of normal saline and served as the control group.

2.4. Color-Doppler ultrasonography

Color-Doppler ultrasonography and serial blood flow volume measurements were carried out in both hindlimbs (thrombin and control) in order to functionally assess collateral artery growth and estimate peripheral vascular resistance of the distal hindlimb. Evaluation began at the 5th post-surgical day and was repeated at 5-days intervals until the final day of DSA, i.e. the 40th post-surgical day. An ultrasonography device (Envisor, Philips) with a direct-contact high-frequency probe (12 MHz) was used. An average of 6 sequential blood flow volume calculations (ml/min) was recorded each time. An experienced radiologist blinded to the infusion protocol performed all the ultrasonographic examinations.

2.5. DSA acquisition protocol

Hindlimb DSA was performed in order to depict the conduit collateral vessels and obtain a morphological assessment of their growth and development in the median thigh of the hindlimb (i.e. the original location of the femoral artery). Direct intraaortic DSA of both hindlimbs of each rabbit was performed 20 days after delivery of the angiogenic agent, i.e. at the 40th post-surgical day. DSA images were obtained with a Philips DVI-S angiography unit. The acquisition protocol was 1 image per second at 40–90 KV and the contrast agent was infused at a rate of 1 ml/s (5 ml total contrast volume) by an automated angiographic injector. The hindlimbs of the rabbits were positioned approximately 25 cm below the X-ray tube and the focal spot to intensifier distance was 110 cm. In order to achieve field homogenization and increase image quality a 15 cm thick water filter was interposed between the subject and the intensifier. Opacification of the vasculature of the hindlimb was observed on a monitor in real time. Care was taken so that almost every small artery present was visualized and recorded. All digital angiographic images were stored in the angiographic unit database. All animal subjects were sacrificed after completion of DSA.

2.6. Image post-processing

DSA images from the angiographic unit database were transferred to the Medical Physics Department through a high performance LAN (Ethernet, Gigabit). Image post-processing was performed with the aid of Analyze PC 5 and custom-made image processing software under the Windows XP environment. A series of processing operations was performed to enhance the digital images. At first, advanced image-processing techniques were applied in order to increase the signal to noise ratio as proposed in the literature [8,9]. In short, a first median filter smooths the image without blurring it and suppresses the background fog. A second sharpening filter accentuates all edges within the image. Next, the linear histogram equalization function is applied to enhance image contrast and redistribute the gray values of the image across the full range of 256 gray levels. Finally a combination of a ‘weak’ low-pass and a ‘weak’ high-pass filter is employed in order to gain visualization of hidden small vessels and minimize artefacts.

The second step was a morphological evaluation of the vascularity according to the gray level of the individual objects within an image. The gray level reflects the vessel diameter, as the intravascular volume of radiopaque contrast depends proportionally to the square of the vessel radius. Based on this concept, vessels are interactively classified through a threshold operation on gray levels, followed by a ‘scraping’ operation that selects the objects in the image on the basis of their area [9]. The ‘scraping’ operation cuts off irregular gray levels created by overlapping small vessels. The clustering of vessels according to their size was carried out by means of the pixel gray level histogram. Finally, the objects selected were measured regarding their total vessel area and, following a
2.7. Hypotheses and statistics

We hypothesized that intramuscular infusion of thrombin induces angiogenesis and arteriogenesis in chronic ischemic rabbit hindlimbs, which could have potential clinical applications of great value. Computerized measurements of total vessel area and total vessel length were expressed in pixels, each pixel corresponding to 212.77 μm in length. Color-Doppler measurements of blood flow volumes were expressed in ml/min. Quantitative values were formulated as mean ± standard deviation (SD). Paired t-test was applied for all statistical comparisons and the level of statistical significance was set at 0.05.

3. Results

Our two groups consisted of rabbit ischemic hindlimbs injected with thrombin (group T) and normal saline (group C). Color-Doppler measurements of blood flow volume of the feeding hindlimb artery indicated a hemodynamic plateau until day 20 in both groups. However, after administration of thrombin (group T), blood flow volume demonstrated a gradual, almost linear increase, up to day 40, when final DSA was performed. Blood flow volumes (ml/min) at baseline and at 20 days after thrombin infusion were 25.87 ± 11.09 and 38.06 ± 11.72 in group T versus 26.57 ± 11.19 and 20.35 ± 7.20 in group C (p = 0.8898 and 0.0007, respectively).

After DSA acquisition and image post-processing the results of each group were firstly analyzed as a total and then they were divided into two subgroups; one representing small and the other representing big diameter vessels (subgroups Small and Big). The threshold for this clustering was a diameter of 500 μm. The threshold diameter was selected taking into consideration the gray-value histogram modes of the respective images [9]. Thus, all our data were first separated into two groups taking into account the treatment technique (groups T and C) and then each of them into two subgroups taking into account the size of the measured vessel diameter (subgroups Small and Big). Computerized algorithms summed up the total vessel length and area (Fig. 1).

Thrombin achieved significantly higher total vessel area and length when comparing the whole vasculature. After the application of image processing techniques total vessel area and length (in pixels) were 14,713 ± 1023 and 5,466 ± 1327 in group T versus 12,015 ± 2557 and 4,598 ± 1269 in group C (p = 0.0062 and 0.1526, respectively). However, focusing distinctly on the Small and Big subgroups, no statistically significant difference was detected.

4. Discussion

Angiogenesis is defined as the sprouting of new blood vessels by the proliferation and migration of pre-existing fully differentiated endothelial cells (ECs) in response to stimuli such as hypoxia and inflammation. Quiescence of endogenous ischemic-based angiogenesis was estimated to occur by day 20 after hindlimb femoral artery resection and an arteriogenic response with a considerable increase of the limb’s functional reserve flow capacity was detected between days 20 and 40 in absence of resting ischemia and with decreased VEGF levels [6]. In the present experimental study we sought to investigate in vivo the angiogenic properties of thrombin, a documented potent angiogenesis activator since 1993 [1]. To our knowledge this is the first experimental study evaluating thrombin’s angiogenic and arteriogenic capacity in the hindlimb ischemia model [7].

DSA represents the gold standard modality of vascular imaging in a clinical setting and its major advantage is its ability to depict evolution of angiogenesis. In order to increase the resolution scale of in vivo angiographic imaging of angiogenesis several modalities have been proposed [10]. We sought to increase to the spatial resolution of standard everyday DSA with the application of a skeletonization procedure that collapses vessel diameter to one pixel, their total vessel length.
of advanced post-processing algorithms and simultaneously provide a methodology for quantitative imaging of angiogenesis and arteriogenesis. Accurate visualization of at least 100 μm microvessels and moderate precision in identifying and measuring microvessels down to the 50 μm scale has already been demonstrated [9,11].

By applying DSA plus computerized image post-processing algorithms we considerably increased the signal to noise ratio and achieved visualization of the microvessel collateral network. Hence, we document effective angiogenesis and arteriogenesis in the rabbit hindlimb ischemia model by intramuscular injections of heterologous thrombin. We applied color-Doppler ultrasonographic measurements of blood flow volumes to the ischemic hindlimbs in order to functionally evaluate the hemodynamics of the revascularization. Indeed, thrombin effectuated an almost 90% increase of blood flow compared to the control contralateral hindlimbs. Changes in Doppler blood flow have been documented to significantly correlate with vascular density of the vessel’s target tissue and have been proposed as an in vivo imaging technique, which could monitor blood flow continuously and serve as a non-invasive surrogate for histologic vessel density [12].

To synopsize, the computerized quantification methodology of our study has already been validated as reliable and accurate down to the 50-μm scale, which is equivalent to alternative high-tech and high-cost experimental modalities [9–12]. Additional advantages are that it requires minimal additional cost and that radiologists can apply it repeatedly in a clinical setting for monitoring of neovascularization evolution. Its clinical orientation may allow for easy embracement by vascular physicians.

5. Conclusions

Intramuscular heterologous thrombin infusion effectuates angiogenesis and arteriogenesis in the rabbit hindlimb ischemia model. DSA plus computerized image post-processing enables accurate quantification of collateral network development in vivo and color-Doppler imaging provides functional assessment of the arteriogenic outcome.

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References