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Thrombin Augments Therapeutic Angiogenesis in the Rabbit Hindlimb Ischemia Model: A Comparative Angiographic and Dynamic Computed Tomography Study

Konstantinos Katsanos[1], Nikos Tsopanoglou[2], Dimitrios Karnabatidis[1], George C. Kagadis[3], Athanasios Diamantopoulos[1], Nikolaos Siasos[4], John Maroulis[4], Panagiota Ravazoula[5], George Nikiforidis[3], Dimitrios Siablis[1]

Departments of [1]Radiology, [2]Pharmacology, [3]Medical Physics, [4]Surgery and [5]Pathology, School of Medicine, University of Patras, Rio 26500, Greece

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S u m m a r y. Purpose: To evaluate and compare the angiogenic and arteriogenic capacity of thrombin with two well-established angiogenic factors (bFGF, VEGF) in the hindlimb ischemia model of the New Zealand White rabbit. Bilateral hindlimb ischemia was invoked by surgical excision of the femoral artery. On day 20, after quiescence of endogenous angiogenesis, thrombin (500 IU or 1,500 IU or 5,000 IU) or VEGF (1 μg or 3 μg or 10 μg) or bFGF (1 μg or 3 μg or 10 μg) were intramuscularly injected in the medial thigh of one ischemic hindlimb per rabbit (n=7 in each group), while the contralateral limbs were injected with equal volume of normal saline and served as the control groups. On day 40, intraarterial DSA followed by computerized quantitative analysis of the depicted vasculature and dynamic-CT at the level of the saphenous artery were performed. Quantitative DSA detected a significantly increased total area of large collaterals (d>500 μm) only in the thrombin 5,000 IU group (p<0.05). Post-processing of dynamic-CT studies documented increased peak enhancement values and shorter time-to-peak periods in all high-dose groups as compared to control hindlimbs. However, the observed difference proved to be statistically significant (p<0.05) only in the thrombin 5,000 IU group. Histopathology of the adductor muscle in the medial thigh documented a significantly increased neovascularization outcome only in the thrombin 5,000 IU group (p<0.05). Thrombin augments therapeutic angiogenesis and ischemic tissue reperfusion and seems to outperform bFGF and VEGF in establishing new vessel conduits with a developed tunica media in the rabbit model of hindlimb ischemia.

INTRODUCTION

Up to 20% of patients presenting with symptoms of Peripheral Arterial Disease (PAD) suffer from heavy atherosclerotic disease and escape eligibility for either surgical or percutaneous revascularization options (1,2). Modern frontiers in the field of cardiovascular research include the application of pharmacologic agents in order to enhance the endogenous angiogenic and arteriogenic pathways and induce therapeutic angiogenesis. A variety of protein-based, gene-based and cell-based angiogenic agents have so far been investigated in vitro and/or in vivo; intraarterially, perivascularly or intramuscularly; with inconsistent results (3,4). Thrombin is a serinoprotease with potent angiogenic properties independent of fibrin formation (5). Thrombin is implicated in both procoagulant and anticoagulant biochemical cascades and modulates stimulation, proliferation and migration of endothelial and vascular smooth muscle cells (6,7). This is an in vivo experimental study evaluating the angiogenic capacity of Thrombin in the well-known New Zealand White rabbit hindlimb ischemia model. Moreover, we compared the angiogenic and arteriogenic properties of thrombin with those of others well-established growth factors: the vascular endothelial growth factor (VEGF) and the basic fibroblast growth factor (bFGF).

METHODS

Bilateral hindlimb ischemia was induced in 63 rabbits (male sex, weight 2.5-3.5 kg and approximately 6 months of age) by means of surgical excision of the femoral artery as described elsewhere (8). After quiescence of endogenous angiogenesis, which takes place approximately 20 days post-surgery (8), different doses of the agents under study were randomly administered in the animals (Table 1). The delivery protocol involved the intramuscular injection of 3 equal amounts of each agent (total volume of dilution: 1ml) into the medial thigh of one hindlimb, whereas the respective contralateral limbs received equal amounts of normal saline (control group).

Table 1
Groups of animals/agents

Agent	Dosage	No of animals	Dosage	No of animals	Dosage	No of animals
bFGF	1 μg	7	3 μg	7	10 μg	7
VEGF	1 μg	7	3 μg	7	10 μg	7
Thrombin	500 IU	7	1,500 IU	7	5,000 IU	7

Digital subtraction angiography (DSA) was performed 20 days after injection of the agents (40th post-surgical day) in order to depict the collateral arterial network and morphologically assess its growth and development. The acquired images were post-processed by the use of sophisticated image analysis algorithms (9). Vessels were interactively classified into two groups: >500 μm and <500 μm, through a threshold operation on gray levels. Focusing in the arteriogenesis process, we only evaluated collaterals with a diameter bigger than 500 μm. In addition, with a purpose to functionally evaluate the hemodynamic result of the newly developed arterial network we performed dynamic-CT at the level of the saphenous artery in the high-dose group of each agent. We calculated the peak enhancement values of the intraarterial space in Hounsfield units and the required time period for the signal to reach the peak value (time-to-peak). The findings were compared to the findings of the contralateral hindlimb, which served as control. Finally, the subjects were sacrificed and histopathological analysis of the adductor muscle in the medial thigh was performed.

RESULTS

After computerized quantification of the total vessel area and length of the large collaterals (d>500 μm) we detected a statistically significant increase up to 30% compared to the control hindlimb only in the high dose group of thrombin (5000 IU). An increase of almost 15% was also detected in the high dose bFGF group (10 μg), which, however, failed to reach the level of statistical significance. Complete results are shown in Graph 1.

Regarding the functional estimation of the blood flow distal to the excised femoral artery, a statistically significant increase of the peak enhancement (up to almost 100%) as compared to the contralateral control hindlimb was observed only in the high dose thrombin group (Figure 1). Although an up to 50% increase was also observed in the high dose groups of the VEGF and bFGF, neither of these differences proved to be statistically significanct. In addition, shorter time-to-peak periods were documented for both the high dose Thrombin and bFGF groups, but only in the thrombin group did the difference exceed the threshold of statistical significance. Histopathological analysis revealed a statistically significant increase in the number of the arterioles present in the adductor muscle again only in the high-dose thrombin group (Graph 2).

DISCUSSION

Angiogenesis is defined as the sprouting of new blood vessels by the proliferation and migration of pre-existing fully differentiated endothelial cells in response to stimuli such as hypoxia and inflammation. New vessels lack the development of tunica media and are typically very small and fragile (3). In contrast, arteriogenesis is identified as the active remodeling and maturing of pre-existing arterioles into large conductance arteries on the basis of elevated pressure and frictional shear forces and irrespective of ischemia (10). Only developed collaterals have the ability to influence vascular resistance and can adequately compensate for severe arterial blockages (3). In this pilot experimental mammalian controlled study and with the scope to evaluate the arteriogenic phenomenon we focused in the quantitative evaluation of collaterals greater than 500 μm. We observed a more pronounced arteriogenic outcome in the high dose thrombin group. We documented significantly effective angiogenesis and arteriogenesis, as well as augmentation of ischemic tissue reperfusion by intramuscular infusion of thrombin. We also documented that thrombin outperformed bFGF and VEGF in establishing new vessel conduits with a developed tunica media in the rabbit model of hindlimb ischemia. Future directions will be the establishment of the most appropriate dose regimen of thrombin in order to achieve the optimal arteriogenic result.

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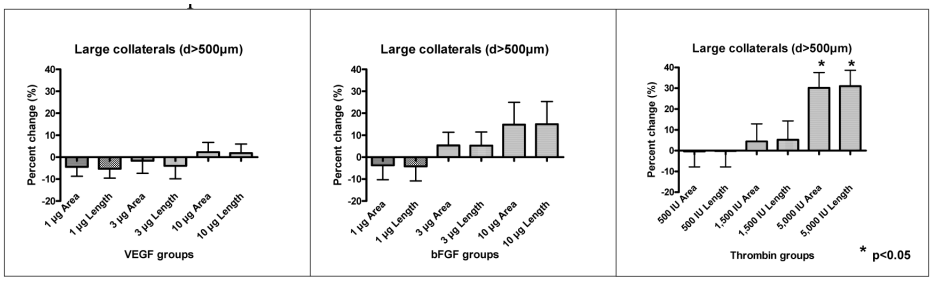
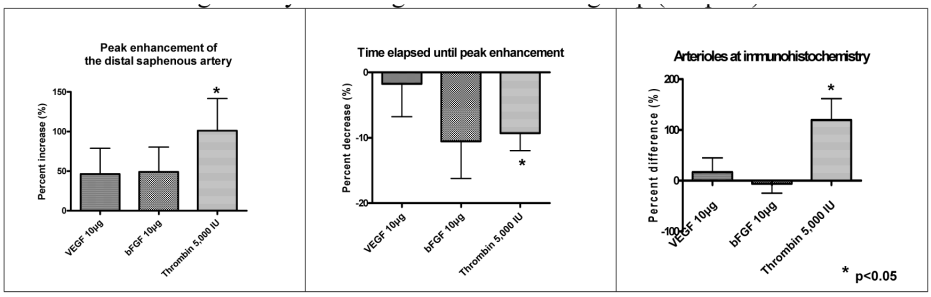


Graph 1. Collateralization results after computerized quantification of DSA images



Graph 2. Results of dynamic-CT and histopathological analysis

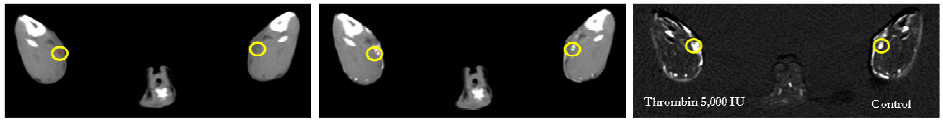


Figure 1. Example of dynamic-CT evaluation. Cross-sectional images at baseline (left image), at peak enhancement (middle image) and after digital subtraction CT of the above (right image). Note the higher enhancement at the level of the distal saphenous artery (asterisks within the yellow circles) in the hindlimb which received an injection of 5,000 IU thrombin as compared to the control ischemic hindlimb