Early in vivo anticoagulation inhibits the angiogenic response following hindlimb ischemia in a rodent model

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Summary
Emerging findings have demonstrated the critical role of blood clotting factors in the formation and stabilization of embryonic blood vessels. Whether a similar role is true during post-natal angiogenesis remains to be determined. Here we sought to determine whether the suppression of thrombin generation with anticoagulant drugs at doses routinely used for therapeutic purposes would affect the angiogenesis pattern following hindlimb ischemia in rats. Animals were treated with r-hirudin or enoxaparin within six hours post induction of hindlimb ischemia, whereas two other groups received oral anticoagulation warfarin beginning at day 3 post-ischemia or saline (as control). The revascularization anatomical and functional responses were evaluated 30 days following tissue ischemia. Chronic administration of the drugs resulted in stable anticoagulation in all animals throughout the experiment. Animals that received drugs with fast anticoagulation effects (i.e. r-hirudin and enoxaparin) presented a significant decrease in capillary density and capillary-to-myocyte ratio compared to control animals. These effects were not associated with changes in relative perfusion of the hindlimb at steady state. These anti-angiogenic effects occur in a time-dependent manner, since delayed inhibition of coagulation (> 72 hours) presents no adverse effect on the angiogenic response. We conclude that the use of anticoagulant drugs immediately after tissue ischemia induction hampers in vivo angiogenic response in a rodent hindlimb ischemia model.

Keywords
Angiogenesis, warfarin, hirudin, heparin, anticoagulant

Introduction
In the last two decades, advances in the understanding of the molecular and cellular events associated with the development of the cardiovascular system made possible the use of vascular growth factors with the aim to increase vascular supply to ischemic tissues. These strategies, collectively known as “therapeutic angiogenesis”, have been already evaluated in phase II clinical trials, and are attractive alternatives for the treatment for patients with arterial occlusive disease (AOD) (1).

More recently, growing evidence demonstrated the essential role of blood clotting factors and their receptors during the normal process of embryonic vascular development. For example, mice engineered by disruption of endogenous procoagulant factors such as prothrombin and factor V, protease-activated receptor-1 (PAR-1) or tissue factor presented embryonic mortality due to bleeding and/or lack of vascular integrity (2–6). Furthermore, detailed in vitro studies have also demonstrated the role of thrombin in different critical steps of angiogenesis (7).

Taken together, these data emphasize the importance of thrombin in angiogenesis during embryonic development, and raise questions on whether there would be an analogous role for thrombin in situations when adult angiogenesis is upregulated such as inflammation, tumour development, and in AOD.

To date, there is limited information on the in-vivo role of thrombin in adult angiogenesis. However, a series of in-vitro studies clearly demonstrated that drugs with different anticoagulant effects and molecular structures such as heparin, heparin-derivatives and hirudin (a thrombin-specific inhibitor) all present inadvertent anti-angiogenic responses that do not depend on the anticoagulant effect. Moreover, inhibition of vitamin K epoxide reductase expression in human umbilical vein endothelial cells
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(HUVEC) by treatment with antisense oligonucleotides also inhibits angiogenesis (8). Based on these findings, we sought to determine whether the use of these common anticoagulant drugs would affect the parameters of therapeutic angiogenesis. Because patients with AOD are likely to require anticoagulant therapy when enrolled in clinical studies on therapeutic angiogenesis, we carried out in-vivo experiments on the effect of anticoagulant drugs in the hindlimb ischemia model in rats.

Materials and methods

Hindlimb ischemia model
All animal experiments were approved by the local Animal Experiments Ethical Committee, and complied with the regulations of the Brazilian Council of Animal Experimentation. The hindlimb ischemia model was performed as previously described (9). Briefly, eight-week-old Lewis rats were anesthetized by intravenous pentobarbital (30–50 mg/kg). All branches of the right femoral artery distal to the inguinal ligament were ligated, and the whole artery was surgically excised to induce limb ischemia. Rats were fed standard rat chow ad libitum, and were evaluated for clinical signs of ischemia according to a previously published clinical ischemia index (10) (abnormal gait, pale feet, pressure sores or tissue necrosis) at days 7, 14, and 28.

Anticoagulation protocol, blood samples and tissue collection
Animals were allocated to one of the three treatment groups: 1) sodium warfarin (Zest Farmaceutica, Rio de Janeiro, Brazil) 0.2 mg/kg diluted in distilled water, given orally from day 3 to day 30 after surgery (n=6); 2) recombinant hirudin (r-hirudin) (Lepirudin, Hoechst Marion Roussel, Kansas City, MO, USA) 2.5 mg/kg s.c. from day 1 (six hours after surgery) to day 30 after surgery (n=5); and 3) the low-molecular-weight heparin (LMWH) enoxaparin (Aventis Pharmaceuticals Inc., Bridgewater, NJ, USA) at the fixed dose of 1,500 U/kg s.c. from day 1 (six hours after surgery) to day 30 after surgery (n=5). Preliminary studies on the use of warfarin prior to hindlimb ischemia were associated with an unacceptable high mortality rate due to surgical bleeding (n=2/2). Similarly, attempts in targeting specifically factor Xa, by injecting tick anticoagulant peptide (kindly provided by Dr. Krishwasnamy, University of Pennsylvania) by intravenous or subcutaneous routes, proved complicated because of the drugs short half-life (<15 min) and high risk of surgical bleeding. In preliminary experiments, the r-hirudin regimen used in the study resulted in an aPTT 1.5- to 2.5-fold longer than baseline (four hours after each dose), which was the target aPTT for r-hirudin-treated animals. Control animals (n=6) received water on the same schedule as warfarin. Blood samples (270 µl of whole blood) were collected into 1.5-ml plastic tubes containing 3.2% sodium citrate solution by tail vein puncture on days 7, 14, 21 and 28 for adjustment of anticoagulant dose from control and warfarin-treated animals. The anticoagulation target level was a prothrombin time (PT) two- to three-fold longer than baseline. The PT and aPTT target levels were chosen for being well established levels of anticoagulation in clinical settings. Two additional blood samples were also collected from r-hirudin-treated animals on days 7, 14 and 28. Capillary density and capillary-to-myocyte ratio
Capillary density was evaluated to determine the angiogenesis response in vivo, represented by proliferation of small capillary vessels in the ischemic hindlimb, would be affected by day 30, all animals were anesthetized as described above, and 3.5 ml of whole blood was collected into 3.2% sodium citrate glass tubes by abdominal aortic puncture. After centrifugation, samples were stored at ~80°C until analysis. Animals were then sacrificed by intravenous infusion of 200 mg/kg of pentobarbital, the abdominal aorta was catheterized, and the hindlimbs were perfused with 20 ml of a 500 U/ml saline solution of sodium heparin to prevent post mortem clot formation. The semimembranosus muscles of the ischemic limbs were harvested, embedded in optimum cutting temperature compound (Sakura Finetek Inc., Torrance, CA, USA), snap-frozen in liquid nitrogen cooled n-hexane, and stored at ~80°C. The semimembranosus muscle was chosen, because i) it is the major muscles of the medial thigh, and ii) it was originally perfused by the deep femoral artery, ligated at the time that the femoral artery was excised (11).

Blood coagulation parameters
The PT assay was performed using 50 µl of sample mixed with 37°C pre-warmed thromboplastin (Simplastin Excel S; BioMérieux Inc., Durham, NC, USA). The aPTT assay was performed using 50 µl of sample, mixed with 50 µl of aPTT reagent (Platelet LS; BioMérieux Inc.). Both assays were performed in a coagulometer (Coag-A-mate MTX; Organon Teknika, Turnhout, Belgium). FVII clotting activity was determined on a similar PT-based assay using 50 µl sample mixed with 50 µl of human FVII deficient plasma (Dade-Behring, Marburg, Germany) at 37°C. FII, FIX and FX clotting activities were determined by aPTT-based assays performed using 50 µl of sample, mixed with 50 µl of human FII (Diamed AG, Switzerland), FIX or FX deficient plasma (Dade-Behring) and 50 µl aPTT reagent (Platelet LS; bioMérieux Inc.). Heparin concentration in enoxaparin treated animals was determined by an anti-FXa based assay (Accucolor Heparin; Sigma, St. Louis, MO, USA).

Hindlimb relative perfusion evaluation
To evaluate the effect of anticoagulant drugs on limb perfusion parameters of treated and control animals, we performed 99mTc-sestamibi scintigraphy of both hindlimbs on day 28, as previously described (12). Briefly, anterior and posterior static images were obtained under pentobarbital anesthesia five minutes after an intravenous injection of 55 MBq (1.5 mCi) of 99mTc-sestamibi using a two-head gamma-camera. Regions of interest were drawn over both hindlimbs on the anterior and posterior images for uptake quantification, and the contra lateral hindlimb was used as a reference. The mean count of the anterior and posterior was calculated, and the ratios between ischemic and normal hindlimbs (I/N) were calculated for each animal. An examiner who was blinded to which group the images belonged performed the evaluation. This method has been previously correlated with blood flow measurements using 15 µm fluorescent microspheres (data not shown), which further support the use of such methodology to assess parameters of limb circulation.
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Figure 1: Coagulation parameters of warfarin-treated and control rats. A) Prothrombin time (PT) ratios for control (dotted line; n=6) and warfarin-treated animals (bold line; n=6) before and at several time points after hindlimb ischemia. B) At day 30, all vitamin K-dependent procoagulant factors were significantly down-regulated in warfarin-treated animals compared to controls (P < 0.05; Mann-Whitney test). Results expressed as percentage of the mean value obtained in control rats ± SEM.

The anticoagulant drugs. On day 30 of the experiment, 7 µm-thick cryosections of the midportion of the semimembranosus muscle from the ischemic hindlimb were obtained and stained for alkaline phosphatase with an indoxyl-tetrazolium method as previously described (13). Briefly, samples were stained for 1 h at 37°C in a solution of nitroblue tetrazolium (Sigma-Aldrich Co., Saint Louis, MO, USA) 30 mg, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma-Aldrich Co., Saint Louis, MO, USA) 6 mg, in 30 ml of a buffer containing 6.9 mM MgSO₄ and 27.5 mM NaBO₃ adjusted to a pH between 9.2 and 9.4 with boric acid. After rinsing and a five-minute post fixation with sucrose (300 mOsml; Sigma-Aldrich Co.) buffered formalin solution (Accustain; Sigma-Aldrich Co.), sections were counterstained with 0.5% eosin. This histochemical staining allows the visualization of capillary endothelial cells in skeletal muscle sections and has been extensively used for the quantification of capillary density in skeletal muscle (14, 15). The muscle sections were then analyzed under 50X magnification, and consecutive fields from each section were digitalized (Kontron KS 300 V2.00 digital imaging system, Munich, Germany), so that the whole area of the section was evaluated (mean number of fields counted per section = 8.2). Image analyses software (Scion Image Beta 4.02 for Windows; Scion Corporation, USA) was used to count the number of capillaries per section, after results in 10 random fields agreed with those obtained with manual counting. Capillary-to-myocyte ratio was also evaluated to prevent an overestimation of capillary proliferation secondary to uneven myocyte atrophy in the experimental groups. The number of myofibers was manually counted in five random fields per tissue section per animal. Finally, the mean number of capillaries/mm² (capillary density) and the capillary-to-myocyte ratio was calculated. Again, the observer was blinded as to which treatment group each animal belonged to.

Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). The statistical significance of differences in the study was evaluated by non-parametric tests (Kruskal-Wallis test or Mann-Whitney test). Differences were considered statistically significant for p-values ≤0.05.

Results

Continuous treatment with warfarin, r-hirudin or enoxaparin resulted in sustained and clinically relevant anticoagulant effect in rats

In warfarin treated animals, PT values ranged from 1.39– to 2.87-fold longer than baseline values by day 7 (baseline PT of six untreated animals was 14.7 ± 0.4 seconds). With a further increase of 25–30% in the warfarin dose per kilo, an anticoagulant effect was uniformly obtained, with long-term maintenance doses ranging from 0.25 mg/kg to 0.37 mg/kg daily (Fig. 1). At day 30, PT values were consistently two-fold higher among treated animals compared to controls, and vitamin K-dependent (VKD) procoagulant protein (factor II, VII, IX and X) levels were significantly reduced (ranging from 2.3– to 6-fold) in all treated animals when compared to controls (Fig. 1). In r-hirudin treated animals, aPTT values obtained four hours after the last dose were consistently 1.5–2.7 longer than baseline values (baseline aPTT six untreated animals was 13.2 ± 0.7 seconds) at day 7 (26.2 ± 2.8 seconds) and day 14 (26.6 ± 1.4 seconds). In enoxaparin treated rats, a mean heparin level of 0.50 ± 0.04 U/ml was obtained at day 30. One animal in the enoxaparin group experienced a minor subcutaneous hematoma in the injection site, and one animal in the r-hirudin group died with diffuse bleeding in the third week of the experiment. An additional animal died of anesthetic complications immediately after the 99mTc-sestamibi scintigraphy evaluation (enoxaparin group).

Effective anticoagulation does not influence functional parameters of the physiological revascularization response that follows hindlimb ischemia in rats

Following the initial surgical removal of the right femoral artery, signs of ischemia were observed in all animals by day 7. These findings slowly returned to normal by day 14, and at day 28 clinical signs of ischemia were completely reversed in all treated and control animals. There were no differences between the groups regarding the pattern of resolution of these signs. In accordance with this data, no difference in relative perfusion was detected at day 28 between animals chronically treated with warfarin (I/N ratio=0.76 ± 0.02; n=6), r-hirudin (I/N ratio=0.72 ± 0.04; n=4) or enoxaparin (I/N ratio=0.81 ± 0.04; n=5) when compared to control animals (I/N ratio=0.72 ± 0.03; n=6).
Early-onset of thrombin inhibition hampers the angiogenic response in the rat hindlimb ischemia model

The capillary density determined for r-hirudin (181.79 ± 7.83 capillaries/mm²; n=4) and enoxaparin (164.83 ± 25.78 capillaries/mm²; n=4) treated animals were significantly lower than that determined for the control group (273.57 ± 25.01 capillaries/mm²; n=6) (P=.001 and 0.04 respectively; Mann-Whitney test). In agreement with these observations, the capillary-to-myocyte ratio in r-hirudin (0.56 ± 0.05) and enoxaparin-treated animals (0.71 ± 0.06) were also significantly lower than in control animals (0.99 ± 0.06). In contrast, no statistically significant difference could be demonstrated between warfarin and control animals when these two parameters were evaluated (Fig. 3).

Discussion

The mechanisms by which thrombin participates in angiogenesis have been well documented in mice deficient in blood coagulation proteases, PAR-1 or tissue factor (2–6). Moreover, a series of detailed in-vitro experiments demonstrated the essential role of thrombin in several steps of angiogenesis required for the development of a fully functional blood vessel (16–23). Here we sought to determine whether down-regulation of blood coagulation would affect parameters of post-natal angiogenesis responses in adult rats undergone to acute hindlimb ischemia. The main finding of our work was that inhibition of thrombin and/or FXa generation in vivo resulted in poor proliferation of small capillaries when angiogenesis is initiated within the first few hours following angiogenic stimuli. Interestingly, no adverse effect on the angiogenic response was detected by delaying the anticoagulation for 72 hours. The time-dependent anti-angiogenic effect could be demonstrated between warfarin and control animals when these two parameters were evaluated (Fig. 3).

Figure 2: Determination of the blood flow by 99mTc-sestamibi scintigraphy following hindlimb ischemia. Relative perfusion expressed as the ratio between the ischemic and contra lateral hindlimb (I/N). At day 28, skeletal muscle perfusion ratios were similar in all treatment groups compared to control animals (P=0.28; Kruskall-Wallis test; n=5 for warfarin and control and n=4 for r-hirudin and enoxaparin groups). Results are expressed as means ± SEM.

Figure 3: Histochemical analyses of muscle sections at day 30 after hindlimb ischemia. Representative sections of ischemic semimembranosus muscle (7 µm) stained by alkaline phosphatase of control, warfarin, r-hirudin and enoxaparin treated animals (Magnification 50X) showing a clear decrease in capillary proliferation among r-hirudin and enoxaparin treated animals, as demonstrated in the bar graph. Results are expressed as means ± SEM; n=6 for control and warfarin groups and n=4 for r-hirudin and enoxaparin groups.
translating this novel therapeutic, i.e. therapeutic angiogenesis, to humans.

The advantage of the current in-vivo model is also demonstrated by experiments with warfarin. When administered 72 hours after ischemia induction, no inhibitory angiogenic effect was documented in animals with sustained long-term anticoagulation. However, Wang et al. recently reported that in vitro the down-regulation of vitamin-K epoxide reductase by antisense therapy inhibits HUVEC proliferation independent from the down-regulation of VKD clotting factors (8). Thus, in the presence of warfarin, the therapeutic angiogenic response could be comprised even in the absence of anticoagulant effect. It is possible that warfarin intake prior to the induction of hindlimb ischemia would affect negatively the angiogenic response as well. Further experiments monitoring levels of vitamin-K epoxide reductase post-warfarin intake during hindlimb ischemia will be required.

The hindlimb ischemia model has been used to assess revascularization of ischemic skeletal muscle in vivo. In this model, the partial restoration of blood flow that follows hindlimb ischemia is dependent on both angiogenesis (the sprouting of new capillaries from pre-existing vessels) and arteriogenesis (the development of larger and more stable collateral vessels) (28). Here, in addition to an anatomical method that evaluates in-vivo angiogenesis represented by proliferation of small capillaries, we took advantage of a non-invasive technique to assess the blood flow to the ischemic limb. This method allows the evaluation of whole depth muscle resting perfusion. In our study, impaired angiogenesis represented by decreased capillary proliferation was not associated with decreased perfusion in animals receiving early anticoagulation. The major limitation of this method is that all tests are carried out under resting rather than challenged by exercise conditions in the hindlimb ischemia model. It has also been shown that isometric exercise (usually induced by electric stimulation of the ischemic muscles) is capable of unmasking the blood perfusion deficit in the model (29, 30). In addition, these animals do not present chronic occlusive vascular disease that will likely influence the ability of restoring blood flow reserve. Similar experiments using animal models with abnormal blood vessels, such as diabetic, senile and atherosclerosis-prone animals, would be necessary to formally exclude this possibility.

In conclusion, we have shown that the use of anticoagulant drugs at the time of induction of angiogenesis in hindlimb ischemia model hampers the pro-angiogenesis response. Therefore, the clinical outcome of protein- or gene-based early-phase pro-angiogenesis trials, which typically consist of testing subtherapeutic or low doses, could be compromised by concurrent use of these anticoagulant drugs.

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References