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Novel Injury Site Targeted Fusion Protein Comprising Annexin V and Kunitz Inhibitor Domains Ameliorates Ischemia-Reperfusion Injury and Promotes Survival of Ischemic Rat Abdominal Skin Flaps

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Abstract

Appropriate antithrombotic therapy is critical for successful outcomes in reconstructive microsurgical procedures involving free tissue transfer. The annexin V-6L15 (ANV-6L15) fusion protein was developed as a targeted antithrombotic reagent. Annexin V specifically binds to exposed phosphatidylserine on apoptotic or injured cells, and prevents coagulation and cell adhesion, whereas 6L15 inhibits tissue factor-VIIa pathway within the coagulation cascade. The treatment efficacy of ANV-6L15 on rat island muscle and pedicled abdominal fasciocutaneous flaps following ischemic injury and ischemia-reperfusion injury (IRI) was evaluated.

Materials and Methods: The effects of ANV-6L15 on survival of rat abdominal fasciocutaneous flaps subjected to 10 hours of critical ischemia were assessed on day 5. Near-IR imaging was applied to evaluate the distribution of ANV-6L15 and flap perfusion. The rat cremaster muscle island flap was used to evaluate the effect of ANV-6L15 on IRI-induced leukocyte-endothelial interactions via intravital microscopy. 2,3,5 triphenyl-tetrazolium chloride assay was used to determine the ratio between live-versus-dead tissue.

Results: ANV-6L15 significantly increased the ratio of viable tissue ($68.5 \pm 9.79\%$ vs $84.8 \pm 5.14\%$, $P < 0.05$), and promoted survival of rat pedicled abdominal flaps (59.3 ± 6.86 vs. 47.0 ± 8.67 , $P < 0.05$). Intravital microscopy demonstrated a significant decrease in the number of adhesive leukocytes (1.8 ± 1.64 vs. 10.0 ± 6.32 , $P < 0.05$), and the percentage change of functional capillaries (16.4 ± 15.1 vs. 47.3 ± 18.3 , $P < 0.05$) in ANV-6L15-treatment group.

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Conclusions: ANV-6L15 promoted survival of ischemic rat cremaster muscle and abdominal fasciocutaneous flaps and ameliorated leukocyte-related IRI. Future evaluation of potential clinical application of ANV-6L15 is warranted as a flap treatment adjunct.

Keywords

annexin v; ischemia-reperfusion injury; anticoagulant

Free tissue transfers using microvascular techniques may encounter unexpected complications that lead to ischemia. Without intervention, global ischemia and complete loss of the flap may occur.¹ Even after revascularization, the prolonged hypoxic injury and ischemia-reperfusion injury (IRI) may still lead to partial or complete flap failure.

Ischemia-reperfusion injury amelioration by administration of free radical scavengers and anti-inflammatory agents has been demonstrated in the microcirculation and animal models.^{2,3} Reversible thrombosis at the microvascular and venous levels is the consequence of both ischemic-hypoxic injury and IRI.⁴⁻⁶ To prevent thrombosis-related ischemic flap failure, thromboprophylaxis using platelet inhibitors, normovolemic hemodilution with dextran and hydroxyethyl starch, and anticoagulation with heparin or direct thrombin inhibitors is a common strategy.⁷ Heparin was shown to act upon antithrombin III, and consequentially attenuated the local effects of IRI on muscle flaps.^{8,9} In addition, the therapeutic benefits of classical anticoagulants seem to be a combined effect of their anticoagulant activity and non-anticoagulant, anti-inflammatory behavior. For example, heparin is proposed to work through complex anti-inflammatory pathways, such as nitric-oxide pathway or the complement cascade.^{10,11} Although systemic anticoagulation generally benefits flap survival, it increases the risk of bleeding.

Annexin V (ANV) is a cellular protein that binds to phosphatidylserine (PS) and phosphatidylethanolamine (PE) in a Ca^{2+} -dependent manner.¹² During cell injury, PS and PE translocate to the cell membrane surface, and lead to acceleration of the coagulation cascade. Binding to ANV to shield the exposed PS and PE molecules and to inhibit thrombosis has been proposed.¹³

ANV-6L15 is one of a series of fusion proteins between ANV and a member of Kunitz protease inhibitor (KPI) proteins,¹⁴ which inhibits serine proteases in the coagulation cascade. ANV-KPIs were demonstrated to specifically bind to PS/PE exposed membranes via the ANV domain.¹² Among them, ANV-6L15 possessed the ability to efficiently inhibit the tissue factor (TF)/VIIa complex at thrombogenic sites.¹⁵ This molecule has been demonstrated to reduce IRI in a rat myocardial infarction model and was 12-fold more potent than human TF pathway inhibitor in plasma clotting assay in vitro.¹⁶

In this work, ANV-6L15 was applied to the ischemic rat fasciocutaneous and muscle flaps. It is hypothesized that it may function as a targeted anticoagulant to promote flap survival while avoiding the detrimental effects of systemic anticoagulation. Additionally, the possible mechanisms of ANV-6L15 treatment were investigated with intravital microscopy and near-IR imaging.

MATERIALS AND METHODS

Animals

A total of 39 Lewis rats were used. Care and surgical procedures complied with Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23) and were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital.

Recombinant ANV-6L15

ANV-6L15 was cloned into the expression vector pET20b(+) (Novagen, Madison, Wis) and expressed in *Escherichia coli* BL21(DE3) pLysS, and the recombinant protein was purified as previously described.¹⁴

Abdominal Fasciocutaneous Flap Model

Male Lewis rats (10 to 12 weeks old) weighting 350 to 400 g were anesthetized with isoflurane. The superior and inferior borders of the flap were a transverse line at the level of the xyphoid process, and at the level of the suprapubic line, respectively. The left and right borders were marked at the midaxillary line. A fasciocutaneous flap based on the left superficial inferior epigastric arteries (SIEA) and proximal femoral system was elevated. The arteries were clamped to induce ischemia. The flap was then sutured back to the wound bed, and animals were allowed to wake and feed normally. After 10 hours of ischemia, rats were anesthetized again, and the inguinal region was opened to remove the vessel clamps.

A total of 24 rats were used (n = 6, 4 groups). The control (vehicle) group was subjected to 10 hours of ischemia without treatment. The sham group received the operation and immediate inset of the flap onto the wound bed with no further ischemia. The posttreatment group received 4 dosages of ANV-6L15 (200 µg/kg) through the penile vein at 12-hour intervals beginning immediately after starting reperfusion. For the pretreatment group, a half dosage was given before and immediately after flap elevation. Three dosages were given after starting reperfusion at 12-hour intervals. The flap necrotic area was evaluated on day 5. Image-J (National Institutes of Health, USA) was used to calculate the area of necrotic tissue versus the total flap size. Necrosis was determined grossly by signs of desquamation, hair loss, skin hardening, and eschar formation.

Cremaster Island Flap Model

Male Lewis rats (4 to 6 weeks old) weighing 200 to 250 g were anesthetized with urethane intraperitoneally based on a dosing regimen of 1000 mg/kg, and if required, incremental doses of 50 mg until hind limb pain reflexes were undetectable. The cremaster muscle and external fascia were exposed with a surgical incision from the anterior iliac spine to the tip of the scrotum. The cremaster muscle was dissected out by blunt dissection. Fascia superficial to the cremaster muscle was cleared under a stereomicroscope. The cremaster muscle was then opened along its least vascular sector (ventral side). The testicle and its appendages were separated from their dorsal connections while preserving the testicular artery and vein, and then reduced into the abdomen. The cremaster muscle was dissected out as an island flap attached to the body through a vascular pedicle that included the pudic-epigastric artery and vein. Finally, it was laid out upon a slide mounted on an in-house

designed acrylic stage and fully spread out by means of five 4-0 silk sutures. The exposed tissue was superfused with normal saline through a temperature controller (Warner Instruments, USA) to prevent dessication throughout the study.

After establishing the island flap model, a 30-minute stabilization period was allowed. During this period, 4 potential postcapillary venules in the central sector of the flap were located and mapped out. Each venule was then recorded for 3 minutes to provide baseline data. Ischemia-reperfusion injury was induced by clamping of the pedicle with a microvascular clamp for 4 hours, followed by 2 hours of reperfusion. After reperfusion, the 4 venules were observed and the venule with the best return of flow was recorded.

A total of 15 rats were used ($n = 5$, 3 groups). The control group was subjected to 4 hours of ischemia and 2 hours of reperfusion without treatment. The ANV-6L15 treatment group received 1 dose of ANV-6L15 (200 $\mu\text{g/kg}$) through the penile vein immediately after starting reperfusion. For the ANV group, 1 dose of ANV (200 $\mu\text{g/kg}$) was given through the penile vein immediately after starting reperfusion.

Intravital Microscopy

Cremaster microcirculation was observed with a Leica MZ16 stereomicroscope (Germany) using a Leica KL2500 light source (Germany) under 200 $\times$ magnification. Recordings were made with a digital camera connected to the side view port of the stereomicroscope. Offline analysis was performed using QuickTime Player 10.

Observation of Leukocyte-Endothelial Interactions and Functional Capillary Density

Several parameters were recorded within the postcapillary venules. They were between 25 to 40 μm in diameter, at least 100 μm in length, and did not have branches in and out of the recorded section. Rolling leukocytes were defined as those moving slower than red blood cells. Adherent leukocytes were defined as those that were stationary for more than 30 seconds along an observed stretch of 100 μm , whereas transmigratory cells were defined as those located exterior to the vessel but within a 50- μm perpendicular distance from the stretch of vessel. Functional capillary density was determined as the total number of functional capillaries in 3 adjacent high-powered fields.

2,3,5-Triphenyltetrazolium Chloride Assay

To evaluate the survival of the cremaster island flap, a 2,3,5-triphenyltetrazolium chloride (TTC) assay was performed. The island flap was divided at the pedicle, and the skeletal muscle flap was incubated in TTC solution (Sigma-Aldrich, USA) at 37°C for 30 minutes to differentiate nonviable tissue (white staining) from viable tissue (brick-red). The tissues were then blotted dry and spread out on acetate sheets.¹⁷ Planimetry was performed using a Canon EOS 5D and Image J to determine the area of viable tissue.

Imaging Study

A novel near-IR imaging agent, Squarticle (patent pending, trademark pending)¹⁸ with specificity for blood vessel visualization was used to observe the vascular perfusion of the abdominal fasciocutaneous flaps at 36 hours after reperfusion. Briefly, after shaving and

depilating the ventral abdominal wall, 1 mL of imaging agent was injected as a bolus through the penile vein. The animal was immediately imaged with the Pearl Impulse system (LI-COR Biosciences, USA). A fluorescent probe, LS288 [14] was conjugated to ANV-6L15 for the study of distribution and specific binding to injury sites. The ANV-6L15/LS288 conjugate with a probe-protein molar ratio of 0.81 was synthesized by a 2-step EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide)/sulfo-NHS coupling process recommended by the manufacturer (Thermo Fisher Scientific, Waltham, Mass). The conjugate (200 µg/kg) was injected through a jugular vein cannula after clamping the SIEA for 30 minutes, and the fluorescence distribution was observed for up to 24 hours.

Prothrombin Time/Activated Partial Thromboplastin Time

Prothrombin time (PT)/activated partial thromboplastin time (aPTT) assay was performed to test for systemic effects of the ANV-6L15. Plasma samples were collected from the ventral abdominal flap group on day 2. Samples collected from the rats treated with unfractionated heparin at 300 µ/kg served as the positive control. Samples were stored at -80°C until testing. Prothrombin time/activated partial thromboplastin time was acquired by the Ceveron alpha (Technoclon GmbH, Austria) automated blood coagulation analyzer with a sample size of 300 µL.

Statistical Analysis

All data are represented as mean ± standard deviation. One-way analysis of variance was used to compare the results for the abdominal fasciocutaneous flap model and PT/APTT tests. Kruskal-Wallis test was used to compare the results for the cremaster flap model due to the non-parametric nature of the data. Post hoc comparisons were performed with Tukey HSD test to identify significant pairs. Statistical analysis was performed using Sigmaplot ver. 12 (Systat Software, USA). Probability less than 0.05 ($P < 0.05$) was considered significant.

RESULTS

Treatment with ANV-6L15 improved the viable flap areas for both flap models (Table 1). Sham group demonstrated a consistent survival pattern (Fig. 1). Both pretreatment and posttreatment groups demonstrated an improvement of approximately 15% in flap survival, although no significant difference was noted between the pair.

Leukocyte-endothelial (LE) interactions were evaluated by intravital microscopy. The treatment group had significantly less newly adherent leukocytes and better preservation of functional capillaries. Annexin V did not show significant effects on either flap survival or LE interaction (Fig. 2).

Vascular perfusion was evaluated with near-IR imaging in the abdominal flap. At 36 hours postoperative, the vascular system can be fully visualized in the sham group with the imaging agent. For the 2 treatment groups, decreased perfusion can be noted at the distal periphery of the flaps. In contrast, the zone of decreased perfusion was greater in the control vehicle group (Fig. 3). To visualize distribution of ANV-6L15 and demonstrate the targeting behavior, conjugated agent of ANV-6L15 and fluorescent probe was administered. Relative

enhancement at the vessel clamp site was noted up to 24 hours compared to surrounding vessels (Fig. 4).

Systemic effects of ANV-6L15 were evaluated with PT/aPTT assay. Heparin was used as a positive control and prolonged the aPTT significantly to 1.5 fold ($P<0.05$). No differences were noted after sham or ANV-6L15 treatment (Fig. 5).

DISCUSSION

In this study, ANV-6L15 showed significant effects on flap survival after ischemia. The extended abdominal fasciocutaneous flap was a random-axial flap based upon SIEA. The sham group exhibited a consistent survival pattern with minimal edge necrosis, demonstrating that our flap design was feasible for this study. No wound dehiscence was noted even with superficial eschar formation. Control group (no treatment) showed a much larger variation in terms of necrotic area, ranging from 40 to 65%. ANV-6L15 rescued distal regions of the flap, and improved perfusion was observed by day 1. This may be attributed to the improvement in early flap perfusion and attenuation of microvascular thrombosis, which rescued zones of borderline flow. In fact, the necrotic or rescued areas followed the distribution of choke zones usually supplied by superior or contralaterally based vessels. Since no flaps went through total pedicle failure, the effects of ANV-6L15 on pedicle thrombus formation cannot be determined in this study.

A significant decrease in the LE system activation with ANV-6L15 was noted, indicating mitigation of IRI. Rapid return of flow and improvement in the microvasculature reflow may have contributed to the decrease in adhesion and loss of functional capillaries. Shear forces are a critical factor in the rolling phase of leukocytes, which is contributed by blood flow.¹⁹ Early prevention of IRI in flaps may help to prevent the detrimental cascade of events that result from capillary plugging and no reflow phenomenon.²⁰ Similar mechanism was suggested by improving of IRI with ischemic preconditioning.²¹

The direct effect of ANV-6L15 was observed by near-IR imaging on vessel perfusion. Squarticle, a near-IR vessel imaging agent was injected to demonstrate the zones of preserved vascularity in the abdominal-fascial flaps. In the sham group, clear vessel architecture could be noted throughout most of the flap with enhancement of the intermediate zones. For both treatment groups, the zone of perfusion extended well past the midline with visible vessels on the contralateral side. In the control group, limited perfusion was noted, mostly limited to the direct axial distribution of the ipsilateral SIEA. For all flaps, a larger zone of perfusion was observed compared to the final surviving flap area, indicating perfusion insufficiency in these areas. Prolonging ANV-6L15 therapy or increasing the dose may provide a solution to this issue. In short, data from our study suggest that the benefits of using ANV-6L15 include amelioration of IRI and promotion of perfusion to distal zones.

The fusion protein ANV-6L15 was created under the premise that the targeted binding of ANV to injured endothelial cells caused by anastomosis, vessel clamping, or ischemic/hypoxic injury would facilitate the inhibition of membrane-associated coagulation complex by 6L15.¹⁴ In addition to anticoagulatory behavior, ANV-6L15 also directly inhibits TF/VIIa

in the TF pathway. Downstream events of TF activation include a TF/VIIa/Xa-mediated protease-activated receptor cell signaling and a thrombin-related inflammatory response that leads to endothelial cell activation, chemokine expression, and leukocyte infiltration. Thrombin contributes to inflammation and tissue injury through the activation of protease-activated receptor-1 signaling, which results in the expression of proinflammatory cytokines, chemokines, and adhesion molecules.²² Application of TF monoclonal antibody was shown to be cardioprotective following myocardial IRI, reducing infarct size by up to 61% in rabbits.²³ Through this mechanism, the targeted inhibition of ANV-6L15 to sites of TF expression may exert an anti-inflammatory effect that promotes survival of the flap.

The usual goal of anticoagulation in flap surgery is thromboprophylaxis. Besides preventing thrombus occlusion of the pedicle, the postoperative hypercoagulable state and injury to the flap initiated by ischemia and IRI may lead to partial loss of the flap through downstream thrombus formation or microvascular emboli.²⁴ Clinically, systemic postoperative heparinization with unfractionated heparin is commonly used, although the risk of systemic bleeding is also increased. Often overlooked is the anti-inflammatory behavior of classical agents such as aspirin or heparin. As previously mentioned, heparin possesses non-anticoagulant properties that are anti-inflammatory.¹⁰ Antithrombin, a direct target of heparin, ameliorated hepatic IRI by working through the COX-1 pathway.²⁵ In contrast, enoxaparin, a Factor Xa inhibitor, was shown to be ineffective in treating limb IRI.²⁶ The introduction of ANV-6L15 and the concept of targeted anticoagulation offers a potentially comprehensive alternative in terms of mechanism of action to current post-operative regimens with the additional benefit of targeted delivery. ANV-6L15 had been administered to mice, rats and rabbits by intravenous bolus injections at 2.5 to 250 µg/kg multiple times in various preclinical models. At these dose ranges, there were little perturbation of coagulation parameters in circulating blood and no apparent increase of surgical bleeding, suggesting that ANV-6L15 may be quite safe for therapeutic applications.

Although ANV did not demonstrate an effect on IRI or cremaster island flap survival in this study, diannexin, a recombinant homodimer of ANV was effective in prevention of LE interaction related to IRI. This was attributed to the increased molecular size and higher affinity for PS binding sites than ANV monomer.²⁷ Interestingly, similar to ANV-6L15, no demonstrable effect on leukocyte rolling was seen with diannexin use. It can be speculated that ANV itself possesses some therapeutic potential although it did not reach an observable level in the current system. The fusion protein concept allows the use of ANV and therapeutic protein agents to successfully promote flap survival.

Clinically, the first 2 postoperative days present the highest risk for thromboses.²⁸ Up to 96% of surgeons use anticoagulants, usually a combination between aspirin, dextran, unfractionated heparin, or low-molecular-weight heparin, in free flap procedures.²⁹ The site-directed anticoagulants provide an alternative to current pharmacological options. Furthermore, ANV-6L15 and its relatives can be considered in different scenarios such as preplanned surgery, unexpected reopenings, traumatic flaps, replantations, and other situations where targeted anticoagulation is potentially beneficial.³⁰

CONCLUSIONS

ANV-6L15, a novel vascular injury site-targeted anticoagulant improves the survival of 2 models of ischemic flaps while avoiding systemic anticoagulation. The possible mechanisms include improving early perfusion, anti-inflammation, and mitigation of IRI, especially in terms of LE interactions. Further studies should be designed to investigate the potential of this fusion protein for clinical use.

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FIGURE 1. Modified abdominal flap model at day 5 postoperative. A, Sham. B, Pretreatment (partial loss at viewer's right upper distal border due to chewing, considered as ischemic region). C, Posttreatment. D, Control.

FIGURE 2.

Change in LE interactions after reperfusion (n = 5). A significant difference was noted for number of increased adhesive leukocytes and percentage decrease in functional capillary density ($P < 0.05$).

FIGURE 3.

Near-IR perfusion imaging with novel perfusion imaging agent (Squarticle) at 36 hours postoperative. Fluorescent regions indicate zones of perfusion. Decreased perfusion was noted in the control group. A, sham; B, pretreatment; C, posttreatment; D, control. White line indicates outer border of abdominal flap.

FIGURE 4.

Distribution of ANV-6L15 to the circulation can be noted by 2 minutes via near-IR imaging of fluorescent ANV-6L15. Positive targeting of ANV-6L15 to sites of endothelial injury is also visible at the clamp trauma site (enhanced fluorescent intensity, indicated by white arrow). The enhanced binding is visible at up to 24 hours. A, Anatomy of the region (notched arrow: SIEA; arrow: groin flap); B, 2 min; C, 1 hour; D, 24 hours following fluorescent ANV-6L15 administration.

FIGURE 5.

PT/aPTT test results ($n = 5-6$). Heparin treatment prolonged the aPTT level to approximately 1.5 times normal range, and was significantly different to all other groups ($P < 0.05$).

TABLE 1.

Viable Flap Areas for Abdominal and Cremaster Muscle

Planimetry for modified abdominal flap survival (n = 6)			
	Sham	Pretreatment	Posttreatment Control
Viable flap (%)	84.7 ± 4.63 *	59.3 ± 6.86 *	61.2 ± 6.88 * 47.0 ± 8.67
2,3,5 TTC assay for cremaster island flap survival (n = 5)			
	Control	Treatment	Annexin V
Viable flap (%)	68.5 ± 9.79	84.7 ± 5.14 *	70.9 ± 8.61

Viable flap areas for both flap models. For both groups, a significant difference was noted through ANOVA analysis. Post hoc tests with Tukey HSD demonstrated significant pairs in the abdominal flap group except between the pre-treatment and posttreatment groups ($P < 0.05$ for control vs pretreatment; $P < 0.01$ for all other pairs). In the cremaster flap study, Tukey HSD demonstrated significance for all pairs ($P < 0.05$) except control vs. Annexin V.

* Significant difference vs control group.
ANOVA, analysis of variance.