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Bolus injections of novel thrombogenic site-targeted fusion proteins comprising annexin-V and Kunitz protease inhibitors attenuate intimal hyperplasia after balloon angioplasty

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ABSTRACT

Background: Systemic administrations of conventional antithrombotics reduce neointima formation after angioplasty in experimental animals. However, clinical translation of these results has not been successful due to high risk for bleeding.
Objectives: We sought to determine whether novel annexin-V (ANV)-Kunitz protease inhibitor fusion proteins, TAP-ANV and ANV-6L15, can specifically target to vascular injury site and limit neointima formation without inducing systemic hypo-coagulation in a rat carotid artery balloon angioplasty injury model.
Methods: Near infrared imaging was carried out after balloon-injury and injection of fluorescent ANV or ANV-6L15 to examine their bio-distributions. For peri-procedure treatment, TAP-ANV or ANV-6L15 was administered as i.v. boluses 3 times: 30-minutes before balloon-injury, immediate after procedure, and 120-minutes post-balloon-injury. For extended treatment, additional i.v. bolus injection was given on day-2, day-3 and every other day thereafter. Carotid arteries were collected on day-7 and day-14 for analysis. Blood was collected for measurement of clotting parameters.
Results: Near infrared imaging and immunochemistry showed that fluorescent ANV and ANV-6L15 specifically localized to injured carotid artery and significant amount of ANV-6L15 remained bound to the injured artery after 24-h. Peri-procedure injections of TAP-ANV or ANV-6L15 resulted in decrease of intima/media ratio by 56%. Extended injections of both yielded similar results. Both decreased the expression of PCNA on day-7 and increased the expression calponin on day-14 in the intima post-balloon-injury.
Conclusions: TAP-ANV and ANV-6L15 can specifically localize to balloon injured carotid arteries after i.v. bolus injections, resulting in substantial attenuation of intimal hyperplasia without inducing a state of systemic hypo-coagulation.

1. Introduction

Arteries [1–3] and veins [4–5] respond to injury by a sequential healing process that includes an acute thrombotic phase (minutes to days), a sub-acute neointima formation phase (weeks to months), and a late vascular remodeling phase (months to years). Such healing process suggests that the mural thrombus formed at the site of vascular injury assumes an important role in subsequent neointima formation by providing a biodegradable matrix with abundant chemokines/cytokines/mitogens into which smooth muscle cell proliferate and elaborate matrix. These concepts led to extensive search for antithrombotic agents to limit intima hyperplasia and restenosis, among of which, however, were mostly ineffective [6–19]. Subsequent studies using genetic [20–23] or pharmacological [24–33] manipulation showed that inhibition of TF/FVIIa by recombinant tissue factor pathway inhibitor (rTFPI) or inactivated FVIIa (FVIIai) were more effective in reducing intimal hyperplasia and restenosis than inhibition of thrombin or FXa by r-hirudin and r-tick anticoagulant protein (r-TAP). However, systemic pharmacological inhibition of TF/FVIIa required continuous infusion of very high doses of rTFPI (25 μg/kg per min for 3 or 14 days) to reduce intimal hyperplasia and restenosis [24,25,28]. Such treatment regimens were

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impractical for clinical application and would render patients at high risk for bleeding.

A series of novel anticoagulant fusion proteins each comprising an annexin-V (ANV) domain linking to a Kunitz Protease Inhibitor (KPI) domain were developed recently [34]. The fusion proteins possess the following distinctive properties: 1) Upon i.v. bolus injection, the fusion proteins can specifically localize to sites of vascular injury due to Ca^{++}-dependent high-affinity binding to phosphatidylserine (PS) on the membrane surfaces of damaged, activated, stressed and apoptotic/necrotic cells [34,35]; 2) the fusion proteins bind to the PS-exposed membranes via the ANV domains and greatly enhance the inhibition of coagulation enzyme/cofactor complexes on the membrane surfaces by the KPI domains. Previous in vitro studies have suggested the fusion proteins may promote endothelial cell membrane repair and reduce inflammation in addition to anti-coagulation [36–39,40,41]. In this study, we hypothesize that fusion proteins with such unique multi-functional properties may potently inhibit thrombo-inflammation at sites of vascular injury, resulting in subsequent attenuation of neointimal hyperplasia without inducing a state of systemic hypo-coagulation.

2. Materials and methods

2.1. Recombinant TAP-ANV and ANV-6L15

TAP-ANV and ANV-6L15 were cloned into the expression vector pET20b(+) (Novagen, Madison, WI, USA) and expressed in *Escherichia coli* BL21(DE3)pLysS as described before [34]. The recombinant proteins were purified by chromatography on Q-Sepharose™ fast flow (GE Healthcare, Piscataway, NJ).

2.2. Rat carotid artery balloon injury model

Animal 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. Adult male Wistar rats weighing 350–400 g were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). Angioplasty of the left external carotid artery was performed using an inflated 2F Fogarty embolectomy catheter. The balloon was inflated with saline (~0.05 ml) with inflation pressure ~2.0 atm. For histology and immunofluorescence study (Figs. 3A, B, 4B and D), the rats were administered with (1) vehicle (PBS), $n = 15$, (2) TAP-ANV at 25, 50 or 100 μg/kg respectively ($n = 15$ for each group) and (3) ANV-6L15 at 25, 50 or 100 μg/kg respectively ($n = 15$ for each group). For PCNA immunofluorescence (Fig. 4A and C), additional rats were treated with PBS (vehicle), TAP-ANV (50 μg/kg), or ANV-6L15 (50 μg/kg) respectively ($n = 10$ for each group). The regimens were given three times via tail-vein bolus injections at 30 min before balloon procedure, immediate after procedure, and 120 min post-angioplasty. After surgery, the treated rats were sacrificed on day 1 for Near infrared (near-IR) fluorescence imaging and immunofluorescence (Fig. 1), on day 7 for immunofluorescence (Fig. 4A and C) and on day 14 for histology and immunofluorescence (Figs. 3A, B, 4B and D) respectively.

For the study of extended treatment (Fig. 3C and D), the rats were administered with (1) vehicle (PBS), $n = 10$, (2) TAP-ANV at 50 μg/kg ($n = 10$) and (3) ANV-6L15 at 50 μg/kg ($n = 10$) respectively by bolus injections via tail-vein for a total of 10 times, including 3 peri-procedure bolus injections plus one bolus injection each on day 2, 3, 5, 7, 9, 11 and 13. The rats were sacrificed on day 14 after surgery.

2.3. Morphometric measurement

The injured segments of the artery were collected and fixed in 4% paraformaldehyde. Then the middle one-third portion of the samples was embedded in paraffin and transverse histological sections (5 μm) were made from each segment and stained with hematoxylin-eosin. Three discontinuous sections (2 mm apart) from each vessel were measured in a rat. Morphometry was performed using a video microscope. The cross-sectional area of the neointima, media and the ratio of the areas of neointima to media (I/M) were measured using IMAGE-PRO PLUS (Media Cybernetics Inc.).

2.4. Immunohistochemistry

The method and quantification of immunohistochemical staining were performed as previously described [48,49]. Immunohistochemical staining was performed using anti-annexin V-Ig (Abcam, USA), anti-calponin-Ig (Dako, Carpinteria, CA), or anti-PCNA-Ig (Abcam, USA) as primary antibodies and Cy3- (red) conjugated secondary antibodies (Chemicon, Temecula, CA), and examined by confocal immunofluorescent microscopy. For quantification, a basic Cy3 intensity was set as threshold to disrupt background noise. The Cys-positive area was calculated and compared to the mean value of control group as relative folds of inductions. All images were processed and analyzed using MetaMorph software (Universal Imaging Corp., West Chester, PA, USA). The results presented from 3 random sections of injured or non-injured arteries of individuals of every experiment group.

2.5. Blood coagulation test

The rats ($N = 6$) received a bolus injection of PBS, heparin (100 U/kg, i.v.), TAP-ANV (50 μg/kg, i.v.) or ANV-6L15 (50 μg/kg, i.v.); then 9 volume of blood was collected into 1 volume of acid-citrate-dextrose at 5, 30, 60, 120 min after injection for measurement of Activated Clotting Time (ACT). Plasma samples were separated by centrifugation at 2000*g* for 15 min for measurement of Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) using GEM PCL® Plus coagulation system (Instrumentation Laboratory, Milano).

2.6. Near infrared (near-IR) fluorescence imaging

A cypate fluorescence probe, LS288 [42], was conjugated to ANV or ANV-6L15 by a two-step EDC/sulfo-NHS coupling process recommended by the manufacturer (Thermo Fisher Scientific, Waltham, MA). ANV-LS288 and ANV-6L15-LS288 conjugates had probe:protein molar ratio of 0.59 and 0.81, respectively. Rats received bolus injections of ANV-LS288 (50 μg/kg) or ANV-6L15-LS288 (50 μg/kg) after balloon injury of the carotid arteries. Fluorescence imaging was performed after 8 h using Pearl® Impulse system (Li-COR Biosciences, Lincoln, NE).

3. Statistical analysis

All values were expressed as the mean ± SEM. Unpaired Student's *t*-test for two groups and one-way ANOVA with post hoc Tukey's tests for multiple comparisons were applied. A value of $P < 0.05$ was considered to be significant.

4. Results

4.1. Binding of ANV and ANV-6L15 to the balloon-injured carotid artery

Previous studies showed that both ANV and ANV-6L15 bound specifically to anionic phospholipids exposed on the membrane surfaces of damaged, activated or apoptotic cells [35,43]. In this study, we conjugated a near-IR fluorescent probe, LS288, to ANV and ANV-6L15 and investigated the binding of these conjugates to the balloon-injured carotid artery in vivo. Fig. 1A showed near-IR fluorescence imaging of the carotid arteries 8 h after balloon angioplasty and injection of ANV-LS288 or ANV-6L16-LS288. ANV-LS288 specifically bound to the injured, but not uninjured, carotid artery 8 h after balloon injury. ANV-6L15-LS288 yielded higher fluorescence intensity than ANV-LS288, partly due to higher avidity of ANV-6L15 for anionic phospholipids than ANV [43]. ANV-6L15 also appeared to have stronger avidity for heparan sulfate proteoglycans on endothelial glycocalyx than ANV (unpublished results), which might account for the higher background fluorescence. The balloon-injured carotid arteries were sectioned and immuno-stained with anti-ANV antibody and Cy3-second antibody (red color). Fig. 1B showed greater extent of ANV-6L15-LS288 binding to the balloon-injured carotid artery than ANV-LS288.

The carotid arteries of rats were subject to balloon angioplasty and treated with vehicle, or a bolus of ANV-6L15 (50 μg/kg) via tail-vein bolus injection. After 24 h, the rats were sacrificed and the uninjured and balloon-injured carotid artery sections were immuno-stained with anti-ANV antibody and Cy3-conjugated second antibody (red). Fig. 1C showed that the uninjured carotid artery (Ctrl) barely stained red color, the PBS-treated/balloon-injured carotid artery (vehicle) lightly stained red, and the ANV-6L15-treated/balloon-injured carotid artery (ANV-6L15) stained red more intensely. These results suggested that a significant amount of ANV-6L15 remained bound to the balloon-injured carotid artery 24 h after bolus injection. Thus, despite relatively short initial circulating half-life ($t_{1/2}\alpha < 2$ min) [35], ANV-6L15 quickly bound to sites

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