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Chapter 8

RGD-ALBUMIN CONJUGATE INHIBITS PLATELET AGGREGATION AND ATTENUATES THROMBUS GROWTH IN A CAROTID ARTERY THROMBOSIS MODEL

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Submitted
Summary

Free RGD-peptides have the potential to inhibit platelet aggregation. However, due to their small size they have a short half-life limiting their usefulness as a medical treatment in atherosclerotic diseases. In an attempt to prolong its half-life, a specific cyclic RGD-peptide was conjugated to human albumin. To test, if platelet inhibitory activity of the conjugate is maintained \textit{ex vivo}, platelet aggregation in rabbit platelet rich plasma, was first measured \textit{in vivo} for the evaluation of the IC$_{50}$, then \textit{ex vivo}. In addition, the conjugate was evaluated in a thrombosis model of the rabbit carotid artery using the ferric chloride method. Thrombosis was assessed by blood flow measurement and histology. Doses of 3.5 mg/kg and 7.5 mg/kg of the conjugate were administered intravenously before thrombus induction and compared to the effect of aspirin and to physiological saline. For platelet inhibition \textit{in vivo} an IC$_{50}$ of the conjugate was found of 1.8 \textmu M. \textit{Ex vivo}, effective inhibition of platelets was still detectable after two hours. Thrombus growth was prevented in 60\% of the animals treated with the 7.5 mg/kg of the conjugate, whereas the lower dose (3.5 mg/kg) failed to show an effect. In conclusion, RGD-albumin conjugate is effective as a platelet inhibitor one and two hours after administration with a 20-to 30-fold prolongation of half-life compared to free peptide.
**Introduction**

Circulating platelets in blood have the potential to adhere and aggregate to the injured endothelium of the arterial and venous wall (1) and, thus, play a crucial role in the formation of cardiovascular and thromboembolic diseases (2, 3). Aggregating platelets become activated and release various substances like ADP, serotonin, coagulation factor V, and thrombin, that will enhance activation of other platelets and trigger the local coagulation system at the site of an endothelial lesion (4,5).

Inhibition of platelet aggregation is, therefore, the target of many pharmacological strategies in the treatment of patients with cardiovascular disease or stroke. Aspirin and other salicylates are frequently used as antiplatelet agents prescribed in patients suffering from progressive atherosclerosis. However, aspirin is not always successful in the prevention of atherosclerotic events, because many fatal events such as myocardial infarction or stroke occur in patients, despite treatment with aspirin. Also, some patients cannot be treated with aspirin, because they suffer from drug intolerance including gastrointestinal side-effects. Moreover, some patients do not respond to the arachidonic pathway inhibition and are therefore not sufficiently protected in secondary prevention for arterial thrombosis (6).

Therefore, a number of new platelet inhibiting agents have been developed. Among these, RGD-peptides are small molecules containing the amino acid sequence Arg-Gly-Asp, which is identical with the sequence of the fibrinogen binding site on the platelet membrane, the glycoprotein IIb/IIIa receptor (GP IIb/IIIa). Platelet aggregation depends critically on fibrin(ogen) binding to activated platelets via the fibrinogen receptor GP IIb/IIIa. RGD-peptides, binding competitively to GP IIb/IIIa, have been shown to be potent inhibitors of platelet aggregation (7-11). However, the clinical application of RGD-peptides has been limited due to their short half-life, which is only a few minutes. Therefore, we constructed a specific cyclic RGD-peptide which was conjugated to human albumin, resulting in a 30-fold prolongation of its half-life compared to free RGD-peptides (Burkhardt et al, in preparation). In the present study we demonstrate, that RGD-HSA conjugate maintains its platelet inhibiting potential in a rabbit carotid artery thrombosis model with an effect comparable to that of aspirin.

**Material and Methods**

*RGD-HSA*

Human albumin was chosen as a carrier for a specific cyclic RGD-peptide mainly for three reasons: first because of its long half-life of 19 days, secondly because it is a natural molecule present in human blood, and thirdly it is easily available. Modelling and synthesis of this
conjugate are described in detail by Burkhardt et al (submitted for publication). Principally, for the peptide, a cyclic analogue of the fibrinogen platelet receptor binding sequence was synthesized and coupled to cysteine in position 34 of loop 1 of human albumin. This position enabled access for the peptide to bind to albumin through a spacer molecule, (sulfo-SMCC, Pierce), without losing its platelet inhibiting capacity in vitro and with an IC₅₀, similar to that of free peptide. The RGD-HSA-conjugate was produced in amounts of 50 to 80 mg and stored as a dry-frozen powder.

Platelet aggregation
Platelet aggregation was standardized in normal rabbit platelet rich plasma (PRP) of New Zealand White rabbits containing 250'000 plt/μl. Platelets were activated by 5 μMol ADP (final concentration), and aggregation was measured using a light transmission aggregometer. Calibration was performed with platelet poor plasma (PPP) for 100% light transmission and with PRP for the 0% line. Aggregation curves were registered on a flow chart for at least 4 minutes or until a steady state plateau was reached. Aggregation was expressed in percent (%) of the maximal value of non-treated healthy controls. The total sample volume of 250 μl consisted of 225 μl PRP, 12.5 μl of ADP, and 12.5 μl of either RGD-HSA solution or NaCl 0.9%. A dose response curve was performed to in vitro to evaluate the dose needed to inhibit 50% of maximal aggregation (IC₅₀), using 0.3, 1.0, 3.0, 10, and 30 μM final concentration RGD-HSA per sample.

The ex vivo measurements were performed before and 5, 60, and 120 minutes after intravenous injection of RGD-HSA or NaCl in the acute thrombosis experiments (see below). Blood samples were drawn from an ear artery into calcified tubes (sodium citrate 3.2%)

Acute arterial thrombosis model
Animal experiments were approved by the Institution Review Board for Animal Experiments of the Academic Medical Center and performed according to the guidelines of the American Physiological Society and the Dutch Law for Animal Experiments.

To test the antithrombotic effect of the RGD-HSA conjugate, we used the ferric chloride (FeCl₃) method for thrombosis induction in the carotid artery. This method was established in other rodents before (12-14), and to our knowledge, has now been applied in the rabbit carotid artery for the first time. New Zealand white rabbits (± 2.5 kg) were anesthetized with 0.5 ml xylazine 2% (Rompun, Bayer AG, Leverkusen, Germany) and 9 mg ketamine/atropin (Aescoket plus, Boxtel, The Netherlands) intramuscularly. A repeat injection of xylazine was administered after 30 minutes, followed by repetitive intravenous injections of thiopental-sodium (Nesdonal) to maintain anesthesia. Body temperature was kept between 36 and 37.5 °C throughout the experiment, using a heating lamp. After a median ventral neck incision, both carotid arteries were
bluntly isolated. A catheter (Baby Feeding Tube, 1.6 mm Ø) was inserted into an ear artery and a second one into an ear marginal vein. Both accesses lines were kept patent with saline. A transonic flow probe (Transonic Systems, Maastricht, The Netherlands) was placed at the distal part of the isolated carotid arteries, and blood flow was measured on both sides simultaneously. To register arterial pulsation at steady state, a short switch from the average to the pulsatile mode on the flowmeter was performed for a few seconds at high paper chart speed. A stripe of filter paper (3 mm width), which was soaked in a 70% solution of FeCl₃, was carefully wrapped around the carotid artery proximal to the flow probe for exactly 5 minutes. However, when applying the method in the first six animals, we found that concentrations up to 70% did not reliably induce thrombosis in every case, as evaluated by blood flow stop. Assuming that clot formation might be prevented in the rabbit carotid artery by the specific hemodynamic conditions, such as a higher average blood flow (15-35 ml/min, i.e. 10 times higher than in mice), and/or a larger vessel diameter, and/or a thicker arterial wall, we decided to add a second factor of Virchow’s triad, namely stasis, in addition to endothelial inflammatory injury. Thus, after 5 minutes of steady state, a vessel clamp was placed proximal to the flow probe and left there for 10 minutes. Immediately after placing the vessel clamp, the filter paper, now soaked with a 35% solution of FeCl₃, was placed again on the artery for 5 minutes. After removal of both, filter paper and vessel clamp, restored blood flow was registered again, and time between setting the stimulus and the first occurrence of ceased blood flow (≤ 0.2 ml/min) was defined as first time to occlusion (TTO₀) as a first endpoint parameter. As occlusion was sometimes followed by a phase of alternating spontaneous recanalisation and reocclusion, final time to occlusion was defined as TTOₙ₀, representing a second endpoint.

Ten minutes following full vessel occlusion, 4.5 mg recombinant tissue-type plasminogen (rtPA, Actilyse) and 300 anti-Xa units of low molecular weight heparin (Fraxiparine) were administered intravenously as an infusion over 10 minutes. Time to recanalisation (TTR) was defined as the time elapsing between start of the lysis and restoration of the blood flow (≥ 1.0 ml/min) within 60 minutes of observation time. When lysis was studied, the same steps were performed 60 minutes after lysis start.

Study design

Animals were treated with antiplatelet agents 20 minutes prior to the application of FeCl₃ with the following doses: 5.0 mg/kg ASA (n = 11), 10 mg/kg ASA (n = 11), 3.5 mg/kg RGD-HSA (n = 4), 7.5 mg/kg ASA (n = 4), physiological saline (n = 8). When only thrombosis formation was studied, the experiment was ended with the excision of the 2 segments of the carotid arteries and an overdose of thiopentalsodium.
**Histological assessment**

The excised carotid artery segments were stored in 10% formaldehyde solution for at least one week and imbedded in paraffine blocks for histological slide processing. Clot and vessel wall analysis was performed from haematoxilyn/eosin stained slides under a conventional light microscope by an independent, blinded investigator.

**Data analysis**

Values for TTO and platelet inhibition are expressed as means ± SEM. For experiments in which two variables were compared, the unpaired Student’s t test was used. Contingency analysis using Fisher’s exact test was performed to test for differences in occurrence of occlusion between the two animal groups. Data were considered as significantly different when p < 0.05.

**Results**

**Platelet inhibition in vitro and ex vivo**

*In vitro* measurements of platelet aggregation as evaluated in a dose-response curve, showed an IC$_{50}$ of 1.8 µMol, for the RGD-HSA conjugate, with an 80% inhibition at a 10 µMol, concentration, and practically full inhibition (8%) at 30 µMol (Figure 1).

![Figure 1](image)

*Figure 1*  *In vitro* measurements of platelet aggregation after administration of RGD-HSA albumin, a dose-dependent inhibition of platelet aggregation was found with an IC$_{50}$ of 1.8 µM.*
Based on the *in vitro* data, doses of 3.5 mg/kg and 7.5 mg/kg RGD-HSA were injected to the rabbits in the acute arterial thrombosis experiments, to achieve an at least 50% inhibition of platelet aggregation *in vivo*, these doses correspond to an estimated plasmatic final concentration of 1 and 2 μM, respectively. Maximally achieved platelet inhibition after administration of 7.5 mg/kg RGD-HSA at 5 minutes was 65%, decreasing to 41% after 60 minutes and 35% after 2 hours (Figure 2). Minutes after administration of 7.5 mg/kg RGD-HSA conjugate (n = 4)

![Figure 2](image_url)

*Figure 2* Platelet inhibition 60 minutes after administration of 7.5 mg/kg RGD-HSA conjugate was still 40% and was only 5% less pronounced after two hours, suggesting a lasting effect of at least several hours (the extrapolated half-life from this curve was approximately 24 hours.

**Thrombus growth**

Flow characteristics and Heart Frequency

Mean basal blood flow before thrombus induction after 10 minutes resting ranged from 19.8 to 28.9 ml/min and did not differ significantly between comparison groups. When thrombosis was induced and blood flow ceased (lower than 0.2 ml/min), a compensatory increase of blood flow was seen in the contralateral artery of 20-30%.

**Time-to-Occlusion and Time-to-Recanalization**

When thrombosis was induced without preliminary injection of a platelet inhibiting agent, mean \( TTO_{\text{fast}} \) was 23.6 minutes, \( TTO_{\text{fast}} \) 27.6 minutes, and recanalization following intervention occurred in only 2 cases (TTR, 10 and 55 minutes, respectively). In the groups treated with
ASA 3 mg and 5 mg, TTO\textsubscript{first} was not significantly increased with 32.7 and 31.9 minutes, respectively, as well as TTO\textsubscript{last} (39 and 44.8 minutes, respectively). Occlusion was seen in all of these animals within 60 minutes of observation time. In the group receiving ASA 10 mg (n = 8) only one transitory occlusion occurred at 33 minutes, followed by spontaneous recanalization at 42 minutes and full patency by the end of the observation time. In the RGD-HSA 7.5 mg group TTO\textsubscript{first} was 39.3 and TTO\textsubscript{last} 43.5 minutes with 2 of 6 animals staying patent, whereas the low dose of RGD-HSA had no effect on TTO\textsubscript{first} and TTO\textsubscript{last}, as presented in Figure 3. None of the occluded vessels in the RGD-HSA group could be recanalized interventionally.

**Figure 3** Treatment with 7.5 mg/kg RGD-HSA conjugate was as efficient as treatment with 5 mg/kg ASA regarding TTO\textsubscript{first} and TTO\textsubscript{last}, with a statistically significant effect (p = 0.02) compared to controls. This effect was not seen in animals treated with only 3.5 mg/kg RGD-HSA conjugate, suggesting a dose-dependent effect of the conjugate.

**Histology**

There was a full conformity between registered flow stop and presence of a thrombus in all groups. Thrombi consisted of red blood cells and fibrin strands with mostly a granulocyte influx.
Thrombocytes could not reliably be identified in haematoxylin/eosin staining. Consistency of thrombi between treatment groups did not reveal any differences in terms of fibrin strands or cellular parts.

**Discussion**

RGD-peptides, known as potent fibrinogen receptor inhibitors, are not suitable for clinical application, because of their short half-life. Therefore, we linked the small agents to recombinant HSA using a new method, as reported in the previous chapter, and tested its *in vitro* and *ex vivo* effect on platelet inhibition and thrombus growth. The data presented indicate that dose-dependent platelet inhibition *in vitro* revealed an IC$_{50}$ of 1.8 μMol. For the *ex vivo* experiments, RGD-HSA was administered in a 2 μMol plasmatic final concentration for the assessment of platelet inhibition resulting in an initial (5 minutes after administration) inhibition of 65%, which declined to 41% after one hour and 35% after two hours. Extrapolation of this curve allows an estimation of the half-life of the RGD-HSA conjugate of about 24 hours. These results are comparable to the half-life evaluated in C57BL/6 mice, as described in the previous chapter. The high initial loss in inhibition activity might be due to the presence of a number of other adhesion proteins presenting the same binding site, which recognizes the amino acid sequence Arg-Gly-Asp (arginine-glycine-aspartic acid, or RGD) and binds to RGD.

The effect of 7.5 mg/kg RGD-HSA on thrombus inhibition in the carotid artery was comparable to the effect of 5 mg/kg ASA, both administered intravenously 20 minutes before thrombus induction. However, drug induced recanalization by rtPA combined with LMW-heparin did not succeed in RGD-albumin treated animals. This apparent failure of RGD-albumin compared to ASA might be due to the low dose of RGD-albumin administered in the present experiments. This is even more probable, when considering the high number of GPIIb/IIIa complexes present on the platelet membrane (approximately 30 to 50'000) and the additional binding sites for the RGD-molecule in other adhesion proteins. Due to high manufacturing expenses and limited feasibility for the production of large amounts of the RGD-HSA conjugate, it was our aim to evaluate, whether the effect on platelet inhibition measured *in vitro* will be maintained after administration of the antiplatelet agent in a mammalian circulation system. Thus, in the present study we showed for the first time, that an RGD-peptide, after conjugation to human albumin, presents a 20- to 30-fold prolongation of its half-life and attenuates thrombus growth with an effect comparable to aspirin. Therefore, RGD-albumin might be be a valuable drug in the prevention of cardiovascular and cerebral arterial disease.
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