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GENETIC ENGINEERING, STRUCTURAL ANALYSIS AND *IN VIVO* CHARACTERIZATION OF THE ANTITHROMBOTIC ANTI-VON WILLEBRAND FACTOR ANTIBODY 82D6A3

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Aims of the study

Haemostasis is a complex process which is necessary for cessation of bleeding at sites of injury. However, uncontrolled haemostasis leads to thrombosis, which is the underlying cause of many cardiovascular diseases, considerably contributing to mortality in the Western world. Huge efforts have been taken in the development of agents that prevent thrombus formation by interfering with platelet-dependent haemostasis or coagulation, however, all antithrombotic agents currently in use increase the risk for bleeding to a more or lesser extent, requiring close monitoring of the patients during treatment. In the search for new and safer antithrombotics, the Laboratory for Thrombosis Research has characterized a monoclonal antibody, 82D6A3, directed to the von Willebrand factor A3-domain. It inhibits the interaction of VWF with collagen, and thus inhibits one of the first steps in platelet-dependent haemostasis. 82D6A3 has anti-thrombotic effects *in vivo*, without significantly prolonging the bleeding time. The aims of this study was to further develop 82D6A3 as an antithrombotic agent which can be used in acute and chronic clinical applications.

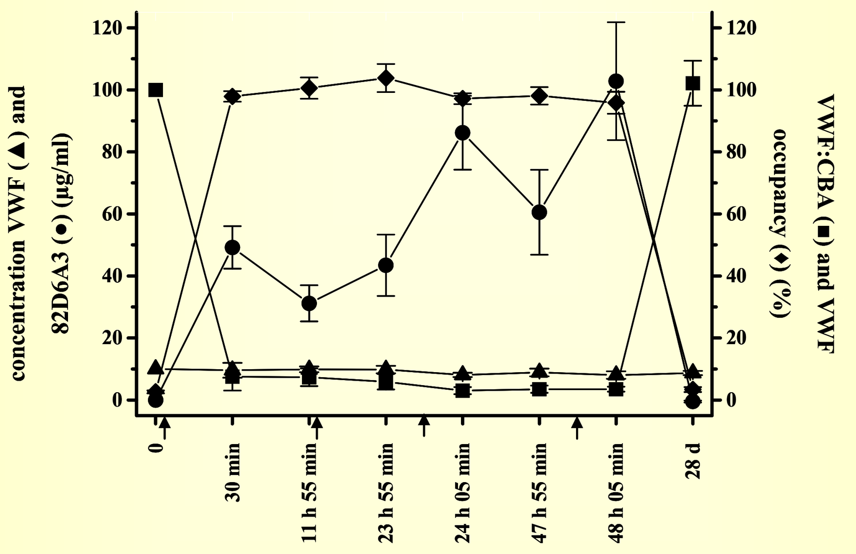
Results

1. Effect of 82D6A3 on in-stent stenosis

Angioplasty and stent implantation, a very common revascularisation technique, leads to a pathological restenosis in the target vessel in >10% of treated patients. The formation of a neo-intima, which mainly consists of smooth muscle cell proliferation and migration, is responsible for this in-stent restenosis. Platelet activation plays an important role in smooth muscle cell activation and therefore, we studied the effect of 82D6A3 on in-stent stenosis in a baboon in-stent stenosis model.

a. optimisation of 82D6A3-treatment

- the VWF-collagen binding was inhibited during three days after stenting (the critical period for platelet adhesion and activation) by injecting the baboons with 1mg/kg 82D6A3 before stenting and at 12h, 24h and 48h after stenting



Pharmacokinetics of 82D6A3 in baboons injected with multiple boli. Baboons were injected with 1 mg/kg 82D6A3 10 min before PTCA and stent implantation and at 12 h, 24 h and 48 h after the first injection. Blood was drawn before the first injection, after surgery and 5 min before and after each of the following 82D6A3 injections. Each data point (mean ± SEM) is the result of double measurements in one experiments for 5 baboons.

b. analysis of stented arteries after 28 days

- the degree of injury to the coronary arteries induced by angioplasty and stenting was the same for control and 82D6A3-treated baboons

- the mean percent stenosis in control animals was 31.2 ± 5.9 (n=4), however, no significant reduction in neo-intima formation could be observed in animals treated with 82D6A3 where a mean percent stenosis of 36.2 ± 4.0 was observed

Cross-sections of stented arterial segments. Representative cross-sections of stented arterial segments at 28 ... (A) and ... (B) baboons. The cross-sections ...

2. Humanization of 82D6A3

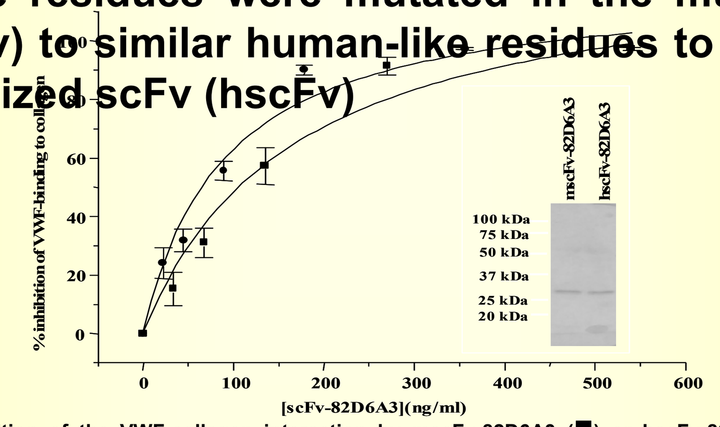
For short term antithrombotic treatment in a clinical environment, an injectable agent is preferable. 82D6A3 is a monoclonal antibody and can thus be injected intravenously. However, repeated injections are very likely to induce an immune respons, since it is a murine antibody. To avoid this, antibodies developed for clinical use are frequently humanized. Here we humanized 82D6A3 by variable domain resurfacing. Furthermore we grafted the humanized variable domains on the constant regions of a human IgG$_4$ to reduce the immunogenicity and to increase the half life of the antibody.

a. variable domain resurfacing

- first a single chain variable fragment (scFv) of 82D6A3 was constructed to facilitate mutagenesis

- next, the non-human like surface accessible framework residues were identified using the homology modelled Fv of 82D6A3

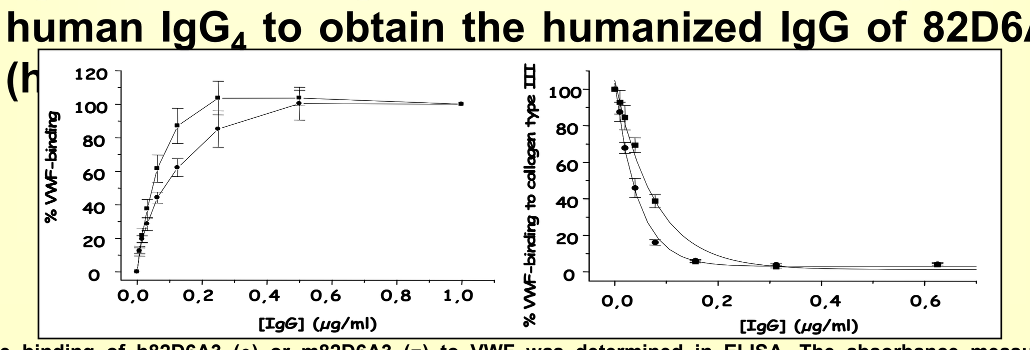
- these residues were mutated in the murine scFv (mscFv) to similar human-like residues to obtain the humanized scFv (hscFv)



The inhibition of the VWF-collagen interaction by mscFv-82D6A3 (■) or hscFv-82D6A3 (●) was determined in ELISA. The absorbance of VWF-binding in the absence of scFv was arbitrarily set as 0% inhibition. Each data point is the mean ± SEM calculated from single measurements in three independent experiments. *(Inset)* The purity of the mscFv-82D6A3 and hscFv-82D6A3 was analysed in SDS-PAGE followed by staining with Coomassie brilliant blue.

b. grafting to human IgG$_4$

- the humanized variable domain of the heavy and the light chain were grafted on the constant region of a human IgG$_4$ to obtain the humanized IgG of 82D6A3 (h...

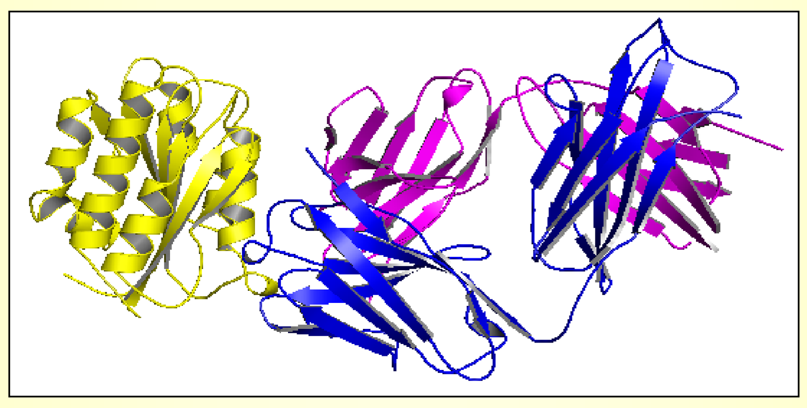


A. The binding of h82D6A3 (●) or m82D6A3 (■) to VWF was determined in ELISA. The absorbance measured at saturation was arbitrarily set as 100% binding. B. The inhibition of VWF-binding to human collagen by h82D6A3 (●) or m82D6A3 (■) was determined in ELISA. The absorbance of VWF-binding in the absence of 82D6A3 was arbitrarily set as 0% inhibition. Each data point in A and B is the mean ± SEM calculated from double measurements in three independent experiments.

3. Determination of the paratope of 82D6A3

Today, there is a high need for safe oral antithrombotics for the prevention of a second cardiovascular event in patients at risk. As a first step in the development of an orally available antithrombotic with the same characteristics as the monoclonal antibody 82D6A3, we determined the amino acids (aa) in 82D6A3 that are important for binding to the antigen, i.e. the paratope.

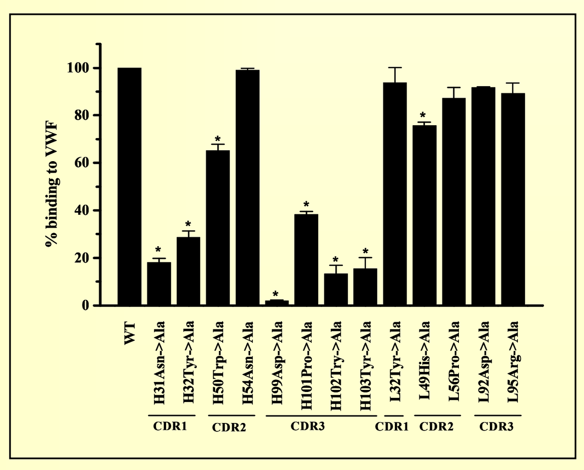
a. crystal structure of the complex between the VWF A3-domain and 82D6A3



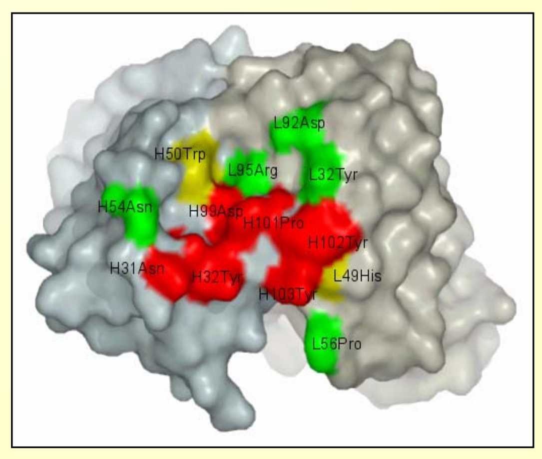
Structure of the complex of the VWF A3-domain and the Fab-fragment of 82D6A3. Ribbon representation of the complex with the VWF A3-domain in yellow and the variable domains of the heavy and the light chain in blue and magenta respectively.

b. binding studies to VWF with mutants of scFv-82D6A3

- all 82D6A3 aa that form hydrogen bonds or salt bridges with residues of the A3-domain as well as all residues that are located within 4.0 Å of the A3-domain and have a buried solvent accessible surface area in the complex of at least 20 Å^2 were mutated to alanine



Comparison of the VWF-binding of scFv-82D6A3(WT) and its alanine mutants. Percentage of binding to VWF of all mutants compared to scFv-82D6A3(WT) at 0.5 µg/ml, the concentration where maximal binding to VWF is obtained for scFv-82D6A3(WT). *, p ≤ 0.005. All data points are the mean ± SEM of single measurements in three independent experiments.



The paratope of 82D6A3. Surface diagram of the variable domains of 82D6A3 with amino acids of the paratope coloured according to the extent of residual VWF-binding observed if mutated to alanine: 0-40%, red; 40-80%, yellow; 80-100%, green.

Conclusions

Development of 82D6A3 as a new antithrombotic agent

1. Next to its antithrombotic effect, 82D6A3 has no effect on neo-intima formation in a clinical relevant in-stent stenosis model in baboons

2. Resurfacing of the variable domains of 82D6A3 and grafting on the constant domains of a human IgG$_4$ did not affect the activity of 82D6A3

3. The paratope of 82D6A3 consists of two short linear sequences in the heavy chain, forming one continuous patch on its surface

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