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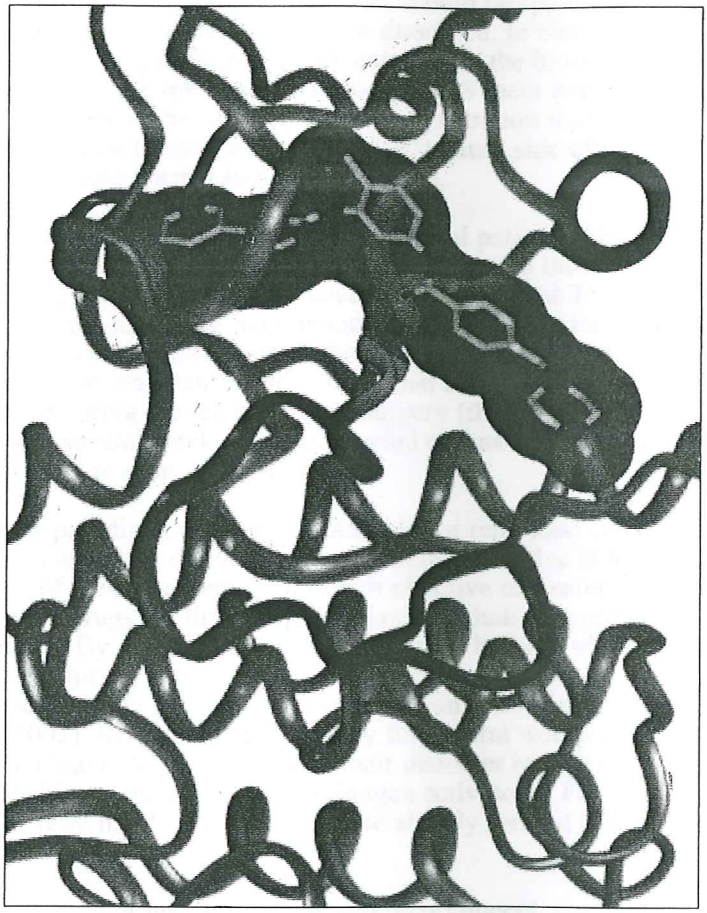

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Coupling of Anti-Thrombotic Agents to Red Blood Cells Offers Safer and More Effective Management of Thrombosis

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When we are wounded, either externally (for instance, when we cut ourselves) or internally (for instance, due to gastric ulcer or brain hemorrhage), blood clots -- sponge-like plugs that are rapidly formed in response to the injury by activated blood platelets and fibrin in a process called coagulation -- prevent profound bleeding. Thus, good or hemostatic clots save our lives.

However, under pathological conditions blood clots can also form inside vessels. Such bad or thrombotic clots occlude blood vessels and cause oxygen starvation of vital organs including the brain (stroke), heart (acute myocardial infarction) or lungs (pulmonary embolism). Thrombosis is one of the leading causes of morbidity and mortality from cardiovascular and other disease conditions.

Diverse anti-thrombotic means are being developed. For instance, anticoagulants (such as heparin) and platelet inhibitors (such as aspirin) help to prevent formation of clots (blood thinners). Fibrinolytics, known as plasminogen activators (such as tissue-type plasminogen activator, or tPA) dissolve formed clots by degrading the fibrin meshwork. Both types of therapeutics are widely used in medical practice, e.g., for treatment of two forms of ischemic heart disease caused by thrombi in coronary vessels -- acute myocardial infarction and unstable angina.

Unfortunately, these anti-thrombotic therapies are not free of serious (sometimes fatal) side effects, the most prominent being bleeding. Thus, fibrinolytics do not discriminate between good, hemostatic clots that should be spared, and bad, thrombotic clots that must be dissolved. In addition, plasminogen activators are eliminated from the bloodstream within a few minutes (which makes them impractical for prophylactic use), in part due to filtration into the extravascular tissues, where they might cause side effects by degrading normal tissue components.

Arguably, the situation is worst in surgical patients. On the one hand, the wounds must be protected against bleeding by good clots. On the other hand, due to the instability of the balance for blood coagulation and the patient's immobilization, clots tend to form in the post-operative period despite anticoagulant means. These bad nascent clots formed during the recovery after surgery (that takes from days to several weeks), embolize vital organs and must be dissolved as soon as possible.

A new paradigm, utilizing erythrocytes or red blood cells (RBC) as a drug delivery carrier, promises to solve at least some of these problems and permit effective and safer use of these drugs for thromboprophylaxis, including surgical patients. By many parameters, RBC could become ideal carriers for the intravascular delivery of drugs, whose prolonged activity in the bloodstream is desirable (Magnani et al., 2002). RBC circulate normally for several weeks and do not leave the blood vessels (their diameter is 1,000 times bigger than that of plasminogen activators). For the same reason, RBC do not permeate already formed blood clots.

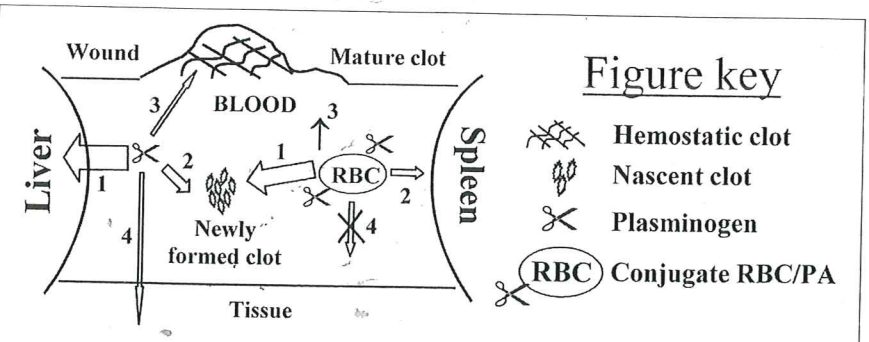

Figure 1. The concept of prophylactic fibrinolysis tPA coupled to carrier RBC with selectivity for nascent intravascular clots. The liver eliminates most of the free plasminogen activators (PA, scissors) within minutes. Residual circulating PA diffuses into pre-existing haemostatic clots and into the tissues (e.g., the brain), causing a high risk of bleeding and tissue damage. RBC-coupled PA has no access to pre-existing clots or extravascular tissues, circulates for a prolonged time (cleared by spleen at a modest rate) and gets incorporated into nascent clots causing their dissolution from within ("Trojan Horse"). Figure is taken from *Nature Biotechnology* 21(8):891-896, 2003.

A new thromboprophylaxis concept, published recently in *Nature Biotechnology* (Murciano et al., 2003) is based on the coupling of tissue plasminogen activators (e.g., tPA) to RBC, in order to optimize their delivery in the vasculature. The idea is that coupling tPA to RBC would prevent its diffusion into good hemostatic clots (sparing them from unwanted dissolution) and extravascular tissues (minimizing side effects). On the other hand, RBC carriage would prolong tPA life-time in the bloodstream, permitting its incorporation into the bad nascent clots, which then would be dissolved from within (a Trojan Horse for fibrinolysis).

This animal study tested whether coupling tPA to the surface of RBC generates a novel fibrinolytic agent (RBC/tPA) capable of dissolving nascent clots from within, while only mini-

mally affecting pre-existing hemostatic clots. After intravenous injection in mice and rats alike, the fibrinolytic activity of RBC/tPA persisted in the bloodstream at least 10 fold longer than did free tPA. Experiments were limited to short-term duration, 3 hours, but the shape of the kinetic curve of RBC/tPA blood clearance strongly suggested that duration of its fibrinolytic activity would persist beyond this time frame. In a model of venous thrombosis induced by aggregation in the pulmonary vasculature of intravenously injected fibrin microemboli (Murciano et al., 2002), soluble tPA lysed pulmonary clots lodged before, but not after the tPA injection. In contrast, RBC/tPA lysed pulmonary clots lodged after, but not before the RBC/tPA injection. Free tPA injected either before or after occlusive thrombosis of carotid artery failed to dissolve clots, whereas RBC/tPA injected before, but not after thrombosis, restored blood flow.

Therefore, RBC-based drug delivery strategy changes the fibrinolytic profile of tPA, providing the basis for prophylactic fibrinolysis. In this particular study, tPA was coupled to isolated RBC chemically, using a biocompatible conjugation technique developed in the prior studies (Muzykantov et al., 1996). In principle, this technique is applicable in the hemotransfusion settings associated with thrombotic disorders (for example, sickle cell anemia) or in elective surgeries where autologous transfusion is a standard practice.

Furthermore, it is possible to couple therapeutic proteins to RBC directly in the bloodstream. Human red blood cells possess the complement receptor 1 (CR1) that binds immune complexes and transfers them to macrophages without opsonization of RBC themselves (Taylor et al., 1991). CR1 monoclonal antibodies and anti-CR1 immunoconjugates injected into animals, bind to RBC and circulate for a prolonged time without RBC damage. Preliminary studies show that, in principle, an anti-CR1/tPA conjugate can be used for thromboprophylaxis without the need for RBC extraction, modification or transfusion.

The proposed strategy may also enhance the safety of cerebral fibrinolysis. Free tPA improves reperfusion in ischemic stroke, but may enhance cerebral bleeding in hemorrhagic stroke. Besides unintended dissolution of hemostatic clots in the cerebral vasculature, diffusion of plasminogen activators into the brain aggravates cerebral edema and matrix remodeling, and causes collateral damage including cytotoxicity to neurons. Therefore, RBC-carried tPA might be safer due to better retention within the vascular compartment.

In summary, animal experiments documented that coupling of a fibrinolytic drug to the surface of red blood cells radically alters its pharmacokinetics and effects, converting it from a somewhat limited therapeutic into a potent prophylactic agent. From a more general perspective, this study for the first time experimentally establishes a concept of intravascular delivery of drugs coupled to the red blood cell surface.

References:

Erythrocyte-mediated delivery of drugs, peptides and modified oligonucleotides.
Magnani M, Rossi L, Fraternale A, Bianchi M, Antonelli A, Crinelli R, and Chiarantini L.
Institute of Biochemistry 'Giorgio Fornaini', University of Urbino, Italy.
Gene Ther 9:749-751, Jun. 2002.
Summary: Magnani et al. described loading of RBC with diverse therapeutics. In particular, steroid drugs loaded inside human RBC provide more sustained effects than free drugs in clinical studies, likely due to prolonged circulation and slow release of the drug in the bloodstream.

Prophylactic fibrinolysis through selective dissolution of nascent clots by tPA-carrying erythrocytes.
Murciano JC, Medinilla S, Eslin D, Atochina E, Cines DB, and Muzykantov VR.
Institute for Environmental Medicine, University of Pennsylvania, Philadelphia, PA, USA.
Nat Biotechnol 21:891-896, Aug. 2003.

Platelets inhibit the lysis of pulmonary microemboli.
Murciano JC et al.
Institute for Environmental Medicine, University of Pennsylvania, Philadelphia, PA, USA.
Am J Physiol Lung Cell Mol Physiol 282:L529-539, 2002.
Summary: This paper described a new model of pulmonary thrombosis based on injection of fibrin microparticles that lodge in the lungs after intravenous injection. These pulmonary emboli are susceptible to fibrinolysis and allow an accurate determination of the efficacy of plasminogen activators and RBC-coupled counterparts.

Regulation of the complement-mediated elimination of red blood cells modified with biotin and streptavidin.
Muzykantov VR, Murciano JC, Taylor RP, Atochina EN, and Herraez A.
Institute for Environmental Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA.
Anal Biochem 241:109-119, 1996.
Summary: This paper described conjugation of model therapeutic proteins to the surface of RBC using the biotin-streptavidin approach. This technique permits attachment of 10^5 protein molecules per RBC without compromising biocompatibility of the RBC and thus provides a prolonged safe circulation of RBC-drug complex in rats.

Use of heteropolymeric monoclonal antibodies to attach antigens to the C3b receptor of human erythrocytes: a potential therapeutic treatment.
Taylor RP, Sutherland WM, Reist CJ, Webb DJ, Wright EL, and Labuguen RH.
University of Virginia School of Medicine, Charlottesville, VA, USA.
PNAS 88:3305-3309, Apr. 15, 1991.
Summary: This paper described the safe use of an antibody against CR1, a complement receptor on RBC, to deliver therapeutic proteins to RBC in the bloodstream without ex-vivo manipulations with RBC.