Exploring Natural Anticoagulation by Endothelial Cells: A novel *In Vitro* Model

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Summary
Acute damage and activation of endothelial cells (EC), the inner lining of blood vessels, is linked to release of heparan sulfate proteoglycans and exposure of a proinflammatory and procoagulant cell surface. To study the pathophysiology of EC activation and the effect of substances which protect EC in vitro we have developed a model with EC cultured on small beads, which are then incubated with whole, non-anticoagulated human blood.

Keywords: human, pig, whole blood, endothelia, coagulation, complement system, transplantation, microcarrier, reduction, replacement, drug screening

Background Information

Endothelial cells in disease

Endothelial cells form the inner lining of blood vessels, the endothelium, which is the interface between circulating blood and surrounding tissue. Endothelial cells are involved in control of blood pressure, coagulation, atherosclerosis, angiogenesis and inflammation. Endothelial activation, leading to dysfunction and damage, has been demonstrated in several different clinical situations:
- Acute vascular rejection in allo- and xeno-transplantation: Endothelial cells are activated due to preformed antibodies and complement.
- Ischemia/reperfusion injury in surgery, transplantation or after myocardial and cerebral infarction: Changes on the endothelial cell surface during ischemia lead to complement activation upon reperfusion.
- Severe sepsis or septic shock: Vascular leakage and disseminated intravascular coagulation due to dysfunctions of the endothelium.

In vivo, endothelial cell activation is generally linked to both the complement system (a bio-chemical cascade of the immune system) and the blood coagulation cascade. By activation-induced shedding of the endothelial surface layer of heparan sulfate proteoglycans, dendritic cells in the blood may undergo maturation and transform into efficient antigen presenting cells. Endothelial cell damage is therefore linked to activation of innate immune defense mechanisms as well as to blood clotting. So far, studying interactions between the endothelium on one hand and the coagulation system, the complement pathways, antibodies, and other players of the innate immune system on the other hand inevitably necessitated the use of in vivo models. This is because conventional ex vivo or in vitro systems can only operate with serum, plasma or anti-coagulated whole blood. With the coagulation system and/or the critical interaction with blood cells "out of bounds", the results derived from such in vitro experiments paint an incomplete picture. The challenge, therefore, was to establish an in vitro model for endothelial cell activation and damage under conditions comparable to in vivo.

The endothelium's natural anticoagulant properties

The resting endothelium effectively maintains an anti-inflammatory and anti-coagulant intravascular environment. This state is mainly upheld by the endothelial surface layer, the glycocalyx, which is composed of glycolipids, proteoglycans and associated glycosaminoglycans. The association of soluble factors from the blood with the glycocalyx secures help to maintain an anti-coagulatory state of the endothelium *in vivo*.

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Preservation of anticoagulant properties *in vitro*

In conventional flatbed culture systems, work with whole, non-anticoagulated blood leads to coagulation as the endothelial surface per blood volume does not suffice to ensure that the endogenous anticoagulant state can be maintained. Accordingly, the interaction between blood cells and derived natural factors and interactions with endothelial cells cannot be studied in conventional cell culture systems. However, replacing flatbed cultures through cultivation of endothelial cells on microcarrier beads increases the surface-to-blood volume ratio twenty-fold to reach that of small arteries and veins (16 cm^2 endothelial cell

surface per ml of blood). This endothelial surface suffices to ensure "natural" anticoagulation in whole blood, rendering the use of anticoagulants unnecessary.

Set-up of whole blood *in vitro* assay

The system is based on endothelial cells (as an example, porcine aortic endothelial cells, PAEC) which are previously expanded *in vitro* in conventional flatbed culture systems. Once a sufficient cell number is available, the cells are transferred to spinner flasks and mixed with collagen-coated Biosilon microcarrier beads (5 ml, equal to 800 cm^2, Nalge Nunc International) and cultured until the cells form a confluent monolayer on the beads. Washed beads are then be incubated with non-anticoagulated, freshly withdrawn whole human blood at a bead-to-blood volume ratio of 1:4. Samples of the bead-blood mixture are taken at regular intervals during the experiment. The beads can be analyzed by immunofluorescence for various cell activation markers, cell integrity etc., while the plasma is used to analyze the coagulation/inflammatory status. In this system potential "endothelial cell protectants", i.e. substances which preserve endothelial integrity and prevent activation of the endothelial cells, can be pre-incubated with the cells in the flask or co-incubated with the whole blood, and their effects assessed.

Search for "endothelial cell protectants"

Conventionally, (pre-)screening of substances with respect to their effects as endothelial protectants needed to be performed *in vivo* in animal models of e.g. ischemia/reperfusion injury, transplantation, or shock. All of them induce a high discomfort to the animals. The novel *in vitro* EC culture system allows for the screening of such substances without the use of animal models. Compounds identified may be candidates for later application *in vivo*.

Preventing activation of cascade systems

One strategy in tackling the problem of endothelial damage is to prevent activation of the cascade systems, mainly complement and coagulation. In collaboration with academic partners in the field of bioorganic chemistry our laboratory has been developing soluble complement/coagulation inhibitors and analyzing their potential role as "endothelial cell protectants". As an example low molecular weight dextran sulfate (DXS), a sulfated polysaccharide which inhibits complement activation in human serum (Laumonier et al., 2003), prevents hyperacute rejection of pig lungs xenoperfused with human blood and significantly reduces the extent of tissue damage in a porcine model of acute myocardial infarction (Fiorante et al., 2001;

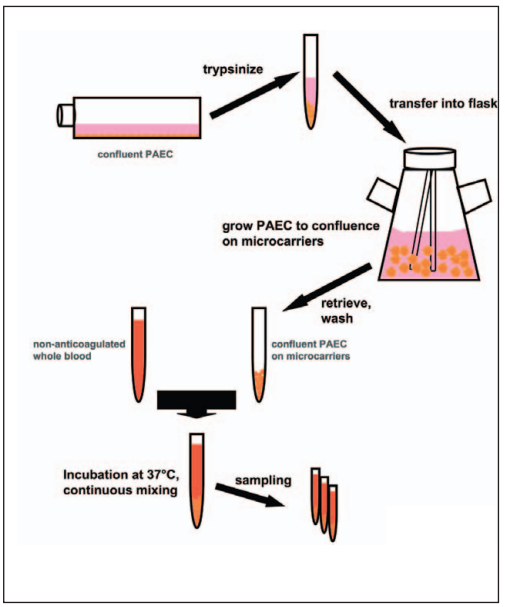

Fig. 1: Schematic representation of the test principle.
Porcine aortic endothelial cells (PAEC, used here as an example of endothelial cells in general) are expanded in conventional flatbed cell culture and then grown to confluence on microcarriers. PAEC-coated microcarriers can then be incubated with whole, non-anticoagulated blood, exploiting the natural anticoagulant properties of the endothelium.

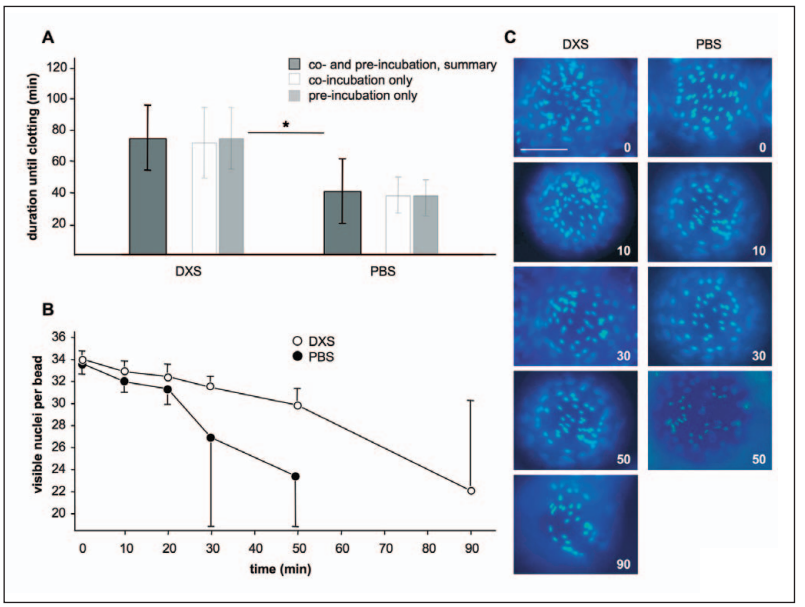

Fig. 2: Effect of the endothelial cell protectant DXS.
(A) Duration of experiments in minutes until occurrence of clotting. Shown are averages and standard deviations for experiments with DXS and PBS controls. *P < 0.05 for DXS vs. PBS. (B) Amounts of nuclei per bead for DXS- and PBS experiments at incubation time, as counted on the surface visible in panel C. (C) Representative images of single beads with DAPI staining nucleiat baseline (0 min), after 10, 30, 50 and 90 min of incubation with human blood. Scale bar represents 100 µm (Banz et al, 2005a).

Banz et al., 2005b). DXS was also tested *in vitro* in our EC-bead system (Banz et al., 2005a) (see below). Several other, fully synthetic substances have been developed and tested for their anti-coagulatory and anti-complement effects using conventional *in vitro* systems. Further testing is now required to see which of these substances act as EC protectants and have the potential for clinical application. This information can now be acquired in the described *in vitro* system before choosing the most promising agents and reverting to *in vivo* animal models for confirmation.

Mimicking xenotransplantation *in vitro*

The addition of non-anticoagulated human blood to beads coated with porcine aortic endothelial cells was used to mimic a xenotransplantation setting in our *in vitro* model. Human blood contains naturally occurring antibodies which bind to galactosyl antigens on porcine cells and activate the complement cascade. The subsequent activation of the endothelial cells leads to a loss of their anticoagulant surface layer of heparan sulfate proteoglycans and therefore initiates the coagulation cascade. *In vivo* this is the hallmark of hyperacute rejection and a major reason for the failure of pig-to-human xenotransplantation. In our *in vitro* system a loss of the PAEC from the polystyrene beads, complement deposition on the beads and a loss of von Willebrand factor expression on the remaining PAEC on the beads were observed. In addition, clotting of the human blood occurred within a few minutes. Time until clotting can be used as an indicator of endothelial cell activation and damage. Addition of our "prototype" endothelial cell protectant DXS significantly prolonged time until clotting as shown in Figure 2a and reduced cell loss from the beads (Fig. 2b, 2c).

3R benefit and limitations

The described *in vitro* model is currently suitable for the analysis of acute endothelial cell damage like antibody- and complement-mediated (hyper)acute vascular rejection or for studying certain aspects of ischemia/reperfusion injury. Most probably due to contact with air, blood clotting occurs within about 90 minutes even with the use of DXS as an endothelial cell protectant. Situations in which endothelial damage takes several hours or days to occur can therefore not be mimicked with the current model. In addition, flow conditions and shear stress which may play an important role *in vivo* are not present in the model. Also the phenomenon of vascular leakage, in which the endothelial cells stay intact but loose their barrier function due to intracellular gap formation, cannot be mimicked. Nevertheless, the possibility to do a first screening with substances aimed at protecting endothelial cells from ischemia/reperfusion injury or antibody- and complement-mediated attack, offered by our new *in vitro* model, has the potential to significantly reduce the number of animals derived from animal models. Furthermore, mechanistic studies can be performed much easier *in vitro*, which in addition reduces the use of animals in the respective areas of research.

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