



Sequential co-immobilization of thrombomodulin and endothelial protein C receptor on polyurethane: Activation of protein C

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ARTICLE INFO

Article history:

Received 27 October 2010

Received in revised form 3 March 2011

Accepted 11 March 2011

Available online 15 March 2011

Keywords:

Thrombomodulin

EPCR

Protein-C

Immobilization

Thromboelastograph

ABSTRACT

In an effort to control the surface-mediated activation of thrombin and clot formation, proteins and molecules which mimic the anticoagulant properties of the vascular endothelial lining were immobilized on material surfaces. When immobilized on biomaterial surfaces, thrombomodulin (TM), an endothelial glycoprotein that binds thrombin and activates protein C (PC), was shown to generate activated PC (APC) and delay clot formation. However, TM-mediated activation of PC on biomaterial surfaces was shown to be limited by the transport of PC to the surface, with maximum activation obtained at a surface density of ~ 40 fmole TM cm⁻². This work investigates surface immobilized with TM and endothelial protein C receptor (EPCR), a natural cofactor to TM which increases the rate of activation of PC on the native endothelium. A sequential and ordered immobilization of TM and EPCR on polyurethane at an enzymatically relevant distance (< 10 nm) resulted in higher amounts of APC compared with surfaces with immobilized TM or with TM and EPCR immobilized randomly and at TM surface densities (1400 fmole cm⁻²) which were previously shown to be transport limited. Ordered TM and EPCR samples also showed increased time to clot formation in experiments with platelet-poor plasma, as measured by thromboelastography. Surfaces immobilized with TM and its natural cofactor EPCR at an enzymatically relevant distance are able to overcome transport limitations, increasing anticoagulant activation and time to clot formation.

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1. Introduction

Implanted medical devices are of increasing importance in the practice of medicine. Even though most biomedical polymers are relatively inert, when most implant materials come into contact with blood they invoke the activation of blood cells as well as the plasma proteolytic enzyme systems, namely, the thrombin-catalyzed conversion of fibrinogen to fibrin, resulting in fibrous clots [2–4]. To overcome the procoagulant processes on the surfaces of biomaterials, surface modifications have been undertaken to achieve hemocompatibility characteristics comparable with native endothelium [5–7].

The vascular endothelium provides several regulatory mechanisms to inhibit and control thrombotic events [8–10]. The endothelium also regulates clot formation through soluble molecules such as prostacyclin and nitric oxide, which inhibit the activation of platelets [11]. While several natural anticoagulant pathways are active in vivo, the protein-C–thrombomodulin (TM) mechanism which regulates hemostasis on the endothelium is relevant,

as it culminates in the generation of activated protein-C (APC), a potent anticoagulant [12,13]. Effective activation of human protein C (PC) to yield APC on endothelial surfaces is catalyzed by surface-bound thrombin (TR), which is sequestered on endothelial surfaces by TM [1], a transmembrane glycoprotein, in a 1:1 complex with high affinity, resulting in > 1000 -fold amplification of the rate of PC activation [14,15]. In addition to the thrombin receptor (i.e., TM), endothelial cells express a protein-C/APC-binding receptor, designated as the endothelial protein-C receptor (EPCR), which binds protein-C and APC specifically and selectively, thus providing a 20-fold higher stimulation of the thrombin–TM mediated activation of PC in vivo, to yield APC (Fig. 1).

In efforts to mimic the anticoagulant attributes of the endothelium, previous attempts have included components present in the native endothelium mainly to sequester TR and render it inactive [6,7,16–21]. For example, heparin, which is known to bind anti-thrombin-III, a potential binding target for TR, has been immobilized on a variety of biomaterials via covalent or ionic linkages, either alone or in conjunction with the other biological compounds or with spacer moieties [22,23]. One of the major anticoagulant components of the endothelial surface with reported thrombin-binding affinity, TM also has been coated onto or immobilized to a variety of biomaterial surfaces, including polyethylene glycol (PEG)-modified glass, polyethylene, polytetrafluoroethylene and

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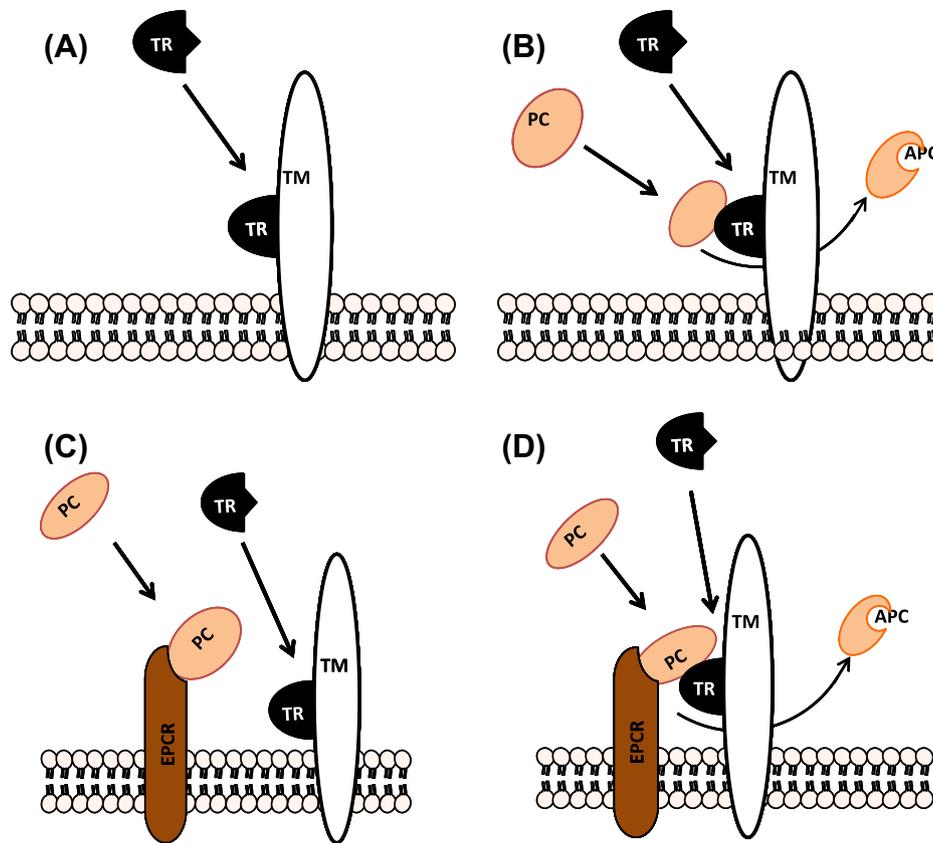


Fig. 1. Schematic representation of proposed PC activation on the endothelial cell surface. In the absence of EPCR, PC activation mediated by the thrombin–TR complex is a relatively low-affinity reaction (A). EPCR, which binds protein-C with high affinity, provides a further 20-fold stimulation of the thrombin–TM-mediated activation of PC, to yield APC (B).

polyether urethane; the bioactivity of immobilized TM has been verified by biochemical assays [17,18,20,24]. Recent studies have shown that the surface concentration of TM in vascular grafts decreases after implantation and that this decrease can be linked to thrombus formation [25]. In the same study, increasing the TM concentration through viral vector to the native arterial concentrations has shown a complete arrest of thrombin formation, demonstrating the importance of this pathway in arterial flow. As a result, surface concentration of TM has been increased on artificial surfaces to attempt to mimic the results. While PC activation initially is linear to the immobilized TM concentration, in vitro studies under flow have shown a maximum reaction occurring at a surface density of 488 fmol cm^{-2} ($\sim 35 \text{ ng cm}^{-2}$) [16], which is thought to be a function of the transport of PC to the biomaterial surface.

While immobilization of TM onto a biomaterial surface mimics one specific pathway—the TM:TR:PC pathway (Fig. 1)—the regulation of hemostasis involves many pathways working in concert. Thus recent studies have attempted to create surfaces which aim to incorporate multiple aspects of the natural regulatory molecules found on the native endothelium [6]. For example, recently, heparin or TM was co-immobilized with a platelet inhibitor such as prostaglandin or a nitric oxide-producing molecule [6]. In another study, either TM alone or a combination of both TM and heparin were immobilized onto artificial biomimetic phospholipid membranes, and the rate of APC generation was quantified [16,26]. However, to the present authors' knowledge, surfaces that mimic the anticoagulant components of the endothelial surface, namely TM and EPCR, have not been described.

The objective of this paper is to integrate the functions of TM and EPCR using tools enabled through biomolecular engineering to generate surface treatments and biomaterial surfaces that (1)

mimic the biomolecular moieties of the endothelium, and (2) will provide efficient regulation of the coagulation by the in situ generation of APC. On endothelial cells, TM is found in close proximity to EPCR so that the active site on PC is held in close molecular proximity to TM-bound thrombin. Because of the cooperative nature of the two proteins, an ordered co-immobilization was desired, to anchor the two proteins at an enzymatically relevant scale, compared with random immobilizations. Biomedical-grade polyurethane (PU) that was surface modified to enable the ordered co-immobilization of proteins via a bidentate moiety was employed in this study. Biomaterial surfaces containing immobilized recombinant human TM [1] and recombinant EPCR (rEPCR) were prepared, and the generation of APC on surfaces displaying rEPCR/hTM, rEPCR, rTM and underivatized controls was assessed using a PC-activation assay which was modified for surfaces.

2. Materials and methods

Sheets of chronoflex AR (HDMI polycarbonate medical grade PU) with a nominal thickness of 0.5–0.75 mm were provided as a generous gift from AdvanSource Biomaterials (Cambridge, MA). Preactivated Ultrabind™ membrane was purchased from Pall Life Sciences (MA). All chemicals and reagents were cell-culture grade and used as received. Pure recombinant human thrombomodulin (rTM, $\sim 72 \text{ kDa}$) was purchased from American Diagnostica (NJ). Heparin ($\sim 202 \text{ U mg}^{-1}$) was purchased from Sigma Chemicals (St. Louis, MO). Recombinant EPCR (rEPCR, $\sim 44 \text{ kDa}$) with a histidine tag for facile purification was expressed in a *Pichia pastoris* system, purified and characterized as detailed elsewhere [27–29]. RCR-2 monoclonal antibody (mAb) which is specific for the EPCR molecule [30] was generously donated by Dr. Kenji Fukudome.

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