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Influence of ethanol on the release of growth factors in human blood-derived platelet gels

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Abstract

Platelet gels (PG) are new topical single-donor blood products which are attracting great interest in regenerative medicine. They are obtained by mixing a platelet-rich plasma fraction with thrombin to generate a fibrin gel enriched in platelet growth factors (GF). The type of thrombin preparation may affect PG reproducibility. We have determined the impact of 14.6% (v/v) ethanol-stabilized thrombin (EHT) on the release of GF by platelets. Various ratios of EHT and platelet concentrates were mixed to obtain from 2.43 to 7.96% ethanol concentration. Platelet-derived growth factor-AB (PDGF-AB), transforming growth factor-ß1 (TGF-ß1), vascular endothelium growth factor (VEGF), epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1) were assessed at 5, 120, and 300 min after PG formation. Protein profiles of thrombin and PG releasates were analyzed by SDS-PAGE. The amount of PDGF-AB, TGF-ß1, and VEGF released per platelet decreased significantly ($p < 0.05$) with increasing ethanol concentrations but, however, not that of EGF. IGF-1 content was stable, consistent with its presence mostly in plasma. SDS-PAGE indicated that ethanol did not affect fibrin formation. In conclusion, ethanol has a significant impact on the amount of GF released by platelets and should be strictly controlled to standardize PG and optimize clinical benefits.

Keywords: Platelet gel; Thrombin; Ethanol; Growth factors; PDGF-AB; TGF-β; EGF; VEGF; IGF-1

1. Introduction

Human blood is a source of important cellular or protein-based biological products used to treat bleeding, immunological, and metabolic disorders. Among the cellular components, platelets are playing important physiological functions. They are anucleate particles that circulate in an inactive state until they come into contact with areas of endothelial damage or activation of the coagulation cascade. Upon activation they adhere to endothelial defects, change shape, release their granule contents, and stick together to form aggregates. Activated platelets express and release molecules that modulate localized inflammatory response through the activation of leukocytes and endothelial cells. The pathophysiological role of platelets goes beyond regulation of hemostasis and thrombosis and encompasses many other physiological and pathological processes including inflammation, host defense, and tissue repair and regeneration. Increasingly, platelets are seen as a source material for the discovery of a wealth of proteins that are potential therapeutic agents [1], in particular for soft and hard tissue healing [2].

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One of the most recently developed therapeutic products prepared from human blood is called platelet gel (PG). PG is a combination product obtained by mixing a plasma fraction rich in platelets with a preparation enriched in thrombin [3–5]. Upon mixing both components, thrombin converts soluble fibrinogen present in plasma into insoluble strands of fibrin, mimicking the last stage of the coagulation cascade, and activates the platelets to release a myriad of growth factors (GF) from the alpha-granules. These physiological reactions lead to the formation of a fibrin-based, GF-enriched biomaterial that generates great interest in regenerative medicine [2].

The main GF released by activated platelets include platelet-derived growth factors (PDGF-AA, -AB, and -BB), transforming growth factor-ß (TGF-ß1 and TGF-ß2), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF). GF exhibit chemotactic and mitogenic properties, thereby promoting and modulating cellular functions involved in soft and hard tissue healing and regeneration [2,4,6–9] as well as cell proliferation [6,9,10]. By mimicking the wound healing and reparative tissue processes [2], exogenous GF found in PG can stimulate cell growth and differentiation. PG has been used successfully for the treatment of hard-to-heal wounds, such as recalcitrant leg ulcers in diabetic patients or severe burns [7,11,12], and various applications in maxillofacial [4,13] and orthopedic [14,15] surgery.

A current drawback of PG is linked to the fact that its production is not standardized. Many variables prevail in the preparation of both the platelet concentrate (PC) and the thrombin, indubitably affecting PG reproducibility and possibly explaining some inconsistency in clinical outcomes [16–18]. One important factor that has an effect on the clinical efficacy of PG is the actual amount of GF released by the platelets upon thrombin activation and the kinetics of such GF release [19–21]. In particular, it is likely that the characteristics of the thrombin preparation used may impact on the platelet activation mechanism. Until recently, the thrombin used in PG preparation was from bovine origin (BT). However recurrent use of BT can lead to immunological reactions and serious bleeding problems in humans due to the development of antibodies to bovine Factor V and/or bovine thrombin [22–24]. In addition, BT may expose patients to the prion that causes bovine spongiform encephalopathy and its human form, the variant Creutzfeldt-jacob disease (vCJD) [25–27]. This is the reason why human thrombin (HT) preparations made from single plasma donations have been developed [28]. Such HT can be generated up to a concentration of about 50 IU/ml by adding calcium chloride to plasma at a dose sufficient to counterbalance the anticoagulant effect of citrate solutions used during blood or plasma collection. However, the HT generated under those conditions is unstable and should be used within 20 to 30 min after preparation, which is not practical for many surgical applications. To circumvent this limitation, a method to prepare stable thrombin has been developed in which 13–15% ethanol is added at the time of addition of the calcium chloride [29]. Such concentration of ethanol, which is thought to inactivate antithrombin, the physiological inhibitor of thrombin, has been shown to stabilize HT for several hours. It has further been demonstrated that ethanol at this concentration does not alter the capacity of thrombin to convert fibrinogen into fibrin [30,31]. However, the interference that ethanol may exert on the activation process of platelets and on the release of GF from PG has not been studied thoroughly yet. In this paper, we have studied the impact of a range of ethanol concentration on the kinetics of release of GF from PG when using a thrombin preparation stabilized with 14.6% (v/v) ethanol.

2. Material and methods

2.1. Apheresis collection

PCs were collected from volunteer donors after informed consent using a multiple component system (MCS+, Haemonetics, Braintree, MA) following the manufacturer's standard procedure. Whole blood was withdrawn through a venous catheter, with an intermittent flow, and mixed with an anticoagulant solution (1 mL of anticoagulant citrate dextrose solution Formula A per 9 mL of blood). The PC was automatically separated from other blood components by centrifugation and collected into a sterile, single-use disposable bag. The cycle was repeated until a predefined platelet yield target was reached (corresponding to an approximate volume range between 225 and 315 mL per donation). The red blood cells (RBCs) were returned to the donor. 100 mL of platelet-poor-plasma (PPP) was also collected for thrombin preparation. PCs were processed within 24 h after collection as described below.

2.2. Blood cell counts

Platelets, white blood cells (WBCs), and RBCs counts were determined with a cell counter (ABC Vet automatic blood counter, ABX Diagnostics, Montpellier, France).

2.3. Human thrombin preparation

EHT was prepared following a procedure similar to that used by surgeons to generate thrombin. Plasma was activated in the presence of calcium chloride (CaCl2) and glass beads to provide a procoagulant surface. 85 mL of PPP was introduced into a plastic bottle that contained 50 g of glass beads. 3 mL of 1 M calcium chloride (CaCl2; final concentration of 23 mM; batch 056k0688, Sigma, St Louis, MO) and 15 mL of absolute ethanol (final concentration of 14.6%; Panreac Chimica S.A.U., Barcelona, Spain) were then added. The mixture was put under mild rotating mixing at room temperature (20–25 °C) until the formation of a fibrin clot, which typically occurred within 30 to 50 min as a result of the generation of thrombin. The thrombin-rich supernatant was separated by decantation from the fibrin clot. The mean volume of supernatant recovered was approximately 90 percent that of the PPP. The thrombin-rich supernatant was used immediately to study the time-course of the concentration and release of the GF at the 5, 120 and 300 time-points, as described below. As a control, thrombin was also prepared in the absence of ethanol.