Biomaterials 34 (2013) 2492–2500

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

A mechanistic investigation of the effect of keratin-based hemostatic agents on coagulation

Mária B. Rahmany [a], Roy R. Hantgan [b], Mark Van Dyke [c,*]

[a] Molecular Medicine and Translational Science Program, Wake Forest University School of Medicine, Medical Center Boulevard, Winston Salem, NC 27157, USA
[b] Department of Biochemistry, Wake Forest University School of Medicine, Medical Center Boulevard, Winston Salem, NC 27157, USA
[c] Department of Orthopaedic Surgery, Wake Forest University School of Medicine, Medical Center Boulevard, Winston Salem, NC 27157, USA

ARTICLE INFO

Article history:
Received 18 October 2012
Accepted 13 December 2012
Available online 16 January 2013

Keywords:
Hemostasis
Keratin
Blood clotting
Coagulopathy
Trauma

ABSTRACT

Uncontrolled bleeding continues to be one of the leading causes of death in individuals following traumatic injury. Prognosis is worsened with the onset of acute coagulopathy characterized by metabolic acidosis, hypothermia and hemodilution, which consequently perpetuates blood loss and increases mortality. While there are several limitations to biomaterials employed as hemostatic agents, keratin biomaterials have demonstrated efficacy in mitigating blood loss in an animal model of hemorrhage in prior studies. Here we investigate the hypothesis that keratins actively participate in coagulation and that a potential mechanism of action is independent of temperature and dilution of clotting factors. Data from this study show that keratins appear to contribute to hemostasis by significantly decreasing plasma clotting lag times and are able to maintain activity under simulated conditions of coagulopathy. Moreover, a system of isolated fibrin polymerization provided evidence of increased fibril lateral assembly in the presence of keratin. The data provided here provides a platform for further development of keratin biomaterials as hemostatic agents.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Traumatic injury is the leading cause of death in individuals under the age of 44, and of these, uncontrollable bleeding accounts for 39% [1]. Mitigating massive blood loss is a critical step for first aid and trauma care. An ideal hemostatic agent is one that is non-immunogenic, simple to apply, durable, effective against severe bleeding and sustains hemostasis to allow for transport to critical care centers [2]. Unfortunately there are several limitations to current treatment options for managing blood loss. For example, while natural products such as fibrin-based materials provide sufficient bioactivity, there is an associated risk of negative immune responses. Compressive hemostatic products such as regenerated cellulose gauze are effective in controlling blood loss if the site of injury can be identified. However, both agents lack the ability to function in coagulopathic patients [2,3]. These limitations have provided motivation for research on new hemostats, particularly those that are effective under coagulopathic conditions. As these advanced hemostatic materials evolve from simply filling space and/or creating pressure to seal damaged tissue to presenting surfaces and other factors that actively participate in aspects of hemostasis, an understanding of their interaction with the coagulation cascade and clot formation is essential.

The coagulation system can be divided into two parts: 1) primary hemostasis, initiated by platelet adhesion to the underlying extracellular matrices (ECM) of damaged tissue and 2) secondary hemostasis that is subsequent activation of the clotting cascade. Primary hemostasis is descriptive of formation of the soft platelet plug that forms post injury to the endothelium. The initial response to injury is vasoconstriction, which functions to slow local blood flow and enhances platelet adhesion to the exposed extracellular matrices. Platelet adhesion is followed by platelet activation, characterized by release of dense granule contents and pseudopod extensions. The release of platelet agonists contributes to receptor mediated platelet aggregation, which ultimately forms the primary platelet plug. Secondary hemostasis involves a cascade of enzymatic reactions that predominantly occur on the surface of platelets and results in thrombin mediated fibrin generation [4]. Rapid consumption of clotting factors is a necessary response to traumatic injury. However, the body's mechanism for controlling blood loss becomes impaired as a result of trauma induced acute coagulopathy. Patients presenting with trauma

* Corresponding author. Tel.: +336 716 1738.
E-mail address: mavandyk@wakehealth.edu (M. Van Dyke).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2012.12.008

induced acute coagulopathy have higher mortality rates as well as significantly greater transfusion requirements, organ injury, septic complications and time spent in critical care units [5–8]. Coagulopathy results from the "lethal triad", a vicious cycle of acidosis, hypothermia and hemodilution that effects clotting enzyme functions.

Hypothermia occurs as a result of both environmental (lack of blood flow, infusion of crystalloids, enviromental temperature) and physiological factors [9]. Clinical studies have shown that traumatic injury with hypothermia has a significantly worse prognosis than either trauma or hypothermia alone [10–12]. For example, patients with admission temperatures <35 °C had significantly higher mortality than patients with temperatures ≥ 35 °C and those that fell below 32 °C with Injury Severity Scores (ISS) > 25 had a 100% mortality [11,13]. In general, for every 10 °C drop in temperature the activity of an enzyme is reduced by approximately 50% [14], implicating decreased clotting factor activity when temperature control is impaired. In addition to lower thrombin production and factor deficiencies, hypothermia results in platelet activity abnormalities. Shear induced platelet activation is depressed at temperatures below 34 °C, further disrupting physiological coagulation [15].

Massive blood loss also results in decreased oxygen distribution to cells and increased use of anaerobic metabolism. The subsequent accumulation of lactic acid leads to acidosis with pH values below physiological levels. Acidosis causes loss of enzyme activity, primarily through protein denaturation, thereby disrupting the biochemical reactions required for coagulation. While acidosis can be an important component of coagulopathy, the work presented here addresses the other determinants of coagulopathy: hemodilution and hypothermia.

Keratin biomaterials have demonstrated efficacy as a hemostatic agent in several animal models including a rabbit hemorrhage model in which the keratin hydrogel performed as well as, or better than the commercialized hemostatic agents in controlling blood loss [16]. Keratin biomaterials are known to produce substrates to which cells have been shown to readily adhere [17]. It has been hypothesized that this is due to peptide motifs that act as ligands for cell surface receptors and indeed some specificity has been demonstrated [18,19]. It follows then that receptor recognition may play some role in keratin's ability to adhere and activate platelets, but a specific mechanism has not been further studied. Keratin proteins in the form of a crosslinked hydrogel network are thought to simulate an ECM-like structure, providing a site for receptor-mediated platelet adhesion that affords a catalytic surface for the clotting cascade. In pilot experiments prior to this study, fibrin polymerization was observed to "nucleate" from a keratin hydrogel surface in contact with a drop of whole blood. Moreover, it was determined through staining and microscopy of this interface that keratin had leached out of the hydrogel and into the blood clot (data not shown). Therefore, the hypothesis in this work was that keratin biomaterials have the capacity to enhance coagulation by contributing to both primary *and* secondary hemostasis. This postulate was tested by investigating the effect of soluble keratin proteins on coagulation.

2. Materials & methods

2.1. Blood & plasma collection

Informed consent was obtained from healthy volunteers in accordance with a protocol approved by the Wake Forest University Institutional Review Board. Whole blood was collected in Vacutainer tubes (BD Franklin Lakes, NJ) containing either no additive or sodium citrate by venipuncture; to avoid the effects of tissue thromboplastin the first tube was discarded. Plasma was isolated by first spinning the whole blood at 800 rpm for 15 min followed by a second centrifugation of the isolated supernatant at 7000 rpm for 10 min. Samples were stored at −20 °C if not used immediately.

2.2. Keratin preparation

Lyophilized Keratin Proteins (KeraNetics LLC, Winston-Salem, NC) were used as received and solubilized in Hepes-Tyrode buffer as a 2% (w/v) stock solution. Samples were prewarmed to experimental temperatures prior to use.

2.3. Scanning electron microscopy

Blood collected in Vacutainer tubes containing no additive were immediately transferred to tubes containing HEPES-Tyrode buffer or soluble keratin at a final concentration of 0.1% (w/v) and allowed to clot at 37 °C for 60 min. The resulting clot was removed and placed in a chambered slide and fixed with 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer containing 2 mM $CaCl_2$ for 2 h at room temperature (RT). Clots were then washed with sodium cacodylate buffer and counterstained with osmium tetroxide for 1 h at RT. After a final wash with deionized water, samples were put through ethanol dehydration and critical point dried. Samples were mounted and gold sputter coated prior to Scanning electron microscopy imaging (Hitachi High Technologies America, Schaumburg, IL)

2.4. Plasma clotting assay

Plasma clot formation was measured in the presence and absence of keratin proteins using a turbidity assay [20]. Plasma was thawed at 37 °C and equilibrated to the experimental conditions prior to recalcification with 13 mM $CaCl_2$. Recalcified plasma constituting 94% of the total reaction volume was then immediately added to wells of a 96-well microtitre plate containing 0.1% soluble keratin proteins (final concentration) prepared in Hepes-Tyrode buffer (HT, pH 7.4, 0.05%). An equal volume of HT buffer alone was used as a negative control. As a positive control, samples without keratin were prepared and initiated with 0.5U thrombin. After a 5-sec manual shaking step for mixing, the optical density at 340 nm was measured every 30 s over 60 min using a plate reader at 37 °C (Molecular Devices Sunnyvale, CA). Lag times were determined using linear regression formulas to calculate the point of intersection between the initial phase of optical density and rapid increase in optical density. Plasma clots were then prepared for SEM imaging.

2.5. Fibrin clotting assay

Fibrin clots were formed with and without 0.1% (w/v) soluble keratin in a 96-well microtitre plate by the addition of 0.2U/mL thrombin (Sigma Aldrich, St. Louis, MO) to 2.2 mg/mL human fibrinogen and 13 mM $CaCl_2$. Experiments were also done in the presence of $CaCl_2$ and 0.1 mg/mL FXIII (Hematologic Technologies Inc. Essex Junction, VT) for SEM as described above. Fibrin polymerization was assessed as turbidity of the solution by measuring absorbance at 340 nm every 10 s for 60 min at 37 °C.

2.6. Mass/length ratio

For determination of mass/length ratio of fibrin fibers in recalcified plasma clots, turbidity measurements were made using a modified spectrophotometer based method [21–23]. Briefly, recalcified plasma gels or fibrin gels prepared from purified fibrinogen and thrombin were allowed to clot in the presence and absence of keratin (as previously described) for 2 h at 37 °C. The gels were then scanned from 400 to 800 nm and the weight average mass per length ratio (μ) of the fibrin polymers were calculated according to the equation:

$$\tau = \left[(88/15)\pi^3 n(\mathrm{d}n/\mathrm{d}C)^2 C\mu\right] \Big/ N\,\lambda^3$$

where τ is the sample turbidity, n is the refractive index, C is the concentration in g/L (3 g/L for plasma samples, 2.2 g/L for fibrin clots), μ is the mass/length ratio, N is Avogadro's number and λ is the wavelength of light. Carr and Hermans demonstrated that for turbid gels where the diameter of the fibers may not be considered small relative to the wavelength, a plot of $C/(\tau\lambda^3)$ *versus* λ^{-2} gives a line for which the inverse of the intercept is the mass/length ratio. The absolute values for μ are not the same as those obtained under varying experimental conditions such as measurements in a cuvette or high-end spectrophotometer [24]. However, the calculated values for μ can be used to compare structural characteristics of clots formed under different conditions, that is, a high value of μ indicates thicker fibers than those with lower values.

2.7. Plasma clotting: hypothermia

To assess clotting under hypothermic conditions, additional experiments similar to those described in Section 2.4 were carried out at physiologic (37 °C), mild hypothermic (35 °C), normal hypothermic (33 °C) and severe hypothermic (28 °C) temperatures [25].

Download English Version:

https://daneshyari.com/en/article/10228434

Download Persian Version:

https://daneshyari.com/article/10228434

Daneshyari.com