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Relevance of hemolysis-induced tissue factor expression on monocytes in soft clot formation in alcohol-containing blood

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ABSTRACT

The fluidity of cadaveric blood is an important characteristic in the post-mortem examination of cases of asphyxial death. Although it is empirically known that soft blood clots are present in cadaveric blood containing alcohol, the relationship between such clots and blood alcohol is unclear. We addressed this issue through *in vitro* studies using blood collected from healthy volunteers. Assessment of global hemostasis by rotational thromboelastometry revealed that ethanol treatment enhanced the procoagulant activity of whole blood. However, ethanol inhibited epinephrine-induced platelet aggregation, whereas plasma levels of von Willebrand factor and the activity of coagulation factors VIII and IX were unaffected. In contrast, tissue factor (TF) activity was higher in plasma obtained from ethanol-treated whole blood than that in plasma from untreated blood. Ethanol induced hemolysis of red blood cells, and the consequent hemoglobin (Hb) release promoted *de novo* synthesis of TF in isolated monocytes, as determined by real-time reverse transcription PCR, western blotting, and flow cytometry. However, ethanol itself did not induce TF expression in monocytes. Given that TF activates the extrinsic coagulation pathway and amplifies hemostatic reactions, Hb-induced TF expression in monocytes might contribute to soft blood clot formation.

1. Introduction

The fluidity of cadaveric blood is an important characteristic for the post-mortem examination of cases of sudden death by asphyxia [1]. Fluidity results from the release of tissue plasminogen activator (tPA) by endothelial cells, which is induced by catecholamine stimulation during the agonal period [2–4]. In contrast, soft blood clots are found in cadaveric blood with specific blood alcohol concentrations (BACs) in cases of asphyxial death. One study reported a positive relationship between BAC and soft blood clot formation [5], but the underlying mechanism remains to be elucidated.

Alcohol has been found to attenuate platelet function via the inhibition of phospholipase A_2 activity or protease-activated receptor 4-mediated signaling [6–8]. Epidemiological studies have also demonstrated that alcohol consumption reduces mortality by reducing the incidence of ischemic heart disease [9,10]. These findings contradict the observation of soft blood clots in alcohol-containing cadaveric blood.

To clarify this point, we performed comprehensive *in vitro* studies using blood collected from healthy volunteers. Our investigation introduces a possible mechanism for soft blood clot formation in alcohol-containing cadaveric blood, which involves the induction of tissue factor (TF) expression in monocytes by hemoglobin (Hb).

2. Materials and methods

All experiments were approved by the Nara Medical University Ethical Committee and were performed in accordance with the Declaration of Helsinki.

2.1. Reagents

Absolute ethanol, phosphate-buffered saline (PBS), and Roswell Park Memorial Institute (RPMI)-1640 medium were purchased

Abbreviations: BAC, blood alcohol concentration; BSA, bovine serum albumin; CD, cluster of differentiation; CFT, clot formation time; CT, clotting time; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FVIII, coagulation factor VIII; FVIII:C, FVIII activity; FIX, coagulation factor IX; FIX:C, FIX activity; f.c., final concentration; Hb, hemoglobin; PBS, phosphate-buffered saline; PE, phycoerythrin; PRRs, pattern recognition receptors; RPMI, Roswell Park Memorial Institute; ROTEM, rotational thromboelastometry; RT, reverse transcription; TF, tissue factor; TLR, toll-like receptor; tPA, tissue plasminogen activator; VWF, von Willebrand factor.

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from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), and human Hb were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biowest (Nuaillé, France). Epinephrine was purchased from Arkray (Shiga, Japan). Owren's Veronal buffer was purchased from Dade Behring Marburg GmbH (Marburg, Germany). Normal human plasma (Coagtrol N), coagulation factor (F) VIII- and FIX-deficient plasma, and phospholipid activator were purchased from Sysmex (Kobe, Japan). anti-human cluster of differentiation (CD)142 (also known as TF) antibodies for western blotting were purchased from American Diagnostica (Stamford, CT, USA). Phycoerythrin (PE)-conjugated anti-CD142 antibody and its isotype control for flow cytometry were purchased from BD Pharmingen (San Diego, CA, USA).

2.2. Blood sampling

Blood samples were drawn from healthy volunteers who had not taken any medication for at least 2 weeks before the experiments. Informed consent was obtained from each participant. Collected blood was treated with 3.8% sodium citrate (9:1 v/v) as an anticoagulant.

2.3. Rotational thromboelastometry

Whole-blood coagulation profiles were obtained by rotational thromboelastometry (ROTEM) (Pentapharm GmbH, Munich, Germany), which is a viscoelastic hemostatic assay that measures global viscoelastic properties of whole blood clots formed under low-shear conditions resembling sluggish venous flow [11]. The assay was performed as previously described, with slight modifications [12]. Briefly, PBS (control) or ethanol [final concentration (f.c.) 50 or 100 mM] was added to citrated whole blood (300 μl), followed by incubation at 37 °C for 10 min. ROTEM measurements were initiated after addition of 20 μl of 0.2 M $CaCl_2$ in the non-activated TEM mode.

Clotting activity was determined based on clotting time (CT) and clot formation time (CFT). CT (time until the detection of clot firmness at 2-mm amplitude) is similar to whole blood clotting time, whereas CFT (time until the detection of clot firmness at 20-mm amplitude) is a measure of the speed at which a clot forms with a certain viscoelastic strength, and reflects the rate of increase of elastic shear modulus in the sample; thus, CT + CFT is an index of procoagulant activity.

2.4. Platelet aggregation

To obtain platelet-rich plasma (PRP), blood samples were centrifuged at 150*g* for 10 min. The platelet concentration of PRP was adjusted to 3×10^8/ml, and residual blood was centrifuged at 400*g* for 10 min to obtain platelet-poor plasma (PPP). To measure epinephrine-induced platelet aggregation, 250 μl of PRP was transferred to a cuvette. PBS or ethanol (f.c. 50 or 100 mM) was then added, followed by incubation at 37 °C for 10 min. After addition of epinephrine (f.c. 50 μM), the increase in light transmission at 37 °C was measured using a dual-channel aggregometer (NKK Hematracer 1; Nikko Bioscience, Tokyo, Japan) with stirring at 1000 rpm. The light transmission values of PRP and PPP were set at 0% and 100%, respectively.

2.5. Measurement of von Willebrand factor levels by enzyme-linked immunosorbent assay

Citrated whole blood was aliquoted into individual polypropylene tubes. PBS (control) or ethanol (f.c. 50 and 100 mM) was then added, followed by incubation at 37 °C for 2 h. Plasma samples were obtained by centrifugation at 400*g* for 10 min, and plasma von Willebrand factor (VWF) levels were determined using a VWF enzyme-linked immunosorbent assay (ELISA) kit (Assaypro, St. Charles, MO, USA) according to the manufacturer's instructions.

2.6. Determination of FVIII, FIX, and tissue factor activity

Plasma samples were obtained in the same manner as for the VWF assay. FVIII and FIX activities (FVIII:C and FIX:C, respectively) were measured with a one-stage activated partial thromboplastin time clotting assay using a coagulometer (KC4; Amelung, Lemgo, Germany). To measure FVIII:C and FIX:C, a standard curve was generated using Coagtrol N in serial doubling dilutions (1:10 to 1:1280) in Owren's Veronal buffer. TF activity was evaluated using the Tissue Factor Chromogenic Activity kit (Assaypro) according to the manufacturer's instructions.

2.7. Evaluation of hemolysis

Plasma samples were obtained as described above. The degree of hemolysis was determined using an Hb colorimetric detection kit (Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.8. Preparation of monocyte samples

Peripheral blood mononuclear cells were separated from erythrocytes and granulocytes by centrifugation on a density gradient using Lymphoprep (Axis-Shield, Oslo, Norway) and then resuspended in isolation buffer (Ca^{2+}- and Mg^{2+}-free PBS supplemented with 0.1% BSA and 2 mM EDTA). Human monocytes were isolated by the depletion of non-monocytes (negative selection) using Dynabeads Untouched Human Monocytes (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and were incubated in a 12-well culture plate in RPMI-1640 containing 10% FBS with PBS (control) or Hb (f.c. 5 mg/ml) in the presence or absence of ethanol (f.c. 50 or 100 mM) at 37 °C for 4 h.

2.9. RNA isolation

Total RNA was extracted from monocyte samples using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and treated with DNase I (Qiagen) to eliminate contaminating genomic DNA. RNA concentration was measured at a wavelength of 260 nm with a DU730 spectrophotometer (Beckman Coulter, Brea, CA, USA).

2.10. Real-time reverse transcription PCR

Total RNA (100 ng) was reverse-transcribed into cDNA using the Superscript RT kit (Life Technologies, Grand Island, NY, USA), and quantitative real-time revere transcription (RT)-PCR analysis was performed on a StepOne system (Applied Biosystems, Foster City, CA, USA) using Taqman probes and primers for the human TF gene (assay ID: Hs01076032_m1). Human hypoxanthine phosphoribosyltransferase 1 (assay ID: Hs02800695_m1) served as an endogenous control.

2.11. Western blotting

Treated monocytes were homogenized with tissue protein extraction reagent containing phosphatase and protease inhibitors (Thermo Fisher Scientific, Rockford, IL, USA). Protein concentration was determined with the bicinchoninic acid assay (Thermo Fisher Scientific), and 10 μg of total protein was used for western blotting