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Substance P–Neurokinin-1 receptor interaction upregulates monocyte tissue factor

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ABSTRACT

Monocytes play an important role in hemostasis. In this study, the prothrombotic effects of the neuropeptide substance P (SP) on human monocytes through neurokinin-1 receptor (NK1-R) were characterized. SP upregulated monocyte tissue factor (TF), the major coagulation cascade stimulator, in a concentration and time dependent manner. Specific inhibition of NK1-R completely blocked TF expression. Monocytes stimulated by SP released cytokines and chemokines. When monocytes were stimulated with cytokines or chemokines, TF was expressed by the cytokines (GM-CSF, IFN-γ and TNF-α). Cytokines may play a major role in the mechanism of SP induced monocyte TF expression. NK1-R antagonists (NK1-RA) may have a role in developing novel therapeutic approaches to patients vulnerable to vaso-occlusive disorders.

1. Introduction

Plasma levels of the neuropeptide SP (Michaels et al., 1998; Douglas and Leeman, 2011; Douglas et al., 2001, 2008; Fundeburg et al., 2010) and TF (Key et al., 1998; Michaels et al., 1998; Fundeburg et al., 2010) are elevated in patients with vaso-occlusive disorders including sickle cell disease (SCD), human immunodeficiency virus (HIV) infection, and other conditions (Douglas and Leeman, 2011). A number of newly identified regulatory molecules including gas6 (Hurtado and de Frutos, 2010), CD40L (Andre et al., 2002), semaphorin 4D (Zhu et al., 2007), semaphorin 3A (Kashiwagi et al., 2005) and ephrins/eph kinases (Prevost et al., 2002) were discovered and have a role in the mechanism of thrombus formation. SP treatment stimulates activation and aggregation of platelets and platelets contain SP immunoreactivity that is released upon activation (Jones et al., 2008). In this study we demonstrate that SP, a member of the tachykinin family, may also contribute to thrombus formation by stimulating the expression of TF on monocytes.

SP is an important neuroimmune stimulator of innate immune functions of monocytes/macrophages (Bremer and Leeman, 2010), released from the nucleus of the solitary tract in the brainstem and other central nervous system (CNS) sites (Bost, 2004). In addition, it is a potent pro-inflammatory mediator which plays an important role in inflammation and HIV infection (Michaels et al., 1998). SP mediates the biologic responses through an interaction with the NK1-R, a G-protein coupled receptor characterized by seven transmembrane domains (Lucey et al., 1994). NK1-R is present on immune cells (Khawaja and Rogers, 1996; Ho et al., 1997) such as monocytes/macrophages (Lucey et al., 1994; Ho et al., 1997), neutrophils (Wozniak et al., 1989), T and B lymphocytes (Payan et al., 1984; Stanisz et al., 1987; Lai et al., 1998), and mast cells (Shanahan et al., 1985). There are two isoforms of NK1-R: full length NK1-R (NK1-RF) composed of 407 amino acids (aa), and a naturally occurring splice variant with a truncated C-terminus which is designated truncated NK1-R (NK1-RT) (Fong et al., 1992; Satake and Kawada, 2006; Zhang et al., 2006), which has a 311 aa sequence with a short carboxyl terminal sequence, extending only 7 aa residues after the end of the seventh transmembrane segment (Lai et al., 2006; Tuluc et al., 2009). The remaining aa sequence of the NK1-RT isoform is identical to that of NK1-R-F21 (Fong et al., 1992). This interaction occurs in several cell systems and is involved in exocrine gland secretion, endocrine secretion, pain transmission, vasodilatation, connective tissue cell proliferation, and neuroimmune modulation (Bost, 2004; Satake and Kawada, 2006). The NK1-RT probably mediates prolonged cellular responsiveness after stimulation and is resistant to homologous desensitization in comparison to the NK1-RF isoform because the missing C-terminal domain is essential for internalization, receptor desensitization and recycling (Tuluc et al., 2009). NK1-RA are a novel therapeutic approach to stress, mood therapy, and control of emesis (Blume et al., 2011; Douglas and Leeman, 2011). NK1-RA

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(n-acetyl-L-tryptophan), significantly reduced edema formation and blood–brain barrier (BBB) permeability at 24 h post-ischemia and significantly improved functional outcome as assessed over 7 days when administered 4 h after onset of ischemic stroke in an animal model (Turner et al., 2011).

Tissue factor, also known as factor III, thrombokinase, or CD142, is a protein present in sub-endothelial tissue, platelets, and leukocytes that is necessary for the initiation of thrombin formation from the zymogen prothrombin. Full-length Tissue Factor (flTF), a 47 kDa transmembrane glycoprotein, is encoded by a mature mRNA transcript that consists of six exons: exon 1 encodes the N-terminal signal sequence, exons 2–5 encode the extracellular domain (aa 1–219), exon 6 encodes the transmembrane region (aa 220–244) and cytoplasmic tail (aa 245–263) (van den Berg and Versteeg, 2010). TF enables cells to initiate the blood coagulation cascades, and it functions as the high-affinity receptor for the coagulation factor VII. In addition, TF promotes the formation of factor VIIa, which converts factor X to factor Xa, which cleaves prothrombin to thrombin. Normally, TF is detectable in the adventitial cells within the vessel wall only (Drake et al., 1993). TF may be totally encrypted, which would allow circulating TF to be present without leading to generalized coagulation.TF, however can be induced by endotoxin on the cell surface of the endothelial cell, monocyte, platelet or the mononuclear cell by cleaved high molecular weight Kininogen (HKa) (Khan et al., 2010).

Mononuclear phagocytes are key inflammatory components of the cellular immune response; they are involved in cytokine production which include IL-1, IL-6, TNF-α and IL-12 (Michaels et al., 1998; Khan et al., 2006; Douglas and Leeman, 2011). Cytokines interact with all phases of the immune system (Douglas and Leeman, 2011) and are known stimulators for TF expression in monocytes (Khan et al., 2010). Cytokines enhance adhesion of leukocytes to endothelium and may play a role in vaso-occlusive events (Michaels et al., 1998). SP is also a known stimulator of TNF-α release and a promoter of IL-8. Plasma SP is increased in SCD (Michaels et al., 1998). SP and its preferred receptor, NK-1R, are central mediators in the interaction between the immune system and the nervous system (Douglas et al., 2001).

Involvement of neuropeptides in the pathophysiology of thrombus formation has not been characterized. We have investigated the effects of SP on the stimulation of human monocyte TF expression through NK1-R. This pathway may be a new stimulator for the mechanism(s) of thrombus formation in vaso-occlusive disorders.

2. Materials and methods

2.1. Human monocytes

Human peripheral blood monocytes were obtained from healthy adult donors (n = 78) from the Penn Center for AIDS Research Immunology Core (Philadelphia, PA, USA). Monocytes were purified from Apheresis products by using RosetteSep Human Monocyte kits purchased from STEMCELL Technologies Inc. (Vancouver, BC, Canada). The cells were suspended in Hanks' balanced salt solution (HBSS), 0.1% Human Serum Albumin (HSA). These cell populations were >97% monocytes, as determined by nonspecific esterase staining and fluorescence-activated cell sorting (FACS) analysis using monoclonal antibodies (MAB) against CD14 (Leu-M3) and low-density lipoprotein specific for monocytes. Before activation with SP, the cell population was checked for activation status at the time of preparation by using CD69, CD16 (BD Biosciences, San Jose, CA.), CD142 (anti-Human TF, American Diagnostica, Stamford, CT.) of the CD45 + CD14 + (Monocyte) population using a BD LSRII flow cytometer and FACS Diva acquisition and analysis software (BD Biosciences, San Jose, CA). The activated population (positive) was measured as a percentage of positive target cell events above the isotype (negative) control signal. Activated cell populations were <0.7%.

2.2. Proteins and antibodies

SP was purchased from Sigma (St. Louis, MO). The NK1-RA, aprepitant (Emend®), manufactured by Merck, was purchased through the Children's Hospital of Philadelphia Pharmacy and purified by chromatography (Chernova et al., 2009). The nonpeptide SP antagonist, CP-96345, and its 2R, 3R inactive enantiomer CP-96,344 were used as previously described (Lai et al., 2006). Recombinant human cytokines: GM-CSF, IFNγ, TNF-α; chemokines: MIP-1α, MIP-1β, RANTES; and neutralizing MAB against GM-CSF, IFNγ, TNF-α and corresponding isotype controls either IgG2a or IgG1 were purchased from R & D Systems (Minneapolis, MN). Imubind Tissue Factor ELISA kit was purchased from American Diagnostica (Stamford, CT).

2.3. Control of lipopolysaccharide (LPS) contamination

Sterile and pyrogen-free working conditions were maintained to minimize any contamination by LPS. LPS assayed using QCL-1000 Chromogenic Limulus Amebocyte lysate kit from BioWhittaker (Walkersville, MD) indicated that all proteins and reagents had <0.01 EU/ml LPS.

2.4. Flow cytometric analysis and ELISA for TF expression

LPS-free SP (0.001, 0.01, 0.1, 1.0, 5.0 and 10.0 μM) was incubated for 4 h at 37 °C with 2×10^6/ml monocytes. Separately, LPS-free SP (5 μM) was incubated for 0, 30, 60, 120, 180, 240 and 360 min at 37 °C with 2×10^6/ml monocytes suspended in HBSS. Following this incubation, the cell suspension was centrifuged at 13,000 *g* for 5 min and the supernatant was used to assay cytokine production using BD Cytometric Bead Array (BD CBA) Flex kits: GM-CSF, IFN-γ, TNF-α, MIP-1α, MIP-1β, and RANTES (BD Biosciences, San Jose, CA). Each capture bead in the array has a unique fluorescence intensity (APC vs. APC-Cy7) and is coated with a capture antibody specific for a single analyte. A combination of the selected beads is mixed with the supernatant and a mixture of detection antibodies that are conjugated to a reporter molecule PE to form sandwich complexes. Following incubation and subsequent washing, the samples were acquired on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). Each bead's detection antibody MFI was measured on the PE channel of the flow cytometer. Quantitative cytokine levels (pg/ml) of each supernatant were determined from a standard curve generated using FCAP Array software (BD Biosciences, San Jose, CA). The pellet was re-suspended in 1% Triton X-100 in 0.05 M Tris and 0.1 M NaCl (pH 8.5) and stirred for 12 h at 4 °C. The suspension was centrifuged at 13,000 *g* for 5 min to separate cell debris. TF antigen levels were measured in supernatant using the Imubind Tissue Factor ELISA kit (American Diagnostica, Greenwich, CT).

2.5. Thrombin generation assay (TGA)

Thrombin generation was measured in platelet poor plasma according to previously described methods with some modifications (Hemker et al., 2003). Severe hemophilia A plasma (<1% FVIII activity; George King Biomed) was used in order to analyze TF-dependent thrombin generation in these assays. Forty μl of plasma was mixed in a black microtiter plate with 10 μl of either SP-stimulated cell lysate or Innovin diluted in phospholipid vesicles (Phosphatidylcholine:PC; Phosphatidylserine:PS: 75:25 PC:PS, 4 μM). Fifty μl of Z–Gly–Gly–Arg–AMC (1 mM in 15 mM $CaCl_2$) was added to initiate coagulation and thrombin generation was measured by monitoring fluorescence for 90 min at 37 °C in a Spectramaz M2e (Molecular Devices) with excitation and emission wavelengths of 360 nm and 460 nm, respectively. Thrombin concentrations were calculated from the raw fluorescence data using a thrombin calibrator (Technothrombin TGA calibrator).