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Recombinant tissue plasminogen activator enhances microparticle release from mouse brain-derived endothelial cells through plasmin

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ABSTRACT

Thrombolysis with recombinant tissue plasminogen activator (rt-PA) is currently the only approved pharmacological strategy for acute ischemic stroke. However, rt-PA exhibits vascular toxicity mainly due to endothelial damage. To investigate the mechanisms underlying rt-PA-induced endothelial alterations, we assessed the role of rt-PA in the generation of endothelial microparticles (EMPs), emerging biological markers and effectors of endothelial dysfunction.

The mouse brain-derived endothelial cell line bEnd.3 was used. Cells were treated with rt-PA at 20, 40 or 80 μg/ml for 15 or 24 h, and EMPs were quantified in the culture media using Annexin-V staining coupled with flow cytometry. Rt-PA enhanced EMP release from bEnd.3 cells with a maximal increase at the 40 μg/ml dose for 24 h (+78% compared to controls). Using tranexamic acid and aprotinin we demonstrated that plasmin is responsible for rt-PA-induced EMP release. The p38 MAPK inhibitor SB203580 and the poly(ADP-ribose)polymerase (PARP) inhibitor PJ34 also reduced rt-PA-induced EMP production, suggesting that p38 MAPK and PARP are downstream intracellular effectors of rt-PA/plasmin. Rt-PA also altered through plasmin the morphology and the confluence of bEnd.3 cells. By contrast, these changes did not implicate p38 MAPK and PARP.

This study demonstrates that rt-PA induces the production of microparticles by cerebral endothelial cells, through plasmin, p38 MAPK and PARP pathways. Determining the phenotype of these EMPs to clarify their role on the endothelium in ischemic conditions could thus be of particular interest.

1. Introduction

The only pharmacological treatment for acute ischemic stroke is currently recombinant tissue plasminogen activator (rt-PA), a thrombolytic administered to achieve clot lysis and thus to allow tissue reperfusion. Unfortunately, rt-PA efficacy in clinical practice remains very low because of a modest recanalization rate and the occurrence of arterial re-occlusion [1–3]. In addition, the use of rt-PA is limited by the risk of post-ischemic intracerebral haemorrhage, also called haemorrhagic transformation, which represents the major complication of this thrombolytic therapy [4]. This well-known vascular toxicity of rt-PA was reported to be related to the direct proteolytic activity of rt-PA and/or to the activation of matrix metalloproteinases (MMP), among the most frequently proposed mechanisms [5,6]. The ubiquitous nuclear enzyme poly(ADP-ribose)polymerase (PARP) is another recently identified downstream effector of rt-PA implicated in its vascular toxicity. Indeed, rt-PA was shown to increase post-ischemic PARP activity [7], and we demonstrated that a PARP inhibitor reduced rt-PA vascular toxicity after experimental cerebral ischemia [8,9].

Further investigation of the mechanisms of endothelial damage occurring after rt-PA treatment represents today a major challenge. In this context, we hypothesized that rt-PA could also mediate its vascular toxicity through an increase in the production of endothelial microparticles (EMPs).

Microparticles are membrane vesicles produced by cell activation or apoptosis. They are characterized by their size (0.1–1 μm) and by the expression of phosphatidylserine on their outer leaflet [10]. Circulating microparticles, emitted by different cells such as platelets, endothelial

Abbreviations: APC, allophycocyanin; BBB, blood brain barrier; DAPI, 4′,6-diamidino-2-phenylindole; EMPs, endothelial microparticles; FITC, fluorescein isothiocyanate; ICAM-1, intercellular cell adhesion molecule; LDH, lactate dehydrogenase; LRP, lipoprotein receptor-related protein; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinases; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; PJ34, *N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-(*N*,*N*-dimethylamino)acetamide hydrochloride; ROCK, Rho-associated protein kinase; rt-PA, recombinant tissue plasminogen activator; TXA, tranexamic acid; VEGF, vascular endothelial growth factor.

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cells, leukocytes and red blood cells are both biomarkers of cell injury and an important emerging class of cell-cell messengers involved in various pathologies, especially in cardiovascular diseases [10–12]. Experimental and clinical data demonstrated that microparticles contribute to inflammation, thrombosis, vascular tone and angiogenesis [13–15].

Although endothelial microparticles represent a small fraction of the overall pool of plasma microparticles, an increase in circulating EMP levels constitute an emerging marker of endothelial dysfunction. With regard to stroke, clinical studies have shown an increase in circulating microparticles, more particularly particles derived from endothelial cells and platelets [16–18]. Moreover, a correlation has been reported between circulating EMPs of particular phenotypic profiles and the severity of the outcome after acute ischemic stroke [19–21].

The aim of the present study was thus to investigate whether rt-PA could enhance the production of EMPs using bEnd.3 cells, a mouse brain-derived endothelial cell line. The involvement of plasminogen/plasmin in such effects of rt-PA was examined. The contribution of the main signalling pathways involved in membrane vesiculation and EMP release (i.e. caspases, Rho-associated protein kinase (ROCK) and p38 Mitogen-Activated Protein Kinase (MAPK)) [22–24], and the potential role of PARP were also studied.

2. Materials and methods

2.1. Materials

rt-PA (Actilyse®) was purchased from Boehringer-Ingelheim France (Paris, France), aprotinin (Trasylol®) from Bayer (Lyon, France), the pan-caspase inhibitor Z-VAD-FMK (FMK001) from R&D systems (Lille, France), Alexa Fluor® 555 Phalloidin (A34055) from Invitrogen (Fisher Scientific, Illkirch, France), and propidium iodide (P16063) from Invitrogen. Annexin-V coupled with fluorescein isothiocyanate (AnnV-FITC; IM3614) was obtained from Beckman Coulter (Villepinte, France), and Annexin-V conjugated to allophycocyanin (AnnV-APC; 550474) from BD Biosciences (Le Pont de Claix, France). PJ34 (P4365), tranexamic acid (857653), the ROCK inhibitor Y27632 (Y0503), the p38 MAPK pathway inhibitor SB203580 (S8307), the *in vitro* toxicology assay kit MTT based (TOX-1) and biochemical reagents unless specified were obtained from Sigma (Sigma-Aldrich, Saint Quentin Fallavier, France).

2.2. Cell culture and evaluation of morphological changes

Transformed mouse brain endothelial cells bEnd.3 (ATCC® CRL-2299™, Manassas, Virginia, USA) purchased from Sigma (Sigma-Aldrich, Saint Quentin Fallavier, France) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal calf serum, 1 mM sodium pyruvate, 1% non-essential amino acids, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified 5% CO_2 incubator at 37 °C. Cell line passages of <30 were used for all experiments.

bEnd.3 cells were cultured in 96 well plates (4×10^4 cells/well) to assess cell viability, and in 24 well plates (2×10^5 cells/well) for the evaluation of EMP release and morphological changes.

Cell morphology was observed with an inverted-microscope (VWR, Fontenay sous Bois, France), and images were taken (Moticam camera 580 5.0 MP, Motic, Barcelona, Spain). For fluorescent cytochemistry, cells were cultured on glass coverslips coated with poly-D-lysine in 24 well plates; 24 h after treatments, cells were washed with phosphate-buffered saline (PBS 0.01 M, pH 7.4) and fixed for 12 min using 4% paraformaldehyde in PBS. Cells were permeabilized for 10 min with 0.5% Triton X-100 in PBS, incubated for 15 min with Alexa Fluor® 555 Phalloidin (1/50) for actin staining, and for 5 min with 4′,6-diamidino-2-phenylindole (DAPI, 0.2 μg/ml) for nuclei staining. Coverslips were mounted with Mowiol® on glass slides and imaged using an epifluorescence microscope (Olympus BX41 Fluorescence Microscope Hamburg, Germany).

2.3. EMP isolation and detection by flow cytometry

EMPs were isolated and identified as elements with a size <1 μm and >0.1 μm that were positively labelled by AnnV in a calcium-dependent manner, according to Vion et al. [25]. All buffers used were filtered through 0.1 μm filters. Media were collected (1.5 ml) and cell debris was eliminated by centrifugation (600 g; 15 min; 4 °C). The resulting supernatant was then ultracentrifuged (20 500 g; 90 min; 4 °C) to pellet EMPs, which were resuspended in 150 μl of PBS and stored at −80 °C until analysis.

Samples were analysed using a highly sensitive flow cytometer (Gallios, Beckman Coulter, Brea, CA, USA) calibrated with fluorescent beads (0.3, 0.5 and 0.9 μm in diameter; Megamix-Plus FSC 7802, Biocytex, Marseille, France) in order to detect events <1 μm diameter (Suppl. Fig. 1). Twenty microliters of EMP resuspended pellet were incubated with 100 μl of AnnV-FITC in AnnV binding buffer (0.04 μg/ml AnnV-FITC, 10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4) 30 min at 4 °C in the dark. As calcium is required for AnnV binding, control experiments were performed in calcium free buffer (10 mM HEPES, 140 mM NaCl, 1,6 mM EDTA, pH 7.4). Flow-count fluorospheres (Spherotech, Illinois, USA) were used to establish EMP concentration in the sample. Events were identified on forward scatter and fluorescence dot representation. Data were analysed using Flow-Jo software (version 10.0.7; TreeStar, Ashland, OR, USA). The experiments were carried out in several series. EMP levels are expressed as a percentage relative to control cells of each series of experiments.

Annexin V negative EMPs together with the background noise of the flow cytometer represented <10% of the total number of events.

Levels of apoptotic bodies were estimated as AnnV+ propidium iodide (PI)+ events. For this purpose, samples were incubated with AnnV-APC for 30 min in the dark at room temperature and PI was added right before the measurement (1 μg/ml). <2% of the events were AnnV+ PI+ (data not shown) and were therefore negligible.

2.4. Determination of cell viability

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, which measures the cell viability by the determination of the mitochondrial activity in living cells, was performed with MTT based TOX-1 kit according to the manufacturer's instructions on cells in 96 well plates. Briefly, 15 h or 24 h after treatment, 10 μl of MTT was added to each well. After a 3 hour-incubation at 37 °C, 100 μl of solubilisation solution was added to each well. The reduction of MTT to formazan by mitochondrial dehydrogenases within viable cells was measured using a microplate reader (SPECTRO star^{Nano}, BMG LABTECH, Champigny sur Marne, France) at a wavelength of 570 nm and a correction of interference at 690 nm. The experiments were carried out in several series. Viability is expressed as a percentage relative to control cells of each series of experiments.

2.5. Cell treatment

After 24 h of culture, confluent cells were incubated for 15 h or 24 h with a serum free medium (control cells) or with the treatments below diluted in the same serum free medium.

In a first study, cells were treated with a range of rt-PA concentrations (20, 40 and 80 μg/ml). The concentration of 40 μg/ml corresponds to the blood concentration of rt-PA reached after a 30-minute intravenous injection of rt-PA at 10 mg/kg in rats, which is the protocol used for experimental thrombolysis [26]. This concentration was framed by two other doses, the lowest of 20 μg/ml being the concentration of rt-PA most often encountered in studies using bEnd.3 cells [27–29]. After