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Induction of Vascular Endothelial Growth Factor and Matrix Metalloproteinase-9 via CD47 Signaling in Neurovascular Cells

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Abstract

Neurovascular injury comprises a wide spectrum of pathophysiology that underlies the progression of brain injury after cerebral ischemia. Recently, it has been shown that activation of the integrin-associated protein CD47 mediates the development of blood–brain barrier injury and edema after cerebral ischemia. However, the mechanisms that mediate these complex neurovascular effects of CD47 remain to be elucidated. Here, we compare the effects of CD47 signaling in brain endothelial cells, astrocytes, and pericytes. Exposure to 4N1 K, a specific CD47-activating peptide derived from the major CD47 ligand thrombospondin-1, upregulated two major neurovascular mediators, vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9), in brain endothelial cells and astrocytes. No changes were detected in pericytes. These findings may provide a potential mechanism for CD47-induced changes in blood–brain barrier homeostasis, and further suggest that CD47 may be a relevant neurovascular target in stroke.

Keywords

Endothelial cell; Pericyte; Astrocyte; Blood–brain barrier; Stroke; Integrin

Introduction

Ischemic stroke remains a challenging clinical problem with limited therapeutic options. Many targets based on excitotoxicity and oxidative stress appear to have narrow treatment windows after stroke onset, which means that reaching patients in time is always a problem [1, 2]. In contrast to acute neuronal cell death; however, some experimental data now suggest that blood–brain barrier leakage and delayed neuroinflammation may mediate prolonged brain injury after ischemia [1, 3]. Hence, understanding the mechanisms of neurovascular injury after stroke is extremely important.

Recently, we showed that CD47 (also known as integrin-associated protein) knockout mice developed less edema, had reduced infiltration of neutrophils, and subsequently suffered less secondary brain injury after focal cerebral ischemia [4]. These findings suggested that CD47 can somehow contribute neurovascular injury in the brain. But the underlying mechanisms are not fully understood. In the present study, we asked how activation of CD47 signaling could affect the three major cell types of the neurovascular interface, comprising brain endothelium, astrocytes and pericytes. We specifically examined responses in two major representative neurovascular mediators, i.e. vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9). Our initial findings suggest that CD47 signaling is active within cells of the neurovascular interface, and these VEGF and MMP responses may partly underlie the ability of CD47 to trigger blood–brain barrier leakage in cerebral ischemia.

Materials and Methods

Reagents

Invitrogen (Carlsbad, CA, USA): RPMI1640, DMEM, trypsin–EDTA, L-Glutamine, fetal bovine serum (FBS), sodium pyruvate, MEM non-essential amino acids, MEM vitamins, and antibiotics. BD Biosciences (San Jose, CA, USA): NuSerum. The 4N1 K peptide corresponding to the cell-binding domain of TSP-1 (KRFYVVMWKK) was from Sigma Genosys, and the control peptide 4NGG (KRFYGGMWKK) was from BACHEM (Torrance, CA, USA). 4N1 K or 4NGG was dissolved in sterile ddH₂O at a concentration of 100 mg/ml as a stock solution. This stock was aliquoted and stored at –80°C. Rabbit anti-CD47 polyclonal antibody was from Santa Cruz (Santa Cruz, CA, USA). Alexa Fluor 488 donkey anti-rabbit IgG was from Invitrogen Molecular Probes (Eugene, OR, USA). Human and rat VEGF ELISA kits were purchased from R&D (Minneapolis, MN, USA). 10% Zymogram (Gelatin) gel, Zymogram Renaturing buffer and developing buffer were from Invitrogen (Carlsbad, CA, USA).

Neurovascular Cell Cultures

A human brain microvascular endothelial cell line (kind gift from Monique Stins, Johns Hopkins University) was used, which was previously confirmed to express brain endothelial phenotypes [5, 6]. Endothelial cells were cultured in RPMI1640 supplemented with 10% FBS, 10% NuSerum, 1 mM sodium pyruvate, MEM non-essential amino acids, MEM vitamins, and 100 units/ml penicillin/ streptomycin. Human brain vascular pericytes, pericyte medium, and pericyte growth supplement were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Pericytes were cultured in pericyte medium containing 2% FBS and pericyte growth supplement. Cells were seeded in poly-L-lysine-coated 6-well plates at 5,000 cells/cm², with medium change every other day until 50% confluence. Then medium is changed daily until 80% confluence. Cells are subcultured when they are over 90% confluent. Primary astrocyte cultures were prepared from cerebral cortices of 2-day-old neonatal Sprague–Dawley rats as previously described [7]. Briefly, dissociated cortical cells were suspended in DMEM containing 10% FBS and plated on collagen-coated 25 cm² flasks at a density of 600,000 cells/cm². Monolayers of type 1 astrocytes were obtained 12–14 days after plating. Remaining microglia and neurons were removed by shaking and

changing the medium. Astrocytes were dissociated by trypsinization and then reseeded on collagen-coated plates at a density of 20,000 cells/cm². In this system, more than 95% of the cells were identified as type 1 astrocytes by GFAP staining and their flattened, polygonal morphology.

Immunocytochemistry

All cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and blocked with 5% normal horse serum in PBS for 1 h. Then the cells were incubated with rabbit anti-CD47 polyclonal antibody (1:200) at 4°C overnight. After washing, the cells were incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:1,000) for 1 h at room temperature.

VEGF ELISA Assay

Cells were incubated with different doses of 4N1 K (25, 50, and 100 µg/ml) or 4NGG (100 µg/ml) for 24 h. The levels of VEGF in culture supernatant were determined by ELISA kits, according to the manufacturer's instructions.

Gelatin Zymography

MMP-2 and MMP-9 in cell culture media were measured by gelatin zymogram following standard procedures. Briefly, after incubation with different concentrations of 4N1 K or 4NGG, conditioned media were collected and centrifuged at 5,000 rpm for 5 min at 4°C to remove cells and debris, and followed by concentrating using Microcon (Millipore) with a 10 kDa pore diameter cutoff. Equal volume of media were loaded and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. After electrophoresis, renaturing and incubation, Coomassie Blue staining and destaining, optical density of gel bands were quantified using NIH Image, and expressed as fold increase versus non-treatment controls.

Statistical Analysis

Three to five independent experiments were performed. Data were analyzed using ANOVA with Tukey post hoc tests (SPSS version 11.5). Statistical significance was set at $P < 0.05$.

Results

Immunocytochemistry confirmed the expression of CD47 in all three types of neurovascular cells that we examined, including a human brain endothelial cell line, primary rat cortical astrocytes, and human brain vascular pericytes (Fig. 1). To assess the comparative effects of CD47 signaling, we used the specific activating peptide 4N1 K, which is derived from the major CD47 ligand thrombo-spondin-1 (TSP-1). All cells were exposed to 4N1 K for 24 h. The inactive scrambled peptide 4NGG was used as controls. Exposure to 4N1 K or 4NGG did not result in any cytotoxicity nor changes in cell number or morphology (data not shown).

First, we measured the response of VEGF. ELISAs were used to quantify the secretion of VEGF into the culture media after 4N1 K treatment. Exposure to different doses of 4N1 K (25, 50, and 100 µg/ml) for 24 h triggered a clear upregulation of VEGF release in brain endothelial cells and astrocytes (Figs. 2a, b). The VEGF response was much higher in

astrocytes compared to endothelial cells (e.g. increased 80% in astrocytes versus 28% in endothelial cells after 100 µg/ml of 4N1 K treatment). No clear changes are detected in brain vascular pericytes (Fig. 2c).

Next, we determined the effect of CD47 signaling on the production of MMPs. Conditioned media were collected from brain endothelial cells, astrocytes and pericytes treated with different concentrations of 4N1 K (0, 50, 100 and 200 µg/ml) for 24 h. MMP levels were quantified using gelatin zymography. Two major gelatinolytic bands at approximately 72 and 92 kDa were detected in the conditioned media from all cells (Fig. 3). Comparison with standards suggested that these likely corresponded to pro-forms of MMP-2 and MMP-9, respectively. Cleaved or active forms of MMPs were not observed. In brain endothelial cells and astrocytes, activation of CD47 with 4N1 K resulted in about threefold and twofold elevation of pro-MMP-9 levels (Fig. 3a, b). There appeared to be no further processing or activation of pro-MMP-9 bands. In contrast, 4N1 K did not appear to trigger MMP-9 release or activation in any of the pericyte experiments (Fig. 3c). Finally, in all 3 cell types, there were no statistically significant changes in MMP-2 levels (data not shown).

Discussion

CD47 (also known as integrin-associated protein) is a member of the immunoglobulin superfamily that plays an important role in blood cell homeostasis and inflammation. By interacting with various beta integrins and its two natural ligands, thrombospondins (TSPs) and signal inhibitory regulatory protein α (SIRP α), CD47 modulates monocyte motility, leukocyte adhesion and migration, platelet activation, phagocytosis, and cell death [8].

Recently, we reported that CD47 knockout mice had less edema, inflammation and secondary brain injury after transient focal cerebral ischemia [4]. In the present study, we sought to investigate the underlying mechanisms involved. We showed that cellular activation of CD47 with 4N1 K, a peptide derived from the major CD47 ligand TSP-1, upregulated neurovascular mediators such as VEGF and MMP-9. The effects of TSP-1-CD47 signaling appeared cell-specific. Only brain endothelial cells and astrocytes increased their production of these mediators, whereas vascular pericytes did not respond. Both VEGF and MMP-9 can profoundly affect blood-brain barrier permeability and homeostasis [9–12]. Hence, our findings provide a possible mechanistic basis for CD47 patho-physiology in cerebral ischemia, and further suggest that blocking TSP-1-CD47 signaling may represent a potential therapeutic approach for blunting VEGF and MMP-9, and thus ameliorating neurovascular injury after stroke. It may also be interesting to speculate in the role of CD47 in a broader spectrum of CNS disease. Increasingly, it is recognized that BBB dysfunction may also be involved in the pathogenesis of neurodegenerative diseases [13, 14]. Neuronal disturbances and cell death may be exacerbated by alterations in tight junctions, neurovascular transport systems and enzymes, aberrant angiogenesis and vessel regression, brain hypoperfusion, and inflammatory responses, thus impairing synaptic function in Alzheimer's disease, Parkinson's disease, multiple sclerosis, and others [14].

In the context of tissue ischemia, the primary ligand for CD47 may be TSP-1 [15]. TSP-1 belongs to a family of multidomain, calcium-binding extracellular glycoproteins which

comprises five genes encoding proteins, TSP-1 through TSP-5 [16]. TSP-1 plays a major role in cell–matrix and cell–cell interactions that influence platelet function, angiogenesis, tumor biology, wound healing, and vascular disease [17]. Blockade of TSP-1-CD47 signaling alleviates tissue ischemia, and may provide a novel and accessible target for the treatment of cardiovascular disease [18]. Similarly, TSP-1-CD47 signaling may underlie brain injury in stroke as well. Acute stroke patients with elevated TSP-1 levels seemed to be more susceptible to further thrombosis and worsening [19].

CD47 may be an attractive target in stroke because it seemed to fulfill the requirement that any target should be manifest in all components of the neurovascular unit. CD47 mediates apoptotic and oxidative cell death in neurons [20, 21]. Activation of CD47 induces cytotoxicity in brain endothelial cells [22]. And CD47 is known to contribute to inflammation and platelet aggregation which could worsen ischemia [18, 23]. However, emerging data now suggest that many mechanisms and targets in ischemic patho-physiology are biphasic in nature [3, 24]. During an acute phase, a particular mechanism might be deleterious. But during the delayed phase, the same mediators may be beneficial. The same may be true for TSP-1-CD47 signaling. The interaction between CD47 and TSP promotes apoptosis and limits inflammation [25]. As a “don’t eat me” signal, CD47 inhibits phagocytosis of host cells and triggers apoptosis, terminating inflammation and providing an important regulator of central nervous system in apoptosis and inflammation [26]. Whether glial activation of CD47 helps regulate scavenging of damaged cells requires more investigation. We previously showed that TSP-1-CD47 signaling may mediate neuronal death [20], brain endothelial injury [22], and delayed inflammation and secondary brain injury after cerebral ischemia in vivo [4]. And our present study suggests that these neurovascular phenomena might be mediated by the ability of TSP-1-CD47 signaling to upregulate mediators such as VEGF and MMP-9. Recently, however, TSP-1 knockout mice seemed to show worsened long-term recovery after focal cerebral ischemia, especially in terms of synaptic remodeling [27]. This is because TSP-1 is known to act as an astrocyte-released factor that enhances synaptic formation and maturation [28]. Hence, the actual role of CD47 signaling may be nuanced, depending on the balance between acute injury versus delayed repair and recovery.

There are also several caveats in this proof-of-principle study. First of all, our data is obtained only in artificial cell culture conditions. We use cells from different species; rat astrocytes versus human pericytes and endothelium. Furthermore, cultures are isolated so that we can define cell-specific responses. But of course, different cell types co-exist in vivo. Endothelial cells behave differently when cross-talk occurs with astrocytes or pericytes [29, 30]. How these various CD47 events unfold in mixed cell environments in vivo remains to be confirmed. Second, our analyses are limited to a single 24 h time point. How VEGF and MMP-9 patterns evolve over longer periods of time remains to be determined. Third, our study used only a single trigger, i.e. 4N1 K as a TSP-1-derived ligand for CD47. Would other CD47 ligands such as integrins and/or SIRP α induce similar responses? And would other TSP-1 receptors (e.g. CD36) play related roles as well? The interplay between all these interconnected mediators may be difficult to dissect. Finally, VEGF and MMP-9 are important neurovascular mediators that have large effects on the blood–brain barrier. But

other trophic factors and proteases will surely be involved too. Whether CD47 signaling can also affect other neurovascular mediators remains to be investigated.

In summary, CD47 activation by TSP-1 may regulate the endothelial and astrocytic production of VEGF and MMP-9, which are two of the major mediators involved in neurovascular injury and barrier leakage after cerebral ischemia. Our data provide a mechanistic basis for further exploring the molecular pathways that underlie CD47 signaling in stroke pathophysiology.

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Fig. 1.
Immunocytochemistry demonstrates the presence of CD47 expression in **a** human brain endothelial cells, **b** human brain vascular pericytes, and **c** rat cortical astrocytes

Fig. 2.

Exposure to 4N1 K (25, 50, and 100 µg/ml) for 24 h induced upregulation of VEGF secretion in the cell culture media in brain endothelial cells and astrocytes (**a, b**). No changes are detected in pericytes (**c**). $N = 3-4$ independent experiments per group. * $P < 0.05$, ** $P < 0.01$ compared with control group

Fig. 3. Gelatin zymograms of MMP responses. Treatment with 4N1 K for 24 h significantly increased the pro-MMP-9 activity in human brain endothelial cells and rat cortical astrocytes (**a, b**). No changes were noted in pericytes (**c**). There was no statistically significant change in MMP-2 levels in all three kinds of cells. *Left panels* show representative gels. *Right graphs* show quantified densitometry results. $N = 4$ independent experiments per group. * $P < 0.05$, ** $P < 0.01$ compared with control group