Angiopoietin-1 reduces vascular endothelial growth factor-induced brain endothelial permeability via upregulation of ZO-2

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Abstract. Brain microvessels possess barrier structures comprising tight junctions which are critical for the maintenance of central nervous system homeostasis. Brain vascular diseases, such as ischemic stroke damage to blood-brain barrier, increase the vascular permeability, and then lead to vasogenic brain edema. Herein, we examined whether angiopoietin-1 (Ang-1) could regulate zonula occludens-2 (ZO-2) expression and counteract vascular endothelial growth factor (VEGF)-induced vascular permeability. When we treated brain microvascular endothelial cells with Ang-1, Ang-1 caused a time- and dose-dependent increase of ZO-2 and down-regulation in endothelial permeability. VEGF, one of the key regulators of ischemia-induced vascular permeability, increased endothelial cell permeability *in vitro*, whereas, Ang-1 reversed this VEGF effect by up-regulating ZO-2 expression. Additionally, the recovery effect of Ang-1 on permeability was strongly blocked by siRNA against ZO-2. Collectively, our results suggest that Ang-1 shows anti-permeability activity through up-regulation of ZO-2.

Introduction

Brain barriers protect the central nervous system (CNS) from harmful components of peripheral circulation (1). In vertebrates, the blood-brain barrier exists at the level of the endothelial cells that form brain capillaries (2), and the presence of tight intercellular junctions between endothelial cells support barrier function with low permeability and high electrical resistance (3-5). Tight junctions are composed of many junctional proteins including occludins, claudins, junctional adhesion molecules (JAMs), which are associated with at least one of the zonula occludens proteins (ZOPs) (6). ZOPs, in turn, establish a link between the junction site and the cytoskeleton by interacting directly with actin filaments (7,8). The observation that ZOPs not only associate with each other but also with components of adherens and gap junctions in cells lacking tight junction structure (9) suggests a universal and important role for ZOPs at cytoplasmic surfaces.

Mammalian ZOPs comprise of zonula occludens (ZO)-1, -2, and -3 (5). ZO-1 was the first protein associated with tight junctions of endothelial cells and well characterized tight junction protein (10). Tight junction associated protein ZO-2 interacts with ZO-1 and binds directly to the COOH-terminal cytoplasmic tail of the transmembrane protein occludin (11).

Pathological conditions, including ischemia, tumors and inflammation, are accompanied by a breakdown of the brain barrier, increase in permeability of the brain microvasculature and conduct the development of vasogenic brain edema (12). Vascular endothelial growth factor (VEGF), known as vascular permeability factor, is a possible candidate for the development of ischemia- and tumor-induced vasogenic brain edema (13,14). During development, VEGF and Ang-1 collaborate to regulate vascular formation (15). Although VEGF initiates proliferation and migration of endothelial cells and can promote the formation of new vessels (15), Ang-1 is required for the stabilization of endothelial contacts with surrounding mural cells to form mature vessels (16). Ang-1, a strong blood-vessel-anti-permeability factor, binds to the endothelial Tie2 receptor tyrosine kinase (17).

Several studies demonstrated that Ang-1 is involved in the reduction of VEGF-induced vascular permeability. *In vivo* mouse ischemia model, coapplication of Ang-1 and VEGF showed a reduction of the ischemic- and edema-volumes in comparison with VEGF-treated mice (18). ZO-1 immunostaining was more complete on the microvessel in the Ang-1 plus VEGF protein treated group compared with VEGF protein

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group in mouse brain (19). We previously reported indirect evidence that Ang-1 might stimulate the formation of blood-brain barrier and likely regulate the expression of several tight junction proteins including ZO-1, -2 and occludin (20). In addition, Ang-1 up-regulates ZO-1 in retina endothelial cells (21). Here, we show that Ang-1 could counteract VEGF-induced vascular permeability by regulating ZO-2 in brain endothelial cells.

Materials and methods

Cell line, recombinant proteins and antibodies. Primary human brain microvascular endothelial cells (HBMECs) were purchased from the Applied Cell Biology Research Institute and grown on 0.3% gelatin-coated dishes in complete serum-free medium (Cell Systems) or in M199 supplemented with 20% FBS (Invitrogen), 3 ng/ml basicFGF (Millipore), 5 U/ml heparin (Sigma) and 1% penicillin/streptomycin. Recombinant human $VEGF_{165}$ (rVEGF) and angiopoietin-1 (rAng-1) were purchased from R&D Systems. ZO-2 was from Santa Cruz. Phopho-AKT and AKT antibodies were from Cell Signaling. Anti-ß-actin antibody was from Sigma. Horseradish peroxidase-conjugated antibodies against mouse and rabbit immunoglobulins were purchased from Dako.

Rhodamine B isothiocyanate-dextran permeability assay. Permeability across the HBMECs cultured on type I collagen-coated transwell units (6.5-mm diameter, 3.0-μm pore size polycarbonate filter; Corning) was measured as described previously (20). After HBMECs became confluent, Human Endothelial-SFM media (Invitrogen) was treated for 15 h, followed by rVEGF (50 ng/ml) and rAng1 (50-100 ng/ml) treatment for indicated time (h). Permeability was measured by adding 0.1 mg of Rhodamine B isothiocyanate (RITC)-labeled dextran (molecular weight, ~10,000)/ml to upper chamber. After incubation for 10 min, 50 μl of sample from the lower compartment was diluted with 50 μl of phosphate-buffered saline and measured for fluorescence at 635 nm when excited at 540 nm with a spectrophotometer (Tecan Spectra Fluor).

SDS-PAGE and immunoblotting. Proteins were extracted with lysis buffer (40 mM Tris pH 7.4, 10 mM EDTA, 120 mM NaCl, 0.1% NP-40, protease inhibitor cocktail (Roche Applied Science) and protein concentration was measured by Bradford assay. Total protein (20 μg/lane) were loaded, separated by SDS-PAGE and transferred onto Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membranes were incubated for 1 h with blocking solution (5% skim milk in 0.1% Tween-20 PBS) and then incubated overnight at 4˚C with appropriate antibody. Membrane was washed with 0.1% Tween-20 PBS and then incubated for 1 h with secondary antibody. The membrane was washed with 0.1% Tween-20 PBS and the signals were developed using ECL plus detection system (Amersham Bioscience). For internal loading control, anti-ß-actin antibody was used.

RNA interference and RT-PCR. HBMECs were grown to 80% confluence, and small interfering RNAs (siRNAs) (50 μM) against ZO-2 were transfected into the cells using MetafectenePro (Biontex). siRNA transfection was performed according to the manufacturer's instructions. ZO-2 siRNA and the control nonsilencing siRNA were purchased from Santa Cruz. Total RNA was isolated using TriZol reagent (Invitrogen). RT-PCR was performed according to manufacturer's instructions (Promega). The following sets of primers were used: human ZO-2, 5'-CTTATTCGGCCCCATA GCTG-3' (forward); 5'-CCATGGCGGTTAAGTAGGAC-3' (reverse); GAPDH, 5'-CCTCTGGAAAGCTGTGGCGT-3' (forward); 5'-TTGGAGGCCATGTAGGCCAT-3' (reverse). The PCR products were separated on 1.2% agarose gels and visualized using ethidium bromide staining under UV trans-illumination.

Data analysis and statistics. Quantification of band intensity was analyzed using TINA 2.0 (RayTest) and normalized to the intensity of ß-actin band. All data are presented as mean ±SD. Statistical significance was evaluated with paired-student t-test for comparison between 2 groups. A probability value of <0.005 was considered significant.

Results

rAng-1 treatment upregulates the expression of ZO-2 and reduces permeability in HBMECs. We first tested the possibility that rAng-1 might regulate the expression of ZO-2 in brain endothelial cells. To evaluate whether rAng-1 activated the angiopoietin signaling pathway in HBMECs, we checked the AKT pathway activation in response to rAng-1 treatment (Fig. 1A and B). The phosphorylated Akt is strongly induced by treatment of rAng-1. This pAKT induction indicated that HBMECs strongly responded to rAng-1. Interestingly, the expression of ZO-2 was time-dependently increased after treatment of rAng-1 (Fig. 1A). Also, ZO-2 mRNA and protein showed a dose-dependent up-regulation (Fig. 1B). Using the passage of RITC-dextran through monolayers of HBMECs as a measure of permeability, we found a parallel result that rAng-1 significantly reduced vascular permeability in a dose response ($p<0.005$) (Fig. 1C).

ZO-2 reduction by rVEGF is recovered by combination treatment of rAng-1 and rVEGF. VEGF is a likely candidate for the development of ischemia- and tumor-induced vasogenic brain edema (13). To examine whether rVEGF affects the ZO-2 protein level, we exposed HBMECs to rVEGF (50 ng/ml) for 3 h (Fig. 2A). ZO-2 expression was markedly decreased by rVEGF. However, when rAng-1 (100 ng/ml) was treated together with rVEGF to HBMECs, the decrease of ZO-2 expression strongly recovered (Fig. 2A). We confirmed this result using RITC-dextran permeability assay (Fig. 2B). Vascular permeability significantly increased by rVEGF ($p=0.001$), yet, it was markedly attenuated by adding rAng-1 into rVEGF treated HBMECs ($p=0.001$) (Fig. 2B). Thus, these results suggest that rAng-1 may recover vessel tightness in part through ZO-2.

Anti-permeability effect of rAng-1 was blocked by siRNA against ZO-2. To conform the mechanism of rAng-1 in anti-permeability effect, we transfected the siRNA targeting ZO-2 (siZO-2) into the HBMECs. As shown in Fig. 3A, we were

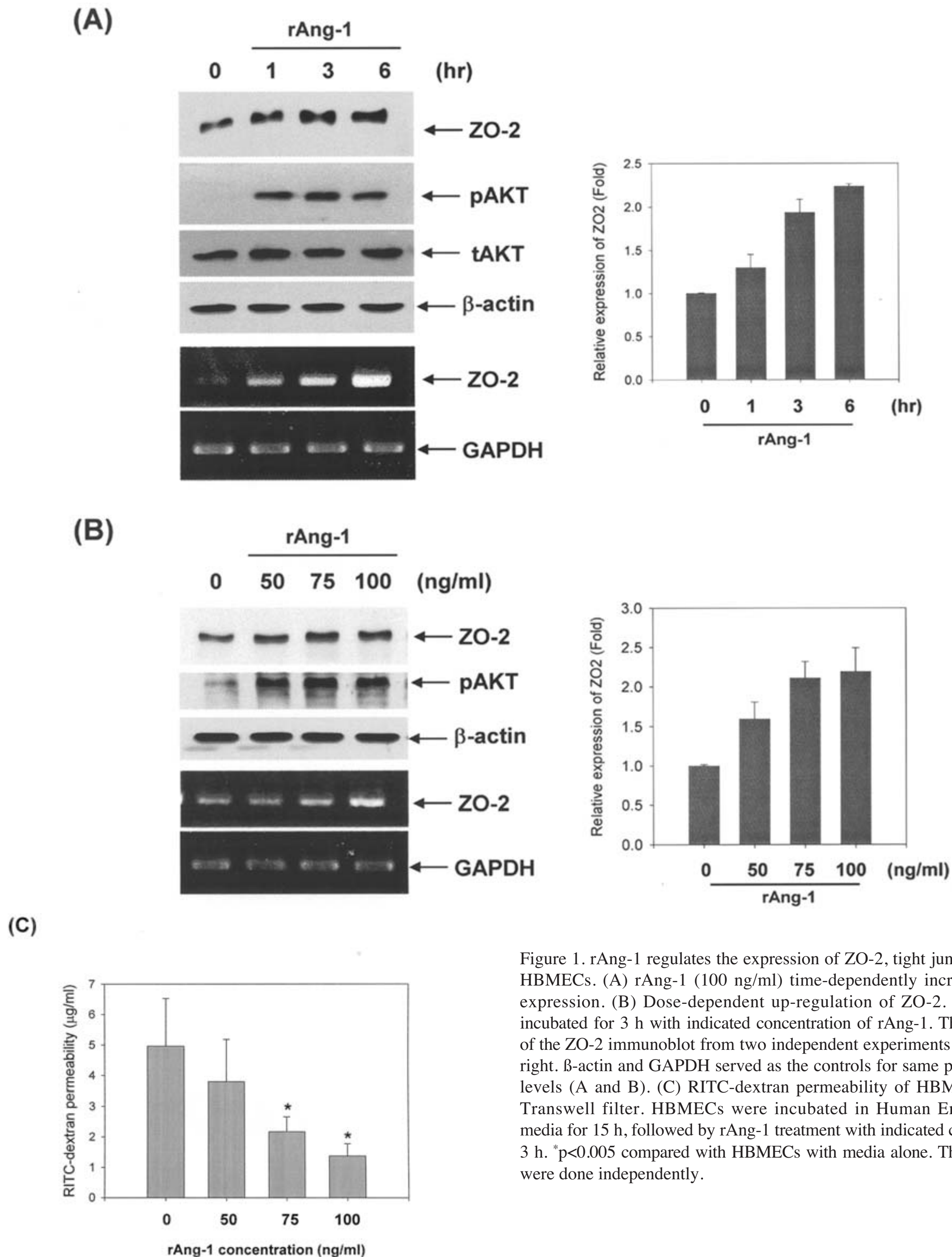


Figure 1. rAng-1 regulates the expression of ZO-2, tight junction protein, in HBMECs. (A) rAng-1 (100 ng/ml) time-dependently increased the ZO-2 expression. (B) Dose-dependent up-regulation of ZO-2. HBMECs were incubated for 3 h with indicated concentration of rAng-1. The quantification of the ZO-2 immunoblot from two independent experiments is shown on the right. ß-actin and GAPDH served as the controls for same protein and RNA levels (A and B). (C) RITC-dextran permeability of HBMECs seeded on Transwell filter. HBMECs were incubated in Human Endothelial-SFM media for 15 h, followed by rAng-1 treatment with indicated concentration for 3 h. *p<0.005 compared with HBMECs with media alone. Three experiments were done independently.

able to detect the complete inhibition of ZO-2 expression in mRNA and protein levels after ZO-2 siRNA (50 μM) transfection. Control siRNA (siCon) transfection had no effect on ZO-2 expression (Fig. 3A). In the permeability assay, rVEGF significantly increased the endothelial permeability ($p<0.005$), whereas rAng-1 plus rVEGF group strongly reduced permeability compared with rVEGF treated group ($p<0.005$) (Fig. 3B). Parallel to Fig. 3A, control siRNA did not alter endothelial permeability compared with rAng-1 plus rVEGF treatment group. However, the recovery effect for endothelial permeability by rAng-1 was completely blocked by ZO-2 siRNA transfection ($p=0.001$) (Fig. 3B), suggesting that anti-permeability effect of Ang-1 in the presence of VEGF may occur via at least the regulation of ZO-2.

Discussion

Tight junctions in brain vessels have a function that reduces the permeability and builds up the CNS barrier, which restricts the free molecular exchange between blood and CNS tissue. This restriction protects CNS from harmful components of the blood and allows the uptake of essential molecules (3). Intact tight junctions between brain capillary endothelial cells are critical for normal brain barrier function. Alterations in

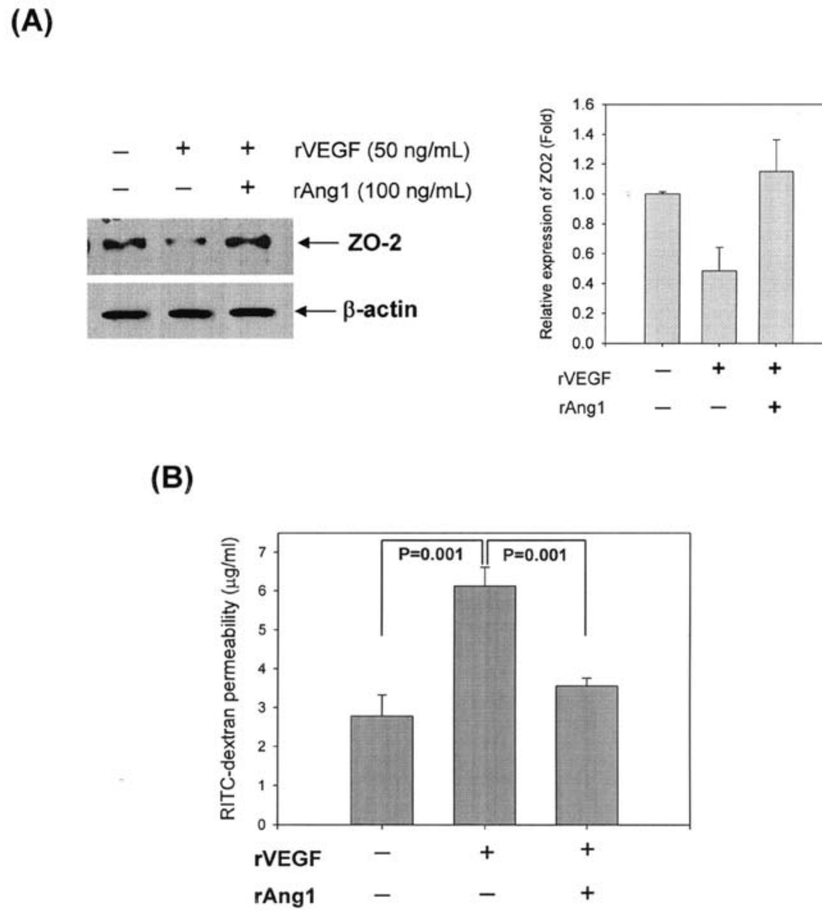
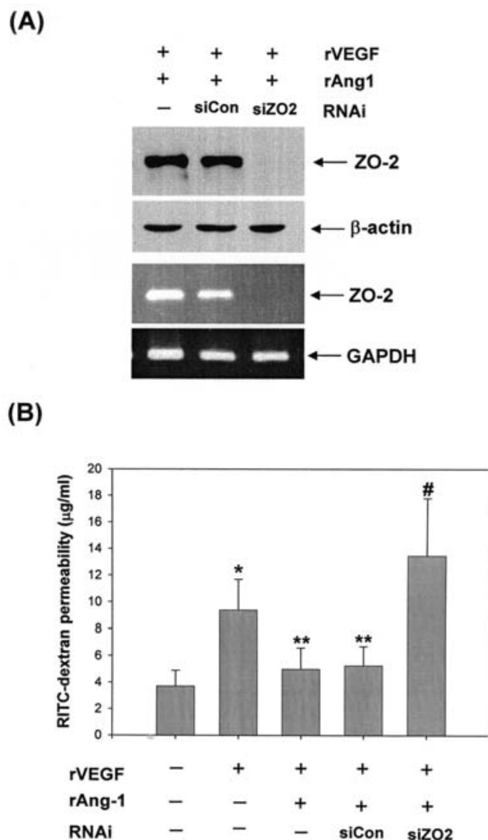


Figure 2. rAng-1 recovered rVEGF-induced ZO-2 reduction. (A) Expression of ZO-2 in HBMECs treated with 50 ng/ml rVEGF and 100 ng/ml rAng-1 for 3 h was analyzed by Western blot (n=4). The quantification of the ZO-2 immunoblot from four independent experiments is shown on the right. (B) RITC-dextran passage was analyzed for vascular permeability in HBMECs under the same conditions as in A. Three experiments were independently performed.



the composition and the distribution of tight junction proteins would result in increased blood-brain barrier permeability.

Notably, several reports suggest that the regulation of expression of tight junction proteins is important in barrier function as well. Reduction of ZO-2, a membrane-associated tight junction protein, was regarded as the cause of human immunodeficiency virus (HIV) entry into the CNS by disruption of the blood-brain barrier (22). Tat protein of HIV decreased ZO-2 expression, whereas total levels of occludin and ZO-1 remained unchanged in brain microvascular endothelial cells (22). Brain edema occurred in fulminant hepatic failure mice. ZO-2 expression was reduced and its distribution was altered in the tight junctions. These effects further preceded the increased blood-brain barrier permeability thus it may cause

Figure 3. siRNA against ZO-2 abolished the anti-permeability effect of rAng-1 on rVEGF treated HBMECs. (A) HBMECs were transfected with siRNA targeting ZO-2 (siZO-2) and control nonsilencing siRNA (siCon), incubated for 24 h, then treated with 50 ng/ml rVEGF and 100 ng/ml rAng-1 for 3 h. ZO-2 protein and mRNA were analyzed (n=3). (B) RITC-dextran passage was analyzed for endothelial permeability in HBMECs under the same conditions as in A. Independent experiments were repeated two times. *$p<0.005$ compared with HBMECs with media only; **$p<0.005$ compared with rVEGF treated HBMECs; #$p=0.001$ compared with rVEGF plus rAng-1 treated- or siCon transfected-HBMECs.

brain edema in fulminant hepatic failure (23). These reports support the importance of ZO-2 in functional blood-brain barrier structure.

Brain tumors, ischemic stroke and trauma are involved with barrier disruption, leading to the development of vasogenic brain edema (24). Hypoxia-induced VEGF is known as a key regulator of these permeability changes (25). VEGF is an angiogenic growth factor whose expression correlates with brain angiogenesis (15). Treatment of VEGF, initially named vascular permeability factor, increased microvascular permeability and decreased occludin and ZO-1 levels (26-28). In our experiment, we found that rVEGF treatment to HBMECs strongly decreased the expression of ZO-2 (Fig. 2A) and increased the endothelial permeability (Fig. 2B), indicating that VEGF-induced vessel leakage might be mediated by ZO-2 protein.

Ang-1 and VEGF have complementary roles during vascular development. VEGF acts early during vessel formation, whereas Ang-1 acts later during vessel remodeling and stabilization (29). Ang-1 is a strong anti-permeability factor that can reduce vascular leakage. When blood-brain barrier breakdown occurs, lesion and perilesional vessels show decreased Ang-1 immunoreactivity (30). Ang-1 suppresses diabetic retinopathy *in vivo* (31) and coapplication of Ang-1 and VEGF showed a reduction of the brain edema in comparison with VEGF-treated mice (18), supporting the leakage resistant role attributed to Ang-1. However, the regulatory mechanism is still poorly understood. Interestingly, treatment of HBMECs with rAng-1 caused a time- and dose-dependent increase ZO-2 expression and caused a dose-dependent decrease in monolayer permeability by RITC-dextran measurements (Fig. 1). Notably, when we treated both rVEGF and rAng-1 to HBMECs, rVEGF-induced endothelial permeability was significantly reduced and ZO-2 expression was augmented (Fig. 2). Moreover, rVEGF-induced vascular permeability was not recovered by rAng-1 in the group of siRNA targeting ZO-2 (Fig. 3B).

Taken together, we conclude that VEGF enhances endothelial barrier disruption but Ang-1 is able to antagonize this VEGF deleterious effect, in association with an up-regulation of ZO-2. A better understanding of tight junction protein and endothelial barrier regulation by Ang-1 could provide useful therapeutics to treat cerebral vascular disorders such as ischemic brain injury.

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