Brain-derived neurotrophic factor induces angiogenin secretion and nuclear translocation in human umbilical vein endothelial cells

Running Title: BDNF and angiogenin

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Abbreviations: BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cells; RT-PCR, reverse transcriptase-polymerase chain reaction; TrkB, tropomyosin receptor kinase B; VEGF, vascular endothelial growth factor.

Abstract

Brain-derived neurotrophic factor (BDNF) is a well-known humoral protein that induces growth of neurons. Recent studies have suggested that BDNF could act as an angiogenesis inducer similar to vascular endothelial growth factor (VEGF). Angiogenin is a strong mediator of angiogenesis. It has particular characteristics both as a secreted protein and a transcription factor. After being incorporated into the cytoplasm, angiogenin is immediately transferred to the nucleus and then mediates the angiogenic effects of angiogenesis inducers, including VEGF. The aim of this study is to determine the association between BDNF and angiogenin. At first, we determined the secretion of angiogenin from human umbilical vein endothelial cells (HUVEC) induced by BDNF with enzyme-linked immunosorbent assay. Next, we determined BDNF-induced nuclear translocation of angiogenin by immunofluorescent staining. In addition, we examined the mRNA expression of angiogenin in HUVEC before and after BDNF stimulation by quantitative reverse transcriptase-polymerase chain reaction. As a result, we noted that BDNF induced angiogenin secretion and nuclear translocation without an increase in the mRNA expression in HUVEC. Furthermore, we demonstrated that BDNF-induced HUVEC proliferation was significantly suppressed when neomycin, a specific inhibitor of nuclear translocation of angiogenin, was administered. These findings indicate that nuclear translocation of angiogenin is critically involved in BDNF-induced proliferation of HUVEC. In conclusion, angiogenin contributes to angiogenesis induced by BDNF.

Key words: BDNF, VEGF, angiogenin, angiogenesis

1. Introduction

Angiogenesis is a physiological phenomenon in which new vascular branches arise from the existing blood vessels [1]. Individually and by interacting with each other, vascular endothelial cells repeatedly conduct the following actions, namely, budding, elongation, and lumen and bridge formation, resulting in the generation of an organized blood vessel network in three dimensions [2]. The cell dynamics in angiogenesis are regulated comprehensively. Though essential for some physiological conditions, such as embryonic development, wound healing, and tissue regeneration, angiogenesis is also involved critically in diverse pathological conditions, including growth and invasion of carcinomas, ischemic heart diseases, and retinal degenerative diseases.

Vascular endothelial growth factor (VEGF), which is a representative angiogenesis inducer, promotes migration and proliferation of vascular endothelial cells [3]. VEGF receptor is expressed specifically on vascular endothelial cells and its expression correlates with tumor progression and poor prognosis in patients with cancer, such as gastric cancer [4-6] and lung cancer [7-9]. Correspondingly, anti-VEGF antibody is used as a therapeutic agent against such cancers.

Brain-derived neurotrophic factor (BDNF) is a humoral protein that binds to a receptor called tropomyosin receptor kinase B (TrkB) on nerve cells and then induces growth of neurons [10]. It has been demonstrated that the BDNF signal through TrkB also induced growth of cells other than nerve cells; e.g., vascular endothelial cells [11-13]. Recent studies have suggested that BDNF could act as an angiogenesis inducer similar to VEGF [14, 15]. Although some reports suggest the activation of VEGF by BDNF [16-18], the precise mechanism of angiogenicity remains unrevealed.

Angiogenin, a strong mediator of angiogenesis, was isolated from the culture supernatant of the HT-29 human colon cancer cell line [16]. It has particular

characteristics both as a secreted protein and a transcription factor. After being incorporated into the cytoplasm, angiogenin is immediately transferred to the nucleus and then mediates the angiogenic effects of angiogenesis inducers, including VEGF [19, 20]. Correspondingly, inhibition of the nuclear translocation of angiogenin has been shown to suppress the angiogenesis.

Several factors are notably involved in the angiogenesis. Although it becomes clearer that angiogenin plays a role in the downstream of the angiogenesis inducers, there is no report that focuses on the association between BDNF and angiogenin. In the present study, we attempted to determine the effects of BDNF on the dynamics of angiogenin.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) purchased from Lonza (Basel, Switzerland) were cultured in HuMedia-EG2 (Kurabo, Osaka, Japan) with supplement of 2% fetal bovine serum at 37 ºC in a humidified atmosphere with 5% CO_2. A fresh medium was replaced every 48 h. Cells within passages guaranteed by the supplier were used for experiments. Prior to the experiments, cells were washed three times with serum-free EBM-2 medium (Lonza) in order to avoid the influences of putative angiogenic factors in the culture medium.

2.2. Reagents

Recombinant human VEGF and BDNF and neomycin were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.3. Enzyme-linked immunosorbent assay (ELISA) to quantify angiogenin

HUVEC were grown up to 60-70% confluency in 24-well plates. After washing, the cells were incubated with BDNF (0, 5, 25, 50, 200, 500, and 1,000 ng/mL) dissolved in EBM-2 for 24 h at 37 ºC. Thereafter, the culture supernatant was collected and subjected to enzyme-linked immunosorbent assay (ELISA) for quantification of angiogenin using Human Angiogenin Quantikine ELISA Kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Similar experiments were repeated more than three times. Each measurement was carried out in duplicates.

2.4. Lactate dehydrogenase (LDH) release assay

HUVEC (2.5×10^4/well of 96-well plates) were exposed to 0, 15.625, 31.25,

62.5, 125, 250, 500, or 1000 ng/mL of BDNF at 37 ºC. After 48 h, lactate dehydrogenase (LDH) release from the HUVEC was determined using Cytotoxicity LDH Release Assay Kit (Dojindo, Kumamoto, Japan). Assay was carried out in triplicates. Percent cytotoxicity was determined according to the manufacturer's protocol.

2.5. Immunofluorescent staining of angiogenin

HUVEC were grown up to about 60% confluency in 4-well chamber slides (Corning, Corning, USA). After washing, the cells were incubated in EBM-2 without stimulant (as negative control), with 10 ng/mL of VEGF (as positive control), or with 1 μg/mL of BDNF for 4 h at 37 ºC. Thereafter, the cells were fixed with cold methanol for 10 min. After removal of methanol, the cells were washed three times with PBS. Next, in order to block non-specific binding of antibodies, the cells were soaked in PBS containing bovine serum albumin (BSA) (30 mg/mL) for 1 h at room temperature. Following these preparations, immunofluorescent staining of angiogenin was carried out. For this purpose, anti-human angiogenin monoclonal antibody (MANG1, mouse IgM) (BMA Biomedicals, Augst, Switzerland) was used as a primary antibody. The primary antibody was diluted to 50 μg/mL in the BSA-containing PBS, and the cells were allowed to react with the diluted primary antibody for 1 h at room temperature (alternatively, overnight at 4 °C). After washing three times with PBS, the cells were next allowed to react with Alexa Fluor 488-labeled goat anti-mouse IgM antibody (Abcam, Cambridge, UK) for 30 min at room temperature in the dark. The secondary antibody was diluted to 10 μg/mL in the BSA-containing PBS. After washing three times with PBS followed by removal of the chambers, the cells were mounted using VECTASHIELD Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI) (VECTOR Laboratories, Burlingame, USA). These slides were observed under a

fluorescent microscopy. Similar experiments were repeated more than three times.

2.6. Photomicrographs

After verifying the general state of staining under low power fields of view, photomicrographs were taken using Nikon NIS-Elements software (Tokyo, Japan). Angiogenin granules in the nucleus were counted concerning 20 cells and then the counts per nucleus were calculated.

2.7. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

HUVEC were grown up to about 60% confluency in 6 cm dishes. After washing, the cells were incubated in EBM-2 without stimulant (as negative control), with 10 ng/mL of VEGF (as positive control), or with 1 μg/mL of BDNF for 4 h at 37 ºC. Alternatively, HUVEC were treated with 1 μg/mL of BDNF at 37 ºC for 1, 4, or 24 h. Total RNA was extracted from the cells using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Conversion to cDNA was achieved using GoScript™ Reverse Transcription System (Promega, Madison, USA). Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for angiogenin was carried out using StepOne®Plus Real-Time PCR Systems (Applied Biosystems, City of Foster City, USA) with the following primers: 5'-ATGGCAACAAGCGCAGCATC-3' as a forward primer and 5'-CGGACGACGGAAAATTGACTG-3' as a reverse primer. After denaturation at 95°C for 10 min, the templates were allowed to amplify with 40 cycles of reaction at 95°C for 15 s and at 63°C for 60 s. The amounts of angiogenin cDNA in the samples were standardized by the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (5'-CCATCACCATCTTCCAGGAG-3' as a forward primer and 5'-CCTGCTTCACCACCTTCTTG-3' as a reverse primer) in the simultaneous RT-PCR.

Relative mRNA expression of angiogenin was determined by the comparative Ct method. Similar experiments were repeated more than three times. Each PCR was carried out in triplicates.

2.8. BDNF-induced Proliferation of HUVEC and effects of inhibitor of angiogenin nuclear translocation

HUVEC were grown up to about 60% confluency in 3.5 cm dishes. After washing, the cells were incubated in EBM-2 without stimulant (as negative control), with 1 μg/mL of BDNF, 100 μM of neomycin, or both BDNF (1 μg/mL) and neomycin (100 μM) at 37 ºC. Neomycin, an aminoglycoside antibiotic, has been shown as a specific inhibitor of nuclear translocation of angiogenin [21]. After 24 h, the cell number was counted. Simultaneously, immunofluorescent staining for angiogenin was carried out as aforementioned. Similar experiments were repeated three times.

2.9. Statistical analysis

Data were compared using Student's *t*-test according to the difference in variance determined by the F-test. The p-values of less than 0.05 were regarded as significant.

3. Results

3.1. Secretion of angiogenin from HUVEC by BDNF stimulation

Secretion of angiogenin from HUVEC by BDNF stimulation for 24 h was determined using the ELISA kit. The concentration of angiogenin in the culture supernatants was increased by BDNF dose-dependently and was significantly higher in the supernatants of HUVEC stimulated by 1 μg/mL of BDNF than in the HUVEC without stimulation (**Figure 1a**). No significant release of LDH from the HUVEC treated with BDNF (even at the concentration of 1 μg/mL for 48 h) was evident (**Figure 1b**). The presence of angiogenin in the supernatant as a result of BDNF cytotoxicity to HUVEC is ruled out. These findings indicate that BDNF can induce secretion of angiogenin from HUVEC.

3.2. Nuclear translocation of angiogenin by BDNF stimulation

In order to determine that BDNF could induce translocation of angiogenin into the nucleus, immunofluorescent staining for angiogenin was conducted on HUVEC stimulated by 1 μg/mL of BDNF for 4 h. For positive control, HUVEC stimulated by 10 ng/mL of VEGF were employed. As shown in **Figure 2a**, fluorescent granules that represented angiogenin were observed in the nuclei of HUVEC stimulated by VEGF. This finding is consistent with the previous report that demonstrates the nuclear translocation of angiogenin induced by VEGF [19]. Similarly, fluorescent granules that represented angiogenin were observed in the nuclei of HUVEC stimulated by BDNF though the cytoplasmic staining for angiogenin was much fainter compared with HUVEC stimulated by VEGF. The numbers of angiogenin granules were 22.05/nucleus of the HUVEC stimulated by BDNF and 15.94/nucleus of the HUVEC stimulated by

VEGF, and they were significantly greater than in the HUVEC without stimulation (9.29/nucleus) (**Figure 2b**).

On the other hand, the mRNA expression level of angiogenin was not significantly altered in HUVEC by stimulation with VEGF (10 ng/mL) or BDNF (1 μg/mL) for 4 h (**Figure 3a**). In addition, the angiogenin mRNA expression was not significantly altered up to 24 h after stimulation with BDNF (1 μg/mL) (**Figure 3b**). The collective findings suggest that both VEGF and BDNF can induce nuclear translocation of angiogenin in HUVEC without a significant increase in the mRNA expression.

3.3. Involvement of angiogenin nuclear translocation in BDNF-induced HUVEC proliferation

In order to determine the involvement of angiogenin nuclear translocation in BDNF-induced angiogenesis, we conducted an experiment using neomycin, a specific inhibitor of nuclear translocation of angiogenin [21]. We demonstrated that BDNF could induce proliferation of HUVEC and the BDNF-induced proliferation was significantly suppressed when the nuclear translocation of angiogenin was inhibited by neomycin (**Figure 4**). These findings indicate that nuclear translocation of angiogenin is critically involved in BDNF-induced proliferation of HUVEC.

4. Discussion

In the present study, we have demonstrated that BDNF could induce angiogenin secretion and nuclear translocation without a significant increase in the mRNA expression and that nuclear translocation of angiogenin is critically involved in BDNF-induced angiogenesis. Kishimoto *et al*. reported the nuclear translocation of angiogenin after stimulation of HUVEC by angiogenesis inducers, such as VEGF, fibroblast growth factor (FGF), and epidermal growth factor (EGF) [19]. They also claimed that inhibition of the nuclear translocation of angiogenin resulted in suppression of the angiogenesis. Regarding the potential to induce nuclear translocation of angiogenin, the strong mediator of angiogenesis, BDNF has property equivalent to other angiogenesis inducers.

The mechanisms of nuclear translocation of angiogenin are discussed as follows. Moroianu and Riordan have demonstrated that the peptide sequence RRRGL (arginine-arginine-arginine-glycine-leucine), which corresponds to the residues 31 to 35 of angiogenin, mediated the nuclear translocation [22]. They have suggested that the arginine R33 could be essential for the nuclear translocation and that the arginines R31 and R32 might regulate this process. On the other hand, Li *et al*. have demonstrated that exogenous angiogenin translocated into the nucleus immediately after incorporation into the cytoplasm [23]. They have also claimed that the nuclear translocation of angiogenin could occur via an independent mechanism on the microtubules and lysosomes without requirement of tyrosine kinase activation because the dynamics was not affected by either destruction of the microtubule system or inhibition of lysosomes and tyrosine kinases. On the contrary, Hu *et al*. have demonstrated that neomycin, an aminoglycoside antibiotic, inhibits nuclear translocation of angiogenin [21]. Based on the finding that another phospholipase C inhibitor, U-73122, has a similar effect, they suggest a pivotal

role of the phospholipase C-inhibiting activity of neomycin on the inhibition of nuclear translocation of angiogenin. Further studies are needed to clarify the mechanisms of nuclear translocation of angiogenin induced by BDNF.

The mRNA expression analysis in HUVEC after BDNF stimulation has revealed that BDNF did not augment the angiogenin mRNA expression in HUVEC similar to VEGF. Although the angiogenin gene locates in the middle of the RNase A superfamily gene cluster, the ribonuclease activity is reported to be very low [24]. In addition, we have observed the secretion of angiogenin from HUVEC stimulated by BDNF. The collective findings suggest that BDNF effects on the protein dynamics of angiogenin rather than the transcriptional regulation. These findings appear to be consistent with the faint immunofluorescent staining of angiogenin in the cytoplasm of HUVEC after BDNF stimulation. In contrast, Arakawa *et al*. have demonstrated that stimulation of VEGF did not induce angiogenin secretion from co-cultured HUVEC and human skin fibroblasts [25]. Therefore, BDNF is considered to lead the nuclear translocation of angiogenin via a pathway different from VEGF.

In summary, this study clearly indicates the contribution of angiogenin to angiogenesis induced by BDNF.

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None.

Author Contributions

Designed study: AM, NH, TM, and AI. Performed the experiments: AM and YN. Analyzed the data: AM, YN, MY, YN, SM, UT, and AI. Wrote the manuscript: AM, UT, and AI.

Conflict of interest statement

The authors declare no competing financial interest.

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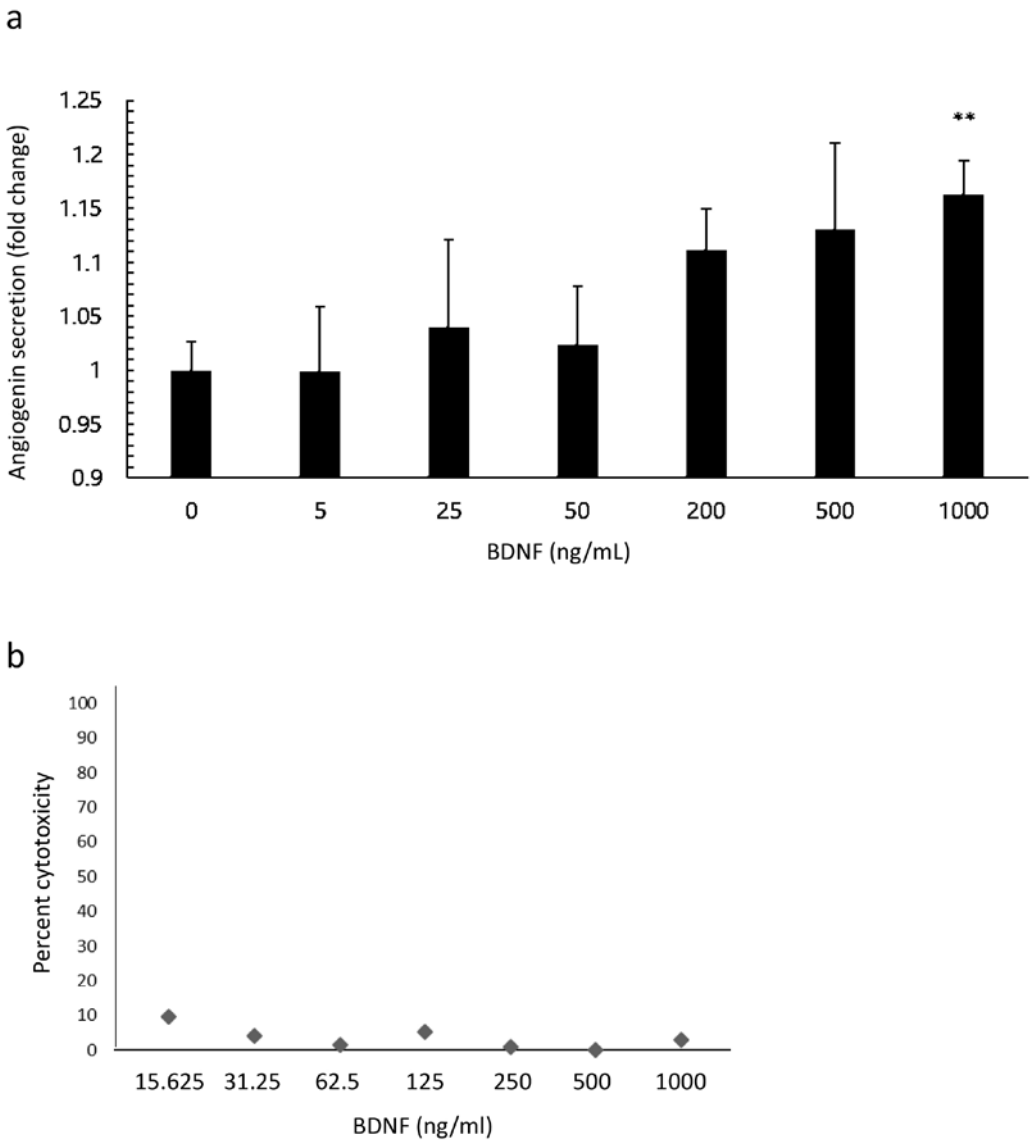

Fig. 1. Secretion of angiogenin from HUVEC by BDNF stimulation

(**a**) Secretion of angiogenin from HUVEC by BDNF stimulation for 24 h determined using ELISA kit is represented as fold change to the concentration of angiogenin in the supernatants of HUVEC without BDNF stimulation. **p <0.01 to 0 ng/mL of BDNF. (**b**) LDH release from HUVEC exposed to BDNF for 48 h determined using Cytotoxicity LDH Release Assay Kit. Data are presented as percent cytotoxicity.

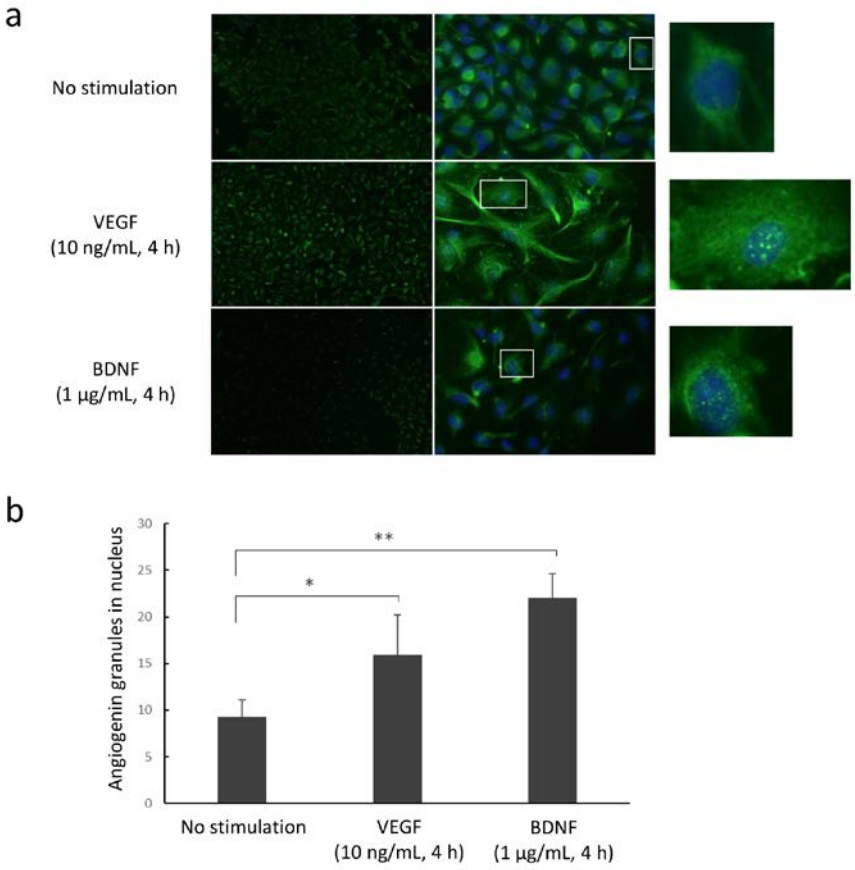

Fig. 2. Nuclear translocation of angiogenin by BDNF stimulation

(**a**) Immunofluorescent staining for angiogenin of HUVEC without stimulation, with VEGF stimulation, and with BDNF stimulation. Angiogenin: green, nucleus: blue. The left and middle panels represent the low and high power fields of view, respectively. The right panels represent the close-up scenes of the insets in the middle panels. (**b**) Number of angiogenin granules per nucleus. *$p<0.05$, **$p<0.01$.

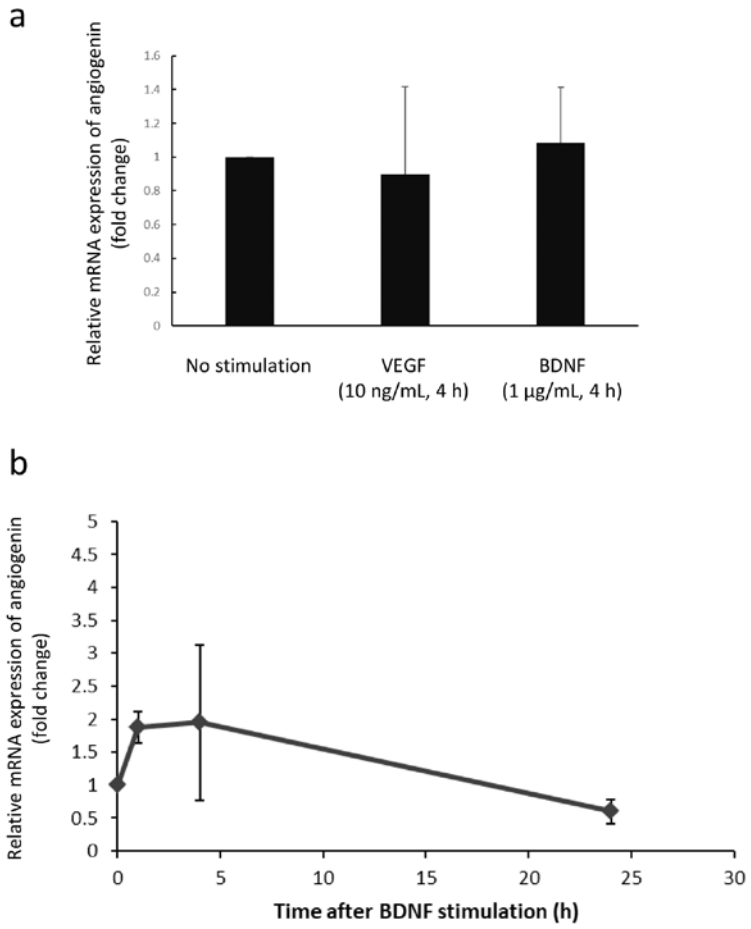

Fig. 3. Angiogenin mRNA expression in HUVEC

(**a**) The relative mRNA expression of angiogenin in HUVEC treated with VEGF (10 ng/mL) and BDNF (1 μg/ml) for 4 h was determined using real-time RT-PCR. (**b**) Time-dependent mRNA expression of angiogenin in HUVEC treated with 1 μg/ml of BDNF. The expression in HUVEC without stimulation was set as 1. Data are presented as mean ± standard error.

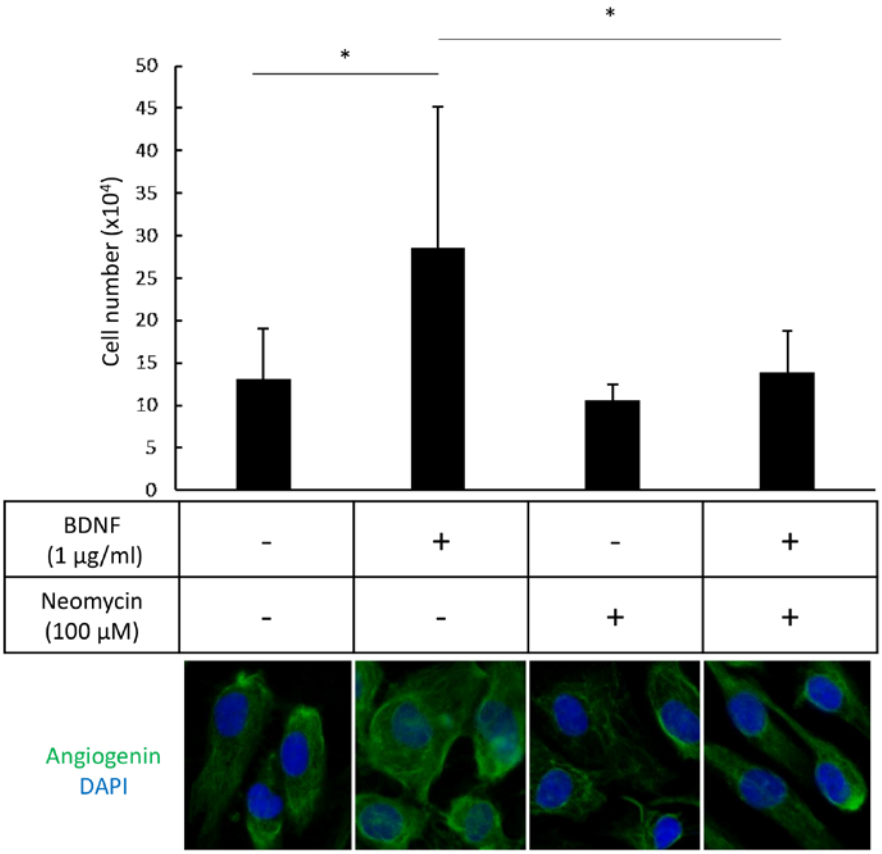

Fig. 4. BDNF-induced proliferation of HUVEC and effects of inhibitor of angiogenin nuclear translocation

HUVEC were treated with 1 μg/mL of BDNF, 100 μM of neomycin, or both BDNF (1 μg/mL) and neomycin (100 μM) at 37 ºC. After 24 h, the cell number was counted. Simultaneously, immunofluorescent staining for angiogenin was carried out.