Biochemical and Biophysical Research Communications 462 (2015) 215–220

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of the proliferation and acceleration of migration of vascular endothelial cells by increased cysteine-rich motor neuron 1

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ARTICLE INFO

Article history:
Received 17 April 2015
Available online 5 May 2015

Keywords:
CRIM1
Endothelial
Proliferation
Migration
Angiogenesis

ABSTRACT

Cysteine-rich motor neuron 1 (CRIM1) is upregulated only in extracellular matrix gels by angiogenic factors such as vascular endothelial growth factor (VEGF). It then plays a critical role in the tube formation of endothelial cells. In the present study, we investigated the effects of increased CRIM1 on other endothelial functions such as proliferation and migration. Knock down of CRIM1 had no effect on VEGF-induced proliferation or migration of human umbilical vein endothelial cells (HUVECs), indicating that basal CRIM1 is not involved in the proliferation or migration of endothelial cells. Stable CRIM1-overexpressing endothelial F-2 cells, termed CR1 and CR2, were constructed, because it was difficult to prepare monolayer HUVECs that expressed high levels of CRIM1. Proliferation was reduced and migration was accelerated in both CR1 and CR2 cells, compared with normal F-2 cells. Furthermore, the transient overexpression of CRIM1 resulted in decreased proliferation and increased migration of bovine aortic endothelial cells. In contrast, neither proliferation nor migration of COS-7 cells were changed by the overexpression of CRIM1. These results demonstrate that increased CRIM1 reduces the proliferation and accelerates the migration of endothelial cells. These CRIM1 effects might contribute to tube formation of endothelial cells. CRIM1 induced by angiogenic factors may serve as a regulator in endothelial cells to switch from proliferating cells to morphological differentiation.

1. Introduction

Angiogenesis is a complex process by which new blood vessels are generated from preexisting microcapillaries in response to angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [1,2]. Angiogenesis involves the sprouting of vascular endothelial cells from preexisting vessels, proliferation of endothelial cells, degradation of the basement membranes and extracellular matrix, cell migration, and tube morphogenesis. Because the suppression of angiogenesis has been shown to inhibit the growth, progression, and metastases of tumors, it has attracted considerable interest and many studies have been conducted to understand the mechanism of angiogenesis.

Cysteine-rich motor neuron 1 (CRIM1) was first discovered as a transmembrane protein expressed in motor neurons that plays an important role in the development of the central nervous system [3]. Further studies have revealed that CRIM1 is expressed in various cell types including vascular endothelial cells, and. implicated in angiogenesis and the development of the vascular system [4–8]. CRIM1 plays a crucial role in tube formation of endothelial cells [4]. CRIM1 regulates the delivery of VEGF from podocytes to endothelial cells in the glomerulus of the kidney [6,7]. Retinal vascular stability is maintained by CRIM1 by enhancing the VEGF autocrine signaling in endothelial cells [8]. However, the molecular activity of CRIM1 in endothelial cells remains unclear.

Abbreviations: CRIM1, cysteine-rich motor neuron 1; HUVEC, human umbilical vein endothelial cell; BAEC, bovine aortic endothelial cells; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

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http://dx.doi.org/10.1016/j.bbrc.2015.04.118
0006-291X/

Expression of CRIM1 mRNA in endothelial cells is upregulated by angiogenic factors only in extracellular matrix gels where endothelial cells form a tubular structure network [4,9]. The increase in CRIM1 expression occurs in the early phase of the tube formation process, followed by the generation of tubular structures. The inhibition of CRIM1 induction by CRIM1-antisense oligonucleotides results in the severe impairment of tube formation of endothelial cells. In contrast, CRIM1 mRNA remains unchanged in monolayer culture despite of the presence of angiogenic factors, and knock down of CRIM1 has no effect on the proliferation of monolayer endothelial cells. These results demonstrate that increased CRIM1 may regulate endothelial functions, including morphological differentiation to tubular structures. Monolayer cultured cells are applicable to determine cell proliferation and migration properly. However, CRIM1 upregulation does not occur in a monolayer culture. Therefore, we constructed CRIM1-overexpressing endothelial cells by transfection with full-length CRIM1 cDNA and investigated the effects of CRIM1 at high expression levels on the proliferation and migration of endothelial cells.

2. Materials and methods

2.1. Materials

The reagents used in this study and their sources are as follows: human VEGF (PeproTech, Rocky Hill, USA); human umbilical vein endothelial cells (HUVECs), EBM-2 medium and culture supplies (Lonza, Basel, Switzerland); bovine aortic endothelial cells (BAECs) (TOYOBO, Osaka, Japan); Lipofectamine2000, fetal bovine serum (FBS), and Stealth siRNAs for human CRIM1 and for nonspecific negative control (Invitrogen; Carlsbad, USA); pCI-neo (Promega; Madison, USA); anti-CRIM1 antibody (Santa Cruz Biotechnology, Santa Cruz, USA); Dulbecco's modified Eagle's medium (DMEM) and anti-actin antibody (Sigma Aldrich, St. Louis, USA); secondary antibody linked to peroxidase (Dako, Glostrup, Denmark); ECL Western blot detection system (GE Health Care, Little Chalfont, UK); collagen type I-C (Nitta Gelatin; Osaka, Japan); G418 and WST-8 (Nacalai Tesque, Kyoto, Japan). All other chemicals were of reagent grade.

2.2. Cell culture

HUVECs were maintained in EBM-2 medium containing supplements consisting of 2% FBS, epidermal growth factor, bFGF, VEGF, insulin-like growth factor-1, heparin, hydrocortisone, ascorbic acid and GA-1000 at 37 °C in 5% CO_2 and 95% air. Cells between passages 5 and 10 were used in the experiments. BAECs, F-2 cells (a mouse vascular endothelial cell line) [10,11] and COS-7 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2 and 95% air. All culture dishes were coated with collagen type I-C.

2.3. RNA interference of CRIM1 in HUVECs

HUVECs were transfected with 50 nM Stealth siRNA targeting CRIM1 or a nonspecific control siRNA using Lipofectamine2000, according to the manufacturer's instruction. After the cells were incubated with siRNA for 4 h, the culture medium was replaced with EGM-2 medium containing the full supplements. The cells were cultured for 24 h unless otherwise stated.

2.4. Transient expression of CRIM1 in BAECs and COS-7 cells

A full-length cDNA of human CRIM1 (GEN BANK Accession AY358372, GI: 37181866) was obtained by amplification of HepG2 cell total RNA by reverse transcription-polymerase chain reaction. The cDNA was inserted into the pCI-neo vector, after the nucleotide sequence was confirmed to be CRIM1. BAECs or COS-7 cells were transfected with pCI-neo without any insert DNA (Mock) or pCI-neo containing CRIM1. Transfection was performed using Lipofectamine2000, according to the manufacturer's instruction. After cells were exposed to the vector for 4 h, the cells were cultured in DMEM supplemented with 10% FBS and 600 μg/ml G418 for 24 h.

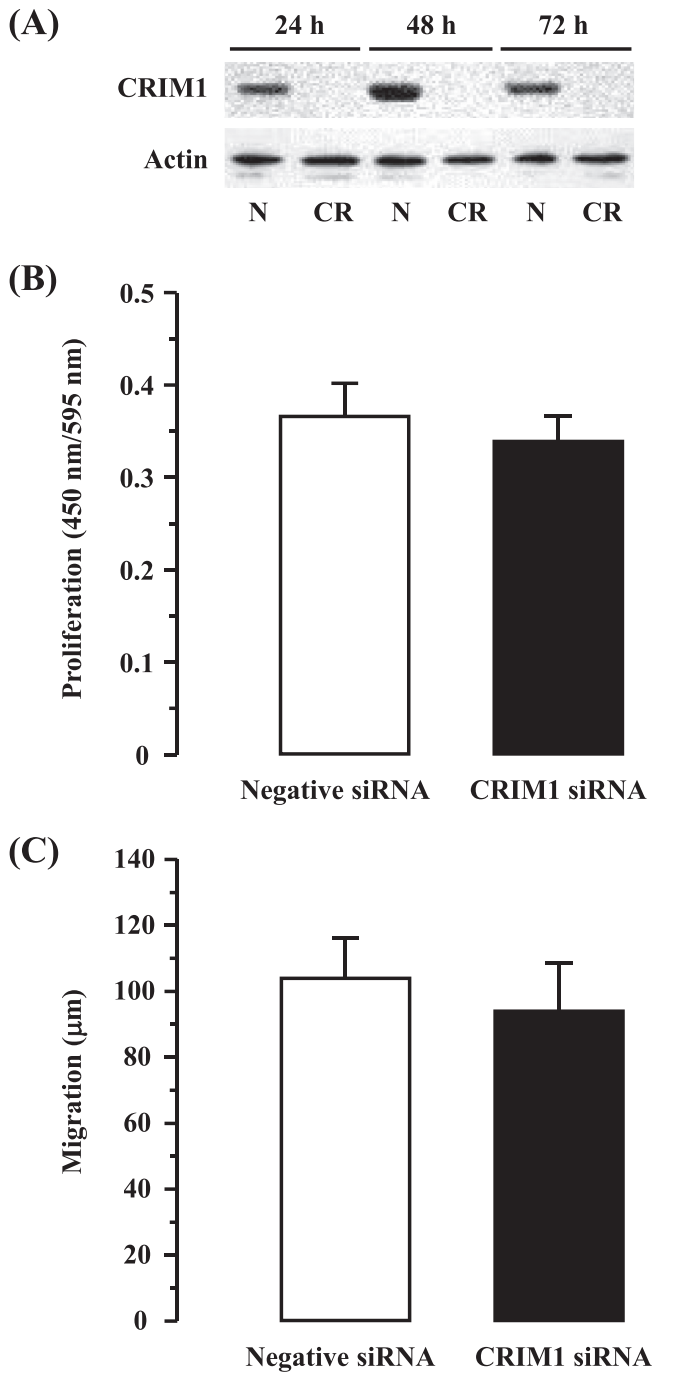

Fig. 1. (A) Negative siRNA (N) or human CRIM1 siRNA (CR) was introduced to HUVECs, and the cells were cultured for the indicated times. Expression of CRIM1 and actin was detected by immunoblotting. (B and C) HUVECs were subjected to RNAi for CRIM1 for 24 h. After the cells were incubated with VEGF for 48 h or 6 h, cell proliferation (B) or migration (C) was determined, respectively. Data are represented as mean ± S.E. (n = 6).

2.5. Construction of stable high CRIM1-expressing F-2 cells

F-2 cells were transfected with pCI-neo containing CRIM1 as described above. Transfected cells were selected by treatment with 200 μg/ml G418 for 3 weeks. Thereafter, several colonies were obtained, and single cells were cloned from the respective colonies by using the limiting dilution method. The cloned cells were maintained in DMEM supplemented with 10% FBS and 200 μg/ml G418 for additional 1 month. Two clones (CR1 and CR2) were randomly chosen and used in the experiments.

2.6. Immunoblotting

Cells were lysed in 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 120 mM NaCl, 5 mM sodium orthovanadate, 10 mM sodium fluoride, 1% NP40, 0.25% sodium deoxycholate, 0.8 μM aprotinin, 15 μM E-64, 20 μM leupeptin and 10 μM pepstatin A. The suspensions were agitated for 20 min and then were centrifuged at 15,000 rpm for 10 min. The supernatants were recovered as lysates. After the lysates were subjected to 7.5% SDS-PAGE, the separated proteins were blotted. The blots were incubated over night with the primary antibody and then probed with the secondary antibody linked to peroxidase. Immunoreactive proteins were visualized using an enhanced chemiluminescence kit.

2.7. Proliferation

Proliferation was assessed using WST-8, according to the manufacturer's instruction. Cells ($2–4 \times 10^3$ cells) were seeded in 96-well plates, incubated for 24 h and then starved in growth factor- or FBS-free medium. The starved HUVECs or the other cells were incubated with 50 ng/ml VEGF or 10% FBS, respectively, for 48 h and then WST-8 was added. Two hours later, the absorbance at 450 nm and 595 nm were measured.

2.8. Migration assay

Cells were seeded in 12-well plates, allowed to grow to confluence and then starved. Scratch wounds were created by scraping the monolayer with a micropipette tip. After changing the medium, HUVECs or the other cells were incubated with 30 ng/ml VEGF or 1% FBS, respectively, for the indicated times. The wounded area was observed under a phase-contrast microscope equipped with a digital camera. Four randomly chosen fields were marked,

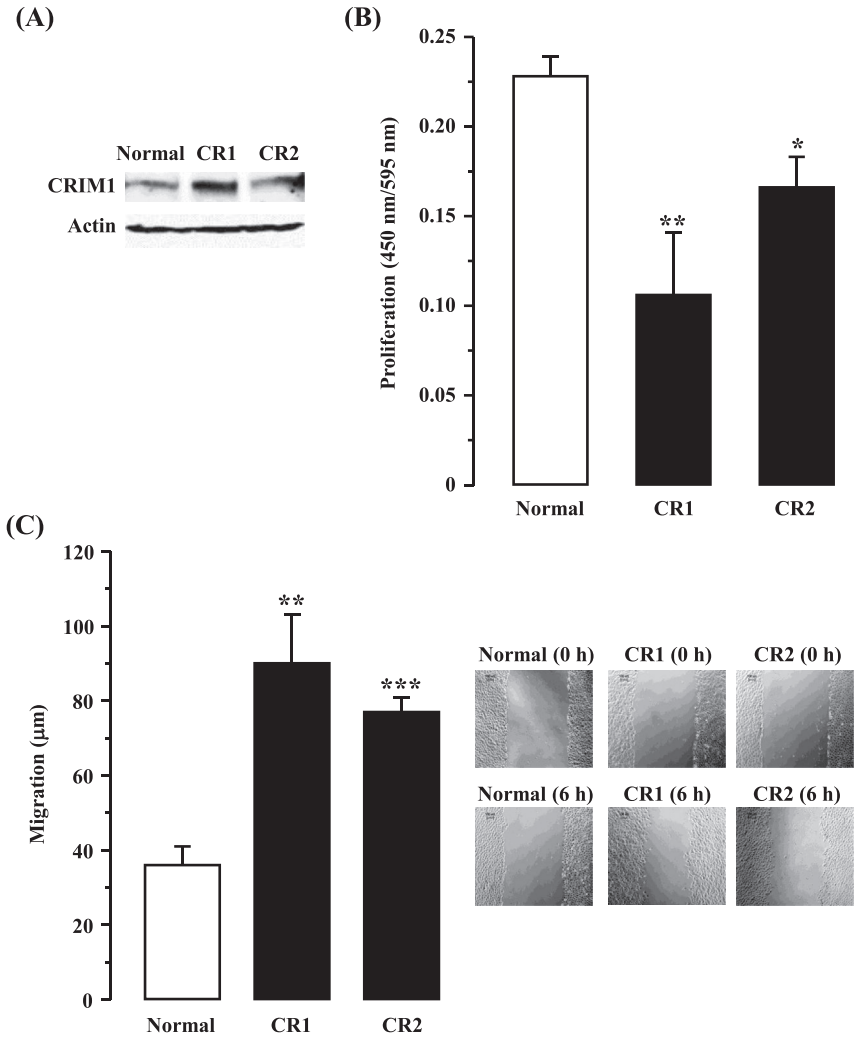

Fig. 2. (A) CR1 and CR2, stable CRIM1-overexpressing F-2 lines, were constructed. Expression of CRIM1 and actin was detected by immunoblotting. (B and C) Normal, CR1 and CR-2 F-2 cells were incubated with FBS for 48 h or 6 h, and cell proliferation (B) or migration (C) was determined, respectively. Data are represented as mean ± S.E. (n = 6). Significantly different from normal cells at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

and the migration of endothelial cells from the edge of the injured monolayer was quantified by measuring the distance between the edges before and after injury.

2.9. Statistical analysis

Data are represented as means ± S.E. Statistical differences were evaluated by one-way ANOVA followed by Student's *t*-test. A *P* value of <0.05 was regarded as significant.

3. Results

To examine the role of CRIM1 at a basal level in the proliferation and migration of endothelial cells, a CRIM1 knock-down study was conducted. Introduction of CRIM1 siRNA caused a marked loss of CRIM1 in HUVECs from 24 h through 72 h, but the negative control siRNA had no effect on CRIM1 expression (Fig. 1A). HUVECs were pretreated with CRIM1 siRNA for 24 h for the following knock down experiments. The proliferation and migration of CRIM1 knock down cells were promoted by VEGF, and were equivalent to those of the negative control cells (Fig. 1B and C).

Overexpression of CRIM1 in HUVECs was attempted, but the transfection efficiency was too low and disproportionate to prepare a sufficient amount of HUVECs with a high expression of CRIM1. Therefore, other endothelial F-2 cells with similar characteristics to HUVECs were used. F-2 cells (a murine vascular endothelial cell line) express CRIM1 endogenously and are capable of forming tubular structures in response to angiogenic factors [10,11]. Stable CRIM1-overexpressing F-2 cells, termed CR1 and CR2, were constructed, as shown in Fig. 2A. When a proliferation assay was performed, proliferation of CR1 and CR2 cells was significantly reduced (Fig. 2B). They decreased by 53.5% for CR1 and 27.2% for CR2, compared with that of normal F-2 cells. In contrast, migration of CR1 and CR2 cells was significantly faster than normal cells (Fig. 2C). The migratory distance of CR1 and CR2 cells increased 2.5-fold and 2.1-fold, respectively, compared with that of normal F-2 cells.

The effects of CRIM1 were also determined in BAECs. Endogenous CRIM1 could not be detected in BAECs, but the cells were susceptible to transfection (Fig. 3A). Expression of CRIM1 markedly inhibited proliferation of BAECs, the decreasing by 56.9% compared with that of Mock-treated cells (Fig. 3B). The migration of BAECs

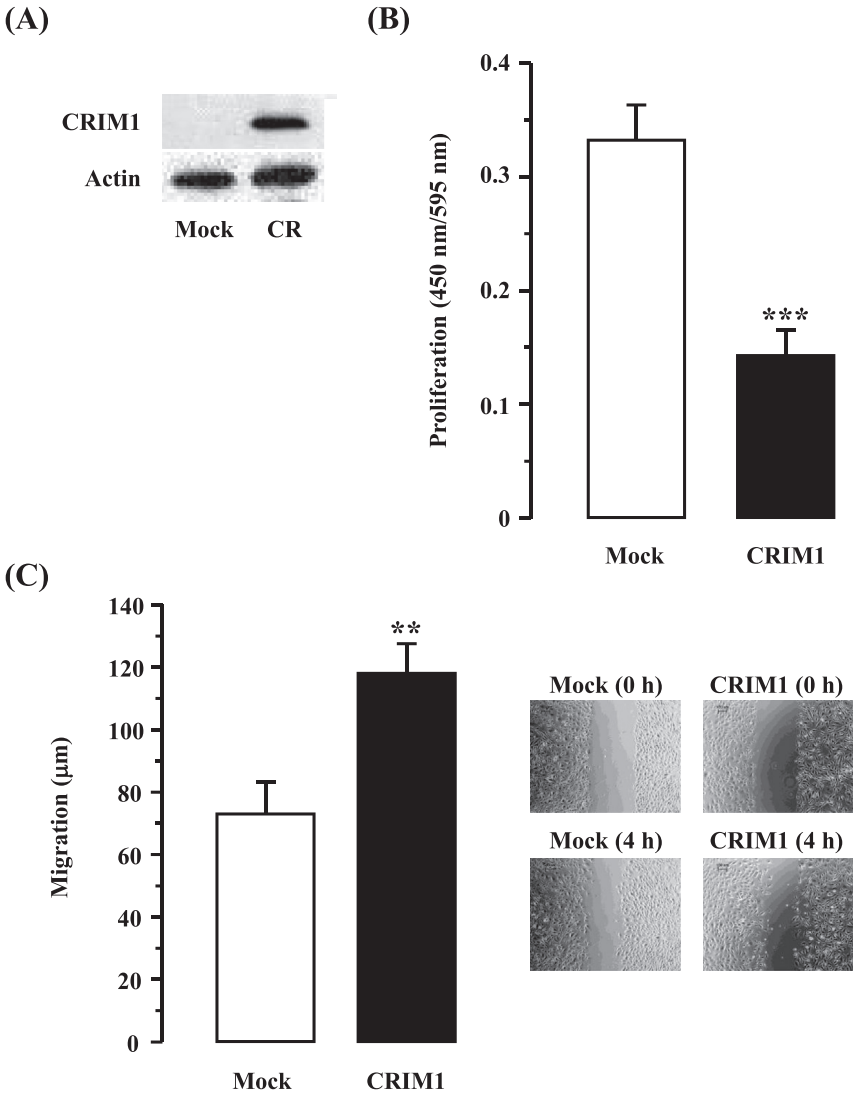

Fig. 3. (A) pCIneo without any insert cDNA (Mock) or pCIneo containing CRIM1 cDNA (CR) was introduced to BAECs, and the cells were cultured for 24 h. Expression of CRIM1 and actin was detected by immunoblotting. (B and C) BAECs were subjected to overexpression of CRIM1 for 24 h. After the cells were incubated with FBS for 48 h or 4 h, cell proliferation (B) or migration (C) was determined, respectively. Data are represented as mean ± S.E. (n = 6). Significantly different from Mock at $^{**}P < 0.01$ and $^{***}P < 0.001$.

was significantly accelerated by CRIM1 (Fig. 3C). The migratory distance of CRIM1-expressing cells was 1.6-fold longer than that of Mock-treated cells.

We examined whether such effects of CRIM1 were specific for endothelial cells. CRIM1 was overexpressed in COS-7 cells, but CRIM1 did not affect the proliferation or migration of COS-7 cells (Fig. 4).

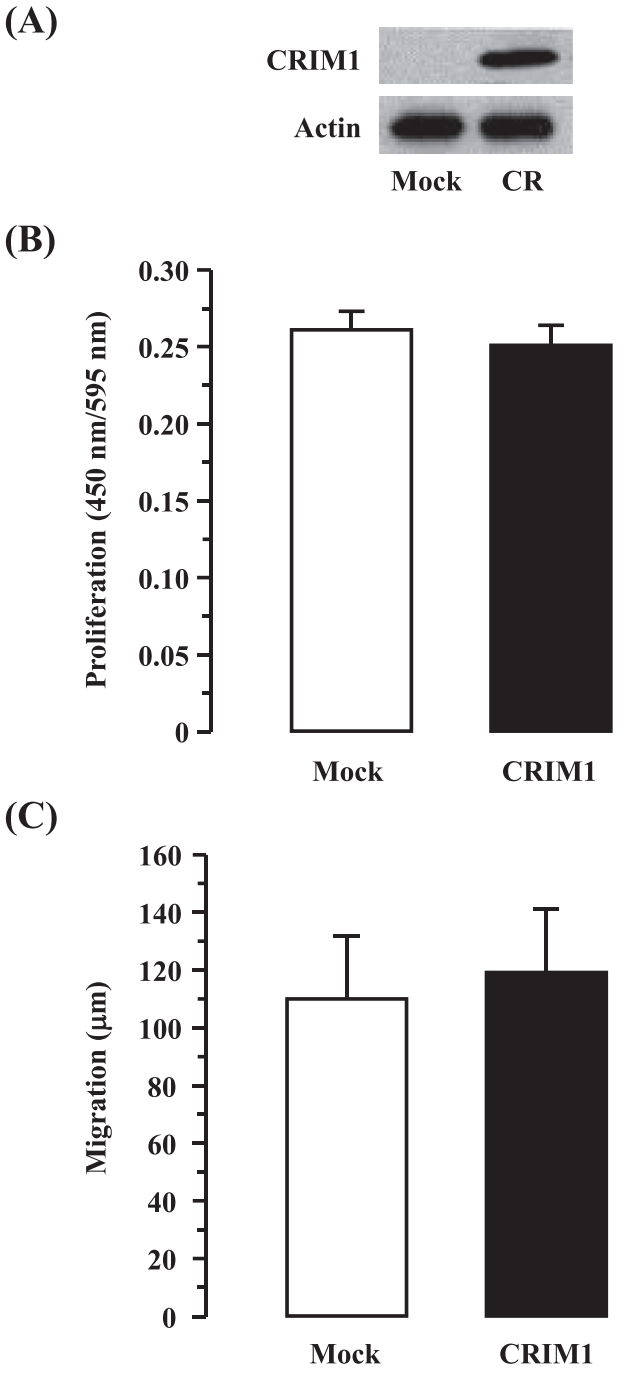

Fig. 4. (A) pCIneo without any insert cDNA (Mock) or pCIneo containing CRIM1 cDNA (CR) was introduced to COS-7 cells, and the cells were cultured for 24 h. Expression of CRIM1 and actin was detected by immunoblotting. (B and C) COS-7 cells were subjected to overexpression of CRIM1 for 24 h. After the cells were incubated with FBS for 48 h or 6 h, cell proliferation (B) or migration (C) was determined, respectively. Data are represented as mean ± S.E. (n = 6).

4. Discussion

Previous studies have demonstrated that CRIM1 is upregulated in response to angiogenic factors, and the increase in CRIM1 expression is a prerequisite for tube formation of endothelial cells [4,9]. Therefore, we assumed that the increased CRIM1 may affect other endothelial cell functions. In the present study, CRIM1 at high expression levels markedly inhibited the proliferation of F-2 cells and BAECs, indicating that increased CRIM1 reduces the proliferation of endothelial cells. Overexpression of CRIM1 in F-2 cells enhanced tubular network formation in collagen gels (Takahashi et al., unpublished data). Angiogenic factors such as VEGF and bFGF stimulate the proliferation of endothelial cells, whereas they induce morphological changes to tubular structures by arresting proliferation [12]. There are no published report concerning tubulogenesis-related endothelial protein to inhibit proliferation. CRIM1 may serve as a regulator in endothelial cells to switch from proliferating cells to morphological differentiation. Furthermore, our results showed that increased CRIM1 accelerates the migration of endothelial cells, contributing to tube formation of endothelial cells.

Interestingly, CRIM1 may exert these activities only in endothelial cells, because the effects of CRIM1 on proliferation and migration were observed in endothelial cells, but not in COS-7 cells. There may be endothelial cell-specific proteins that mediate CRIM1 intracellular signaling. The activation of MAP kinases and Akt was examined in CRIM1-overexpressing endothelial cells, because protein kinases such as MAP kinases and Akt play important roles in the proliferation and migration of endothelial cells [13–15]. The phosphorylation levels of these kinases in CRIM1-overexpressing cells did not differ from those in normal cells (Takahashi et al., unpublished data). Further investigations are needed to clarify the CRIM1-related signals.

Consistent with the report by Glienke et al. [4], knock down of CRIM1 had no effect on proliferation or migration of monolayer HUVECs. These results indicate that CRIM1 at a basal expression level is not involved in the proliferation or migration of endothelial cells. In contrast, Fan et al. [8] reported that basal CRIM1 enhanced receptor signaling of autocrine VEGF that was derived from HUVECs. It remains unclear whether or not basal CRIM1 plays a specific role in endothelial cells.

In conclusion, the present study demonstrates that increased CRIM1 reduces the proliferation and accelarates the migration of endothelial cells, and that these effects of CRIM1 might contribute to tube formation of endothelial cells. CRIM1 induced by angiogenic factors may serve as a regulator in endothelial cells to switch from proliferating cells to morphological differentiation.

Conflict of interest

None declared.

Acknowledgments

We thank Akinori Sugiyama (Iwate Medical University), Mariko Sakanaka, Aya Iehara, Kayo Motomura, Yasuyo Hashimoto, Shizuka Yamagata, Yuki Akiyama, Kana Takimoto (Mukogawa Women's University) and Seiko Hashiguchi (Kyushu University of Health and Welfare) for their assistance. This research was supported by grant from Japan Society for the Promotion of Science (JSPS) [Grant-in-Aid for Scientific Research (C) #23590099].

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.118.

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