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Induction of cysteine-rich motor neuron 1 mRNA expression in vascular endothelial cells

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ABSTRACT

Cysteine-rich motor neuron 1 (CRIM1) is expressed in vascular endothelial cells and plays a crucial role in angiogenesis. In this study, we investigated the expression of CRIM1 mRNA in human umbilical vein endothelial cells (HUVECs). CRIM1 mRNA levels were not altered in vascular endothelial growth factor (VEGF)-stimulated monolayer HUVECs or in cells in collagen gels without VEGF. In contrast, the expression of CRIM1 mRNA was elevated in VEGF-stimulated cells in collagen gels. The increase in CRIM1 mRNA expression was observed even at 2 h when HUVECs did not form tubular structures in collagen gels. Extracellular signal-regulated kinase (Erk) 1/2, Akt and focal adhesion kinase (FAK) were activated by VEGF in HUVECs. The VEGF-induced expression of CRIM1 mRNA was significantly abrogated by PD98059 or PF562271, but was not affected by LY294002. These results demonstrate that CRIM1 is an early response gene in the presence of both angiogenic stimulation (VEGF) and environmental (extracellular matrix) factors, and Erk and FAK might be involved in the upregulation of CRIM1 mRNA expression in vascular endothelial cells.

1. Introduction

Angiogenesis is a complex process by which new blood vessels are generated from preexisting microcapillaries in response to angiogenic factors [1,2]. Angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) stimulate the proliferation, migration and tube formation of endothelial cells. Angiogenesis is a promising target for the treatment of cancers because of its essential role in the growth, persistence and metastases of tumors, and many studies have been conducted to understand the mechanism of angiogenesis.

Cysteine-rich motor neuron 1 (CRIM1) is a type I transmembrane protein with insulin-like growth factor binding protein domain and six repeats of von Willebrand factor C domain [3]. CRIM1 is expressed in various cell types including vascular endothelial cells, and is implicated in angiogenesis and the development of the vascular system [4–8]. CRIM1 regulates the delivery of VEGF from podocytes to endothelial cells in the glomerulus of the kidney [7,8]. CRIM1 stabilizes neuronal morphogenesis by forming a complex with N-cadherin and β-catenin [9]. Retinal vascular stability is maintained by CRIM1 by enhancing VEGF autocrine signaling in endothelial cells [6]. However, the molecular function of CRIM1 in angiogenesis remains unclear. CRIM1 is considered to play a crucial role in tubulogenesis in the angiogenic process, because the expression of CRIM1 mRNA is upregulated in endothelial cells during tubulogenesis, and CRIM1-antisense oligonucleotides have no effect on proliferation, but they abrogates tubulogenesis [4]. The intracellular signaling pathway that elicits the upregulation of CRIM1 is also uncharacterized.

In the present study, we determined the cellular condition required for the induction of CRIM1 mRNA expression and subsequently investigated the protein kinase pathways involved in the upregulation of CRIM1 in endothelial cells.

Abbreviations: CRIM1, cysteine-rich motor neuron 1; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; Erk, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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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); PD98059 and LY294002 (Wako Chemicals, Osaka, Japan); PF562271 (Tocris, Ellisville, USA); HUVECs, EBM-2 medium and culture supplies (Lonza, Basel, Switzerland); Sepasol-RNA (Nacalai Tesque, Kyoto, Japan); Dulbecco's modified Eagle's medium (DMEM), SuperScript III Supermix, Taq polymerase and deoxynucleotides (Life Technologies, Carlsbad, USA); anti-phosphotyrosine antibody and anti-FAK antibody (BD Biosciences, Lexington, USA); anti-Akt antibody, anti-pAkt antibody (phospho-Ser473), anti-Erk1/2 antibody, anti-pErk1/2 antibody (phospho-Thr202/Tyr204), secondary antibody linked to peroxidase and Phototope-HRP Western Blot detection system (Cell Signaling, Beverly, USA); collagen type I-A and type I-C (Nitta Gelatin; Osaka, Japan). Protein kinase inhibitors were dissolved in dimethyl sulfoxide where the final concentration of the solvent was less than 0.5% in culture dishes. All other chemicals were of reagent grade.

2.2. Cell culture

HUVECs were maintained in EBM-2 medium containing supplements consisting of 2% fetal bovine serum, 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. All culture dishes were coated with collagen type I-C, and cells between passages 5 and 10 were used in the experiments.

2.3. Induction of CRIM1 mRNA expression

HUVECs (2×10^5 cells) were seeded onto a 60-mm dish in full EBM-2 medium and were allowed to grow to 60% or 100% confluence. The cells were starved for 6 h in the medium without the growth factors. The cells were pretreated with inhibitors at 10 μM for 30 min and then stimulated with 30 ng/ml VEGF or vehicle at 37 °C for the indicated times.

To induce tubulogenesis, HUVECs were incubated in collagen gel matrix according to our previously reported method [10] with modifications. Seven volumes of 0.5 mg/ml collagen type I-A solution were mixed with two volumes of fivefold concentrated $NaHCO_3$-free DMEM and one volume of reconstitution buffer (4.77 g HEPES and 2.2 g $NaHCO_3$ in 100 ml of 50 mM NaOH), and then the solution was kept on ice. An aliquot (2 ml) of the mixture was added to 60-mm dishes which were incubated at 37 °C for 30 min to allow gel formation. HUVECs (2×10^5 cells) were pretreated with inhibitors at 10 μM for 30 min and seeded onto the gels and incubated with 30 ng/ml VEGF or vehicle at 37 °C for the indicated times.

2.4. Determination of CRIM1 mRNA level

The mRNA expression levels of CRIM1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined by reverse transcription-PCR. Total RNAs were extracted using Sepasol-RNA followed by DNase I digestion according to the manufacturer's instruction. cDNAs were synthesized using SuperScript III Supermix, and semi-quantitative PCR was performed. cDNAs (1 μg) were denatured for 1 min at 95 °C and amplified using 20 cycles of 94 °C for 30 s; 60 °C for 30 s and 72 °C for 30 s with a final 5-min extension at 72 °C. The PCR primers were 5′-CTGCTGCCCACAGTGTACAGAT-3′ and 5′-GCATGCTGTAGAAGCCACTGAATC-3′ for CRIM1, and 5′-TG AAGGTCGGAGTCAACGGA-3′ and 5′-ATTGAGAGCAATGCCAGCC-3′ for GAPDH. The DNA products were resolved on a 1% agarose gel containing ethidium bromide. The sequence of the CRIM1 product (889 bp) was confirmed to be identical to known sequences. The CRIM1 mRNA expression levels were normalized to the GAPDH mRNA levels, and the relative mRNA expression was determined based on comparisons with control cells.

2.5. Detection of phospho-Akt, Erk1/2 and -FAK

HUVECs were lysed in 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM β-glycerophosphate, 1% Triton X-100, 0.5% sodium deoxycholate and the protease inhibitor mixture. The suspensions were agitated for 10 min and then were centrifuged at 10,000 rpm for 10 min. The supernatants were recovered as lysates.

To detect phospho-Akt and -Erk1/2, the lysates were subjected to 10% SDS–PAGE. To detect phospho-FAK, the lysates were incubated with anti-FAK antibody for 2 h and then overnight with protein G-agarose beads, after which the immunoprecipitates were subjected to 10% SDS–PAGE.

The separated proteins were blotted onto polyvinylidene difluoride membranes. 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.

2.6. Statistical analysis

The data are represented as means ± S.E. Statistical differences were evaluated using the Student's *t*-test. $P < 0.05$ was regarded as significant.

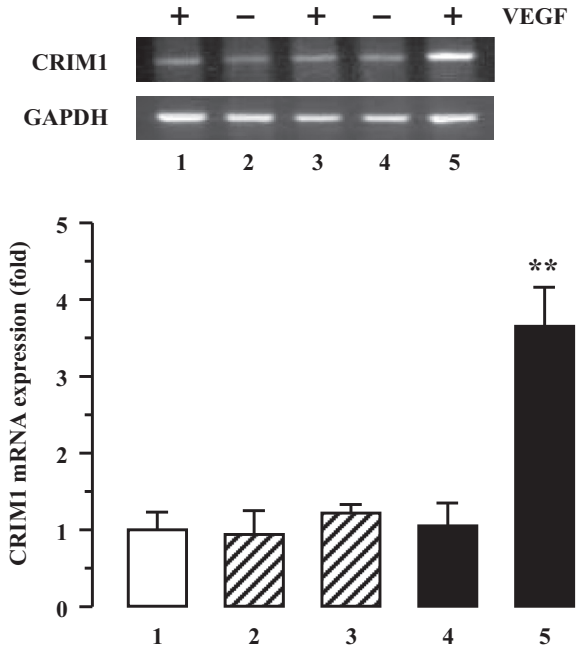

Fig. 1. CRIM1 mRNA expression in HUVECs. HUVECs were incubated in collagen-coated dishes (groups 1–3) or in collagen gels (groups 4 and 5) in the presence of vehicle or 30 ng/ml VEGF for 8 h. The confluence levels of the monolayer culture were 60% for group 1 and 100% for groups 2 and 3. The CRIM1 and GAPDH mRNA levels were measured by reverse transcription-semiquantitative PCR. The CRIM1 mRNA levels were normalized to those of GAPDH, and the relative mRNA expression was determined as the fold difference compared with group 1. The data are represented as mean ± S.E. for four experiments. *Significantly different from group 1 at $^{**}P < 0.01$.

3. Results

Glienke et al. reported that, in the presence of angiogenic factors, CRIM1 mRNA levels are increased in the tubular structures of endothelial cells, compared with that in proliferating monolayer cells [4]. Therefore, we investigated the expression of CRIM1 mRNA in HUVECs in various conditions (Fig. 1). The CRIM1 mRNA levels in a confluent monolayer of HUVECs were equivalent to those in proliferating cells irrespective of VEGF stimulation. When HUVECs were cultured in collagen gels, tubulogenesis did not occur in the absence of VEGF, whereas the cells formed an extended network of tubular structures in response to VEGF. The expression of CRIM1 mRNA was elevated significantly only in tubular HUVECs.

The time course of CRIM1 mRNA expression was examined in VEGF-stimulated HUVECs in collagen gels (Fig. 2A), which showed that the expression of CRIM1 mRNA increased in a time-dependent manner. The CRIM1 expression levels at 2 h were significantly higher than the basal levels at 0 h, and those gradually increased up to 8 h. However, morphological changes in endothelial cells were not observed at 2 h when the CRIM1 mRNA expression levels were upregulated (Fig. 2B).

Next, we examined the intracellular signals that elicit the expression of CRIM1 mRNA. VEGF induced the activation of Erk1/2, Akt and FAK in HUVECs, according to the phosphorylation of these protein kinases (Fig. 3). PD98059 (MEK inhibitor), LY294002 (phosphatidylinositol 3-kinase inhibitor) and PF562271 (FAK inhibitor) abrogated the phosphorylation of Erk1/2, Akt and FAK, respectively.

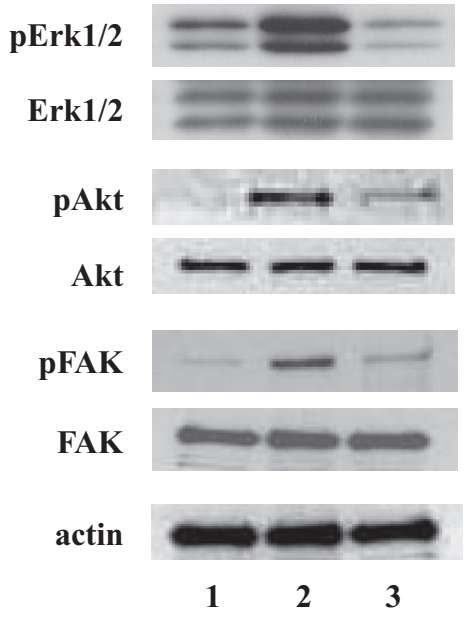

Fig. 3. Activation of Erk1/2, Akt and FAK in HUVECs. HUVECs were pretreated with 10 μM PD98059 for Erk1/2, 10 μM LY294002 for Akt or 10 μM PF562271 for FAK (lane 3) for 30 min and then stimulated with vehicle (lane 1) or 30 ng/ml VEGF (lanes 2 and 3) for 15 min. Phospho-proteins were detected by immunoblotting.

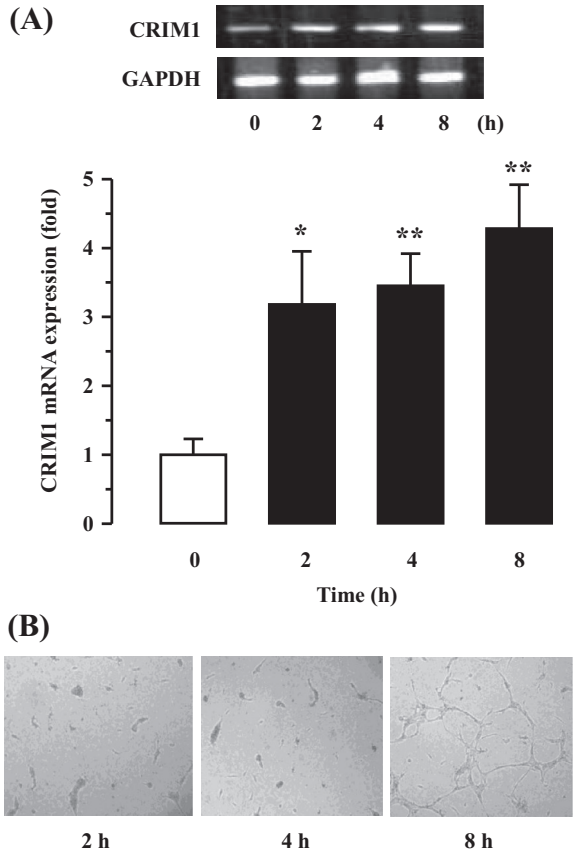

Fig. 2. Time courses of CRIM1 mRNA expression and tube formation of HUVECs. HUVECs were incubated in collagen gels in the presence of 30 ng/ml VEGF for the indicated times. (A) The CRIM1 and GAPDH mRNA levels were measured by reverse transcription-semiquantitative PCR. The CRIM1 mRNA levels were normalized to those of GAPDH, and the relative mRNA expression was determined as the fold difference compared with 0 h. The data are represented as mean ± S.E. for four experiments. *Significantly different from 0 h at $^{*}P < 0.05$ and $^{**}P < 0.01$. (B) Microscopic photographs are shown (magnification: ×100).

The expression of CRIM1 mRNA was examined when HUVECs were pretreated with the inhibitors and then incubated with VEGF for 2 h in collagen gels (Fig. 4). The CRIM1 mRNA expression was markedly suppressed by PD98059, but was not affected by LY294002. PF562271 also significantly inhibited the increase in the expression of CRIM1 mRNA.

4. Discussion

The previous study reported that the CRIM1 mRNA levels in endothelial cells are higher in tubular structures than those in a proliferating monolayer [4]. Endothelial cells do not proliferate during tubulogenesis [11], we therefore examined the expression of CRIM1 mRNA in HUVEC monolayers, which were grown on collagen-coated dishes to cell contact inhibition. However, the CRIM1 mRNA levels were not altered in the monolayer culture. Similarly, the expression levels remained unchanged after placing HUVECs in collagen gels without VEGF. The CRIM1 mRNA levels increased only in VEGF-stimulated HUVECs in collagen gels. These results demonstrate that both angiogenesis stimulation factors such as VEGF and angiogenesis environmental factors such as collagen gel matrix are both required for the upregulation of the CRIM1 mRNA expression in HUVECs.

The CRIM1 mRNA expression levels were elevated from early phase after VEGF stimulation. It should be noted that the generation of tubular structures of HUVECs followed the increase in CRIM1 mRNA expression. The introduction of CRIM1-antisense oligonucleotides into HUVECs resulted in impairment of tubulogenesis [4]. siRNAs for CRIM1 also prevented tube formation of HUVECs (Takahashi et al., unpublished data). These results indicate that an early increase in CRIM1 mRNA expression is a requisite for subsequent tubulogenesis of endothelial cells.

Our results indicate that Erk and FAK might be involved in the induction of the expression of CRIM1 mRNA in HUVECs in collagen gels. These protein kinases were activated by VEGF even in HUVEC

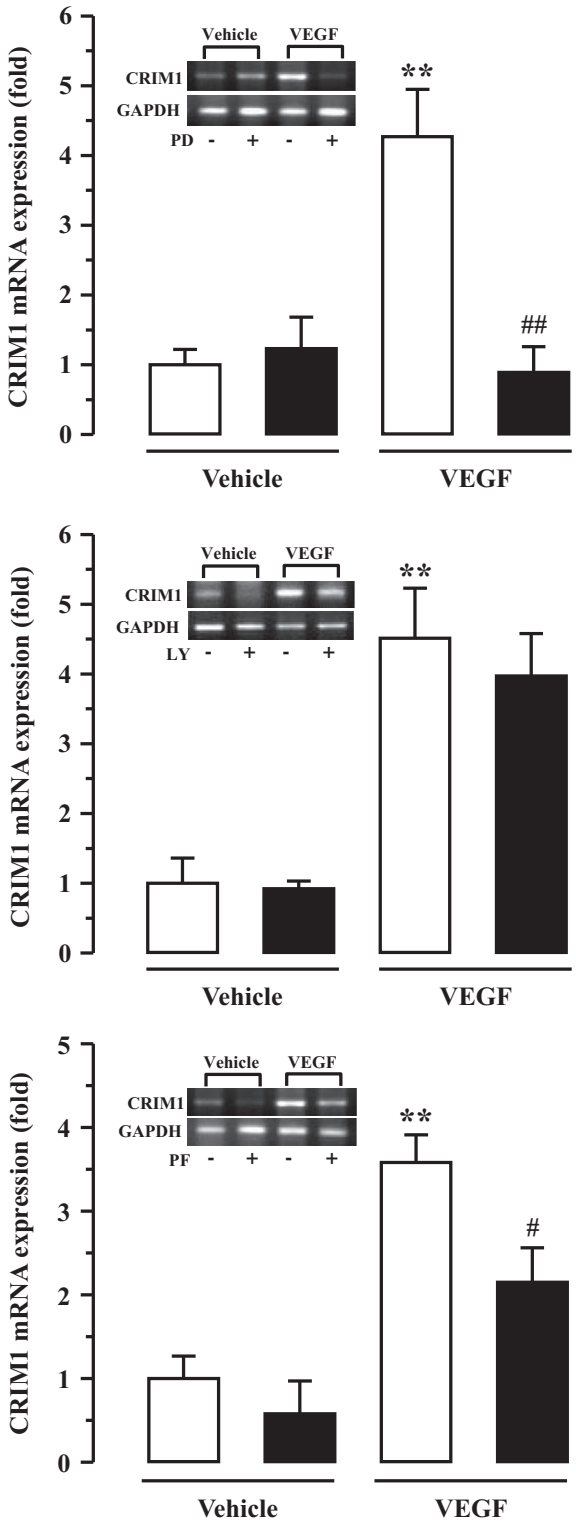

Fig. 4. Effects of protein kinase inhibitors on CRIM1 mRNA expression levels in HUVECs. HUVECs were pretreated with 10 μM PD98059 for Erk1/2, 10 μM LY294002 for Akt or 10 μM PF562271 for FAK for 30 min and then incubated in collagen gels in the presence of vehicle or 30 ng/ml VEGF for 2 h. The CRIM1 and GAPDH mRNA levels were measured by reverse transcription-semiquantitative PCR. The CRIM1 mRNA levels were normalized to those of GAPDH, and the relative mRNA expression was determined as the fold difference compared with vehicle-treated cells without inhibitors. The data are represented as mean ± S.E. for four experiments. *, #Significantly different from vehicle-stimulated cells without inhibitors and VEGF-stimulated cells without inhibitors, respectively, at $^{**}P < 0.01$, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

monolayers where the CRIM1 mRNA levels were not changed. This suggests that Erk and FAK pathways to the induction of CRIM1 might cooperate with unidentified intracellular signals generated by interactions with the extracellular matrix in an angiogenic environment. Further investigations are needed to clarify the unidentified signals.

It is known that Erk, FAK and Akt play important roles in tube formation of endothelial cells [12–15], and we also confirmed that the inhibition of these kinases prevented tubulogenesis of HUVECs (Takahashi et al., unpublished data). CRIM1 may partially contribute to the stimulatory effects of Erk and FAK, whereas Akt pathway is CRIM1-independent.

Overall, we conclude that CRIM1 is an early response gene in the presence of both angiogenic stimulation (VEGF) and environmental (extracellular matrix) factors, and Erk and FAK might be involved in upregulation of CRIM1 mRNA expression in vascular endothelial cells.

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