

CD14 Acts as an Angiogenic Factor by Inducing Basic Fibroblast Growth Factor (bFGF)

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Angiogenesis is the process of generating new capillary blood vessels from pre-existing vessels and a fundamental process in the normal development, reproduction, and wound healing. Since neovascularization in physiological processes is tightly regulated by a balance of angiogenic and anti-angiogenic factors, unregulated angiogenesis may lead to several angiogenic diseases and is thought to be indispensable for tumor growth and metastasis. A growing tumor relies on angiogenesis to receive an adequate supply of nutrients and oxygen. In addition, the newly formed blood vessels provide a way for tumor cells to enter the circulation and to metastasize to distant organs.

Angiogenesis plays an important role in the progression of solid tumors and hematologic malignancies. Acute myelogenous leukemia (AML) is an aggressive disorder characterized by the rapid proliferation of abnormal cells which accumulate in the bone marrow. Recent studies suggest that the increased blood vessel density is observed in AML bone marrow and angiogenesis plays an important role in leukemogenesis.¹ CD14, a mediator of innate pro-inflammatory responses following bacterial lipopolysaccharide (LPS) binding,² is highly expressed on monocytes and macrophages and is found in leukemia cells and peripheral blood endothelial progenitor of monocytic origin.^{3,4} Although CD14 has been shown to be expressed in leukemia cells,^{5,6} it is not clear whether CD14 is capable of inducing angiogenesis. In the present study, we studied the effect of CD14 on angiogenesis.

Since angiogenesis process is composed of various steps including endothelial cell migration through extracellular matrix, we investigated the effect of CD14 on endothelial cell migration. First of all, HEK 293 cells were transfected with CD14 expression plasmid and transfected cells were selected as described in experimental section. After confirmation of protein expression of CD14 (Fig. 1A), conditioned medium (CM) from the selected cells was obtained. In a chemotaxis chamber, human umbilical vein endothelial cells (HUVECs) were treated with CM from stably transfected cells for 2 h and HUVECs that had been migrated through membrane pores were counted under microscope. CM derived from CD14-transfected cells (CD14-CM) showed significantly induced migratory effect, compared to that of control-CM (Fig. 1B). Therefore, this result suggests that CD14-CM has the biological activity that can induce endothelial cell migration.

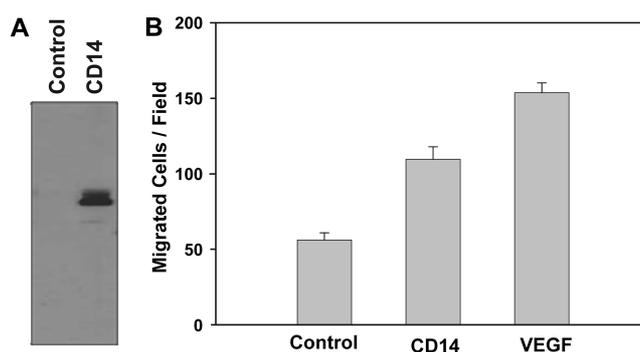


Figure 1. CD14 expression in stably transfected HEK 293 cells and HUVEC migration assays. (A) Stably transfected cells were lysed in a buffer solution, followed by centrifugation at 13,000 rpm for 30 min. Cell lysates were run in SDS-PAGE and transferred to nitrocellulose membrane. The blocked membrane was then incubated with an anti-HA antibody, followed by an appropriate secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were visualized using an ECL system. (B) CD14-CM stimulates endothelial cell migration. Migration assays with human umbilical vein endothelial cells (HUVECs) were carried out in 48-well microchemotaxis chambers. HUVECs were incubated with control-CM or CD14-CM for 2 h. After fixation and staining, the number of migrated cells was determined by counting two regions of each well under a microscope VEGF (20 ng/mL) was used as a positive control.

We next examined the ability of CD14-CM to promote the mesh-like structures of HUVECs on matrigel. Matrigel is generally used for studying HUVEC attachment, migration and differentiation. The extent of mesh-like structure was more enhanced in HUVECs influenced in CD14-CM than that of control CM (Fig. 2A and B). Thus, CD14 has ability of inducing tube formation of endothelial cells.

Among a variety of angiogenic factors, vascular endothelial growth factor (VEGF) and bFGF are produced by many tumor cells and play a role in the growth and neovascularization of tumors.⁷ To examine the effect of CD14 on bFGF secretion into CM, we measured the protein level of bFGF in CM using ELISA assay kit. CD14-overexpressing cells released significantly more bFGF than control cells (Fig. 3). However, change of VEGF secretion level was not observed in CD14-CM (data not shown). This result indicates that the increased level of secreted bFGF may be associated with CD14-induced angiogenic activity.

In conclusion, the results of this study suggest that CD14

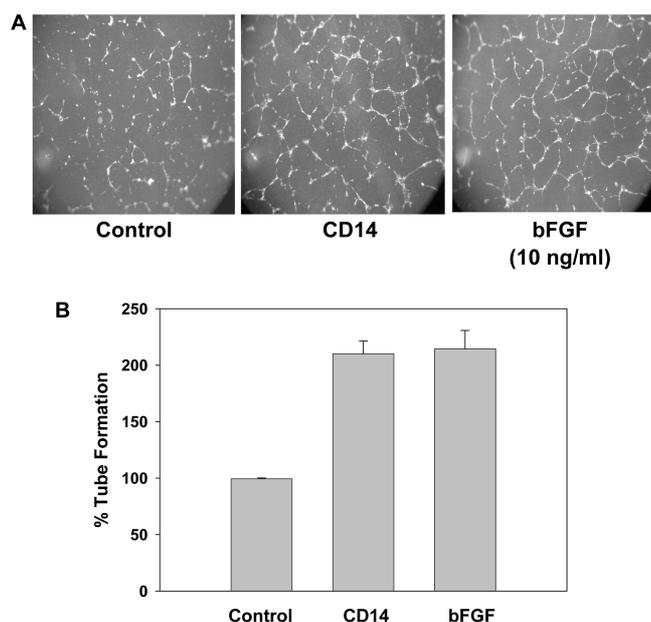


Figure 2. CD14-CM promotes tube formation of endothelial cells. (A) HUVECs were collected and seeded on Matrigel-coated plates at a density of 2.4×10^4 cells/well and then incubated with control-CM or CD14-CM. As a positive control, bFGF (10 ng/mL) was used. After 18 h, fields from each sample were photographed. Representative images of tube formation activity are shown. (B) Total tube areas were analyzed and quantitated by the Scion Image program. The control tube areas were defined as 100% tube formation, and the percent increase in tube formation as compared with control was calculated for each sample.

mediates angiogenesis by inducing bFGF. This observation provides that CD14 might be a candidate for developing anti-cancer agent in tumor growth and angiogenesis.

Experimental section

Cell culture. Human embryonic kidney (HEK) 293 and HUVECs were routinely maintained as previously described.^{8,9}

Plasmid constructs. The N-terminal hemagglutinin (HA)-tagged human CD14 gene for expression in mammalian cells was constructed by polymerase chain reaction, followed by cloning into the pcDNA3.1/Zeo plasmid.

Transfection and Selection of Transfected Cells. HEK 293 cells were transfected by lipofectamine (Invitrogen) with 1 μ g each of pcDNA3.1/Zeo-HA-CD14 or pcDNA3.1/Zeo-empty vector as a control. After 48 h, transfected cells were selected for two weeks in DMEM containing 10% FBS and Zeocin (200 μ g/mL). After 15 days of selection, resistant cells arising in dishes were propagated in low level (150 μ g/mL) of Zeocin. The overexpressed CD14 in stably transfected cells was confirmed by immunoblotting.

Immunoblotting. Immunoblotting were carried out as previously described.⁸

Preparation of CM. Confluent cells were washed and grown in serum free M199 medium. After 20 h of incubation, CM was collected and centrifuged at $500 \times g$ for

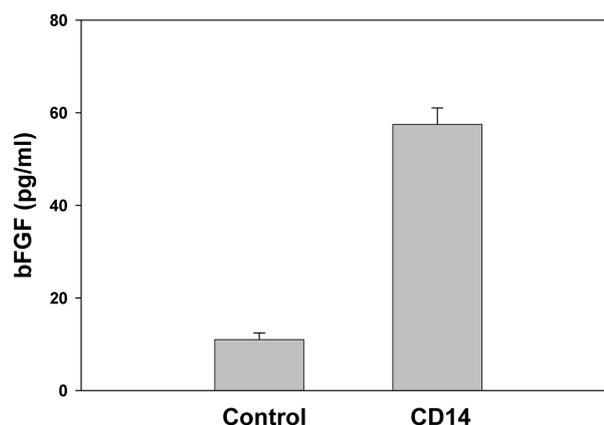


Figure 3. CD14 increases the level of bFGF in CM. Control or CD14 stable cells were incubated in serum-free M199 for 20 h, and aliquots of the CM were collected. The level of bFGF in CM was measured by ELISA as described in experimental section.

10 min and then at $800 \times g$ for 20 min to remove debris. The resultant CM was immediately used for migration and tube formation assays or stored at -70°C until use.

HUVEC Migration assays. Endothelial cell migration assays were carried out as previously described.¹⁰

Capillary-like tube formation assays. Tube formation assays were performed as previously described.⁸

Determination of the VEGF and bFGF level. To determine the VEGF or bFGF protein expression level, we obtained CM from control or stable cells. The amount of VEGF or bFGF protein in the CM from the cells was determined as previously described.^{8,9}

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