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A novel function for dendritic cell: Clearance of VEGF via VEGF receptor-1

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ABSTRACT

It has been reported that the plasma levels of VEGF in tumor patients decreased during dendritic cell (DC)-based immunotherapy, but the underlying mechanism remains unclear. Our current report demonstrates that VEGF levels were significantly decreased in the supernatants of DCs incubated with rhVEGF or tumor conditioned medium (TCM) while the intracellular VEGF in DCs was increased. The increased intracellular VEGF was not due to the *de novo* VEGF synthesis by DCs because exogenous VEGF inhibited the mRNA expression of VEGF in DCs. More direct evidence was provided to demonstrate that Cy3-labeled VEGF could be internalized by DCs specifically and efficiently. In addition, the activity of DCs to internalize VEGF was abolished by neutralizing antibody against VEGF receptor-1 (Flt-1) and inhibitors of endocytosis such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and genistein. This study highlights a novel function of DCs and allows a better understanding of the DC–VEGF interaction.

Tumor growth and metastasis rely on the ability of the tumor to induce its own blood supply. This process is dependent on the induction of angiogenesis mediated by angiogenic factors secreted by tumor cells and stromal cells [1]. Among the identified angiogenic factors, vascular endothelial growth factor (VEGF) is the most potent and representative [2]. VEGF is present in various human tumors and its production in tumor-bearing hosts is directly associated with poor prognosis [3,4]. The important roles of VEGF in tumors are not only because of VEGF-induced angiogenesis but also due to VEGF-related immunosuppression [5]. The mechanisms that regulate expression of VEGF in human tumors are still not well understood.

VEGF mainly binds to two tyrosine kinase receptors, VEGFR1/Flt-1 and VEGFR2/KDR [6,7]. Unlike KDR, Flt-1 undergoes weak tyrosine autophosphorylation in response to VEGF. Soluble form of Flt-1 inhibits the activity of VEGF [8]. Park et al. initially proposed that Flt-1 might negatively regulate the activity of VEGF on endothelial cells by preventing VEGF from binding to KDR [9]. Flt-1 has also been found to be expressed on monocytes and implicated in monocytes migration [10]. It is a common method to generate DCs by culturing monocytes obtained from human peripheral blood with GM-CSF and IL-4 [11], and DCs developed in such way express Flt-1 [12]. The precise function of Flt-1 expressed on DCs has not been fully investigated.

DCs are professional antigen presenting cells that have been regarded as an attractive vehicle for the delivery of specific tumor-associated antigens in immunotherapy protocols against tumors [13]. Increased serum VEGF level is associated with reduced circulating DCs in patients with various malignancies [14,15]. DC infiltration and VEGF expression are inversely correlated in tumor specimens of non-small cell lung cancer [16]. In addition, it has been demonstrated that the plasma levels of VEGF of tumor patients decreased during DC-based immunotherapy [17], suggesting DCs may regulate the expression of VEGF. Many researches have been focused on the inhibitory effects of VEGF on DCs [18–20], however, little is known about the role of DCs in modulation of VEGF.

In the present study, we demonstrated that DCs had capacity to clear VEGF via Flt-1. These results revealed a novel function of DCs and provided a complement for the mechanisms of the DC–VEGF interactions.

Materials and methods

Generation of DCs. DCs generated from human peripheral blood monocytes (PBMCs) were obtained by Ficoll-density gradient centrifugation (Pharmacia, Uppsala, Sweden). The PBMCs enriched fraction was further depleted of lymphocytes by a 2 h plastic adhesion step at 37 °C followed by extensive washing with pre-warmed

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culture medium. Adherent cells were cultured in RPMI1640 (Gibco BRL-Life Technologies, Grand Island, NY) with 10% FBS (Gibco), 50 ng/ml GM-CSF (Leucomax, Roche, Basel, Switzerland) and 50 ng/ml IL-4 (Peprotech, Rocky Hill, NJ) for 6 days to generate immature DCs. Immature DCs were purified by mouse anti-human CD3, CD19, CD56, CD16, and magnetic beads conjugated to anti-pan mouse IgG (Dynal, Oslo, Norway). Purification of DCs was measured on a FACScan analyzer (Becton Dickinson, Mountain View, CA) with mAbs against DC-specific ICAM-grabbing nonintegrin (DC-SIGN), CD1a and HLA-DR. Phenotypic characteristics of DCs were processed for single staining on a FACScan flow cytometer with mAbs against CD80, CD83, and CD86, followed by FITC-labeled goat anti-mouse Ab specific for IgG. All of the antibodies are from BD Biosciences except specifically mentioned.

Preparation of tumor conditioned medium (TCM). Neuroblastoma cells (SK-N-AS) were cultured in RPMI1640 with 10% FBS for 48 h. Supernatants of culture medium (tumor conditioned medium, TCM) were collected by centrifugation to delete cell debris.

ELISA assay. Concentrations of VEGF and TGF-β in cell culture medium and cell lysate were detected by using ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's recommendation. Hepatocyte growth factor (HGF) was detected by

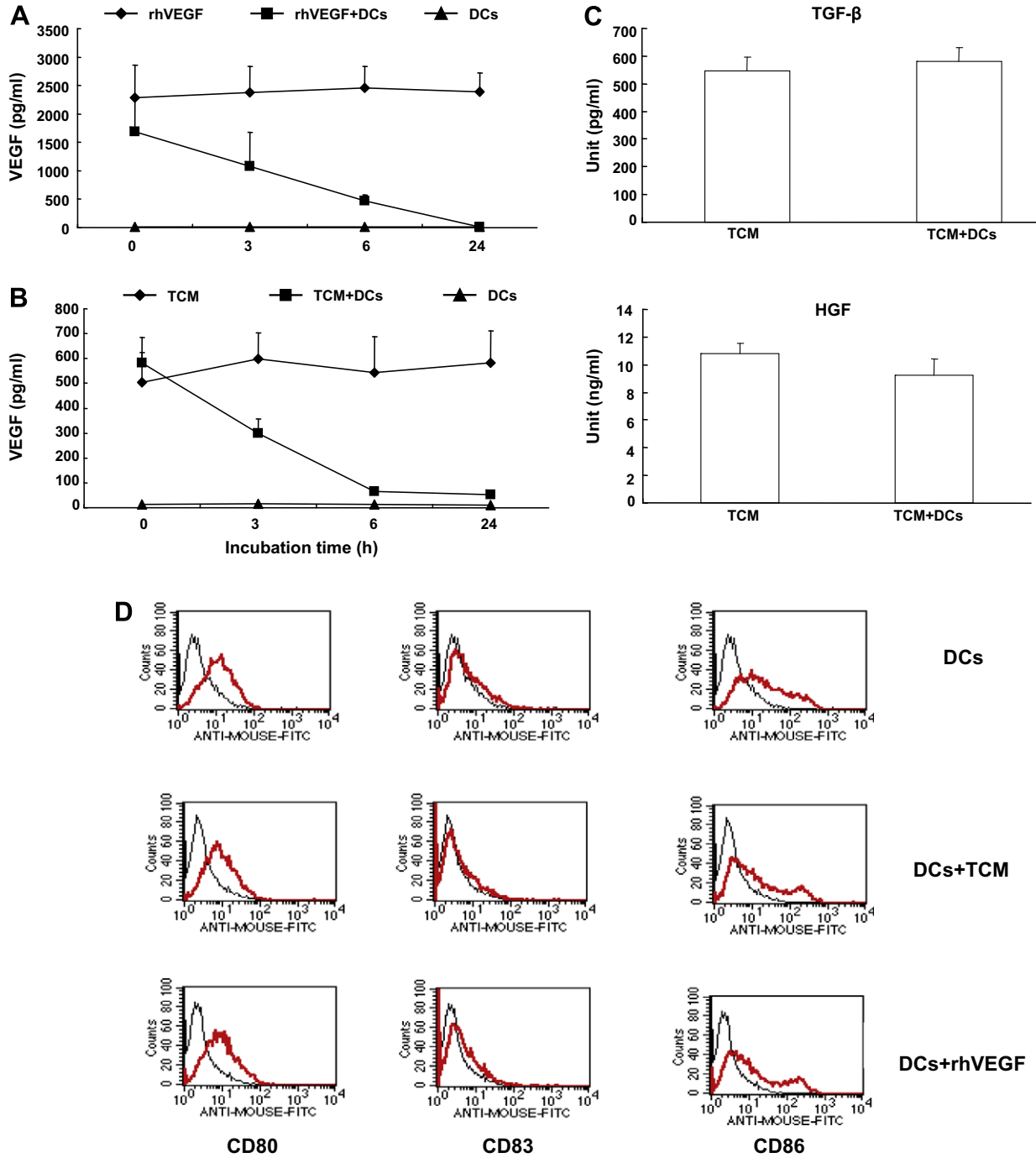

Fig. 1. Concentrations of VEGF in the supernatants in the presence or absence of DCs. DCs were incubated with rhVEGF (A) or TCM (B) for different period of time. The concentration of VEGF in the supernatants was detected by ELISA. Culture medium in the absence of DCs incubating with rhVEGF or TCM (♦); culture medium in presence of DCs incubating with rhVEGF or TCM (■), and DC culture medium alone (▲). (C) Concentrations of HGF and TGF-β in the TCM in the presence or absence of DCs. The data were expressed as means ± SD. (D) The expression of mature markers on DCs incubated with TCM or rhVEGF.

ELISA using 96-well plates coated with 2.5 μg/ml HGF in coating buffer (0.2 M Na_2CO_3/$NaHCO_3$, pH 9.6) overnight at 4 °C. Wells were blocked for 1 h in PBS containing 1% BSA overnight at 4 °C and then incubated with cell culture medium for 2 h at room temperature (RT). Plates were washed twice and then incubated with alkaline phosphatase-coupled goat anti-mouse IgG (Sigma–Aldrich, St. Louis, MO) for 2 h at RT. After washing four times in washing buffer, phosphatase substrate CP-nitrophenyl phosphate (Kirkegaard & Perry Laboratories) was added for 30 min, and absorbance was measured at 405 nm.

VEGF fluorescent labeling. Recombinant human VEGF (rhVEGF) was labeled with a red fluorescent dye Cy3 (Amersham Biosciences, Little Chalfont Buckinghamshire, England) according to the manufacturer's instruction. The molar dye/protein ratio estimated by absorbance at 552 and 280 nm was 4:1. DCs were incubated with 5 ng/ml Cy3-labeled rhVEGF (VEGF-Cy3) in culture medium containing 1% FBS for 3 h at 37 °C. Cy3-labeled BSA (BSA-Cy3) at the same concentration was used as a negative control. After being washed with cold PBS for three times, cells were stained with DC-SIGN Ab (BD Bioscience Pharmingen) followed by FITC-labeled goat anti-mouse Ab specific for IgG (BD Biosciences Pharmingen), and then investigated under fluorescent microscope (Nikon Eclipse E600, Japan), laser scanning spectral confocal microscope (Zeiss LSM 510 Meta, Germany) and FACScan analyzer.

RNA extraction and real time quantitative RT-PCR (qRT-PCR). Total RNA was extracted using Trizol reagent (Invitrogen, NY, USA). cDNA was synthesized using random hexamer (Invitrogen) and qRT-PCR was performed according to the instruction described in ABI assay-on-demand VEGF kit.

Western blotting. Cells were lysed in RIPA-buffer (0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40 and PBS) with phenylmethanesulfonyl fluoride (PMSF) and proteinase inhibitors (Roche, Germany) for 30 min on ice. Flt-1 expression was detected by anti-Flt-1 antibody (Invitrogen).

Blocking assay. Purified DCs were pre-treated with 1 μg/ml anti-Flt-1 Ab or control IgG at 37 °C for 1 h, and then treated with 2.5 ng/ml rhVEGF or Cy3-VEGF for 3 h. VEGF level in supernatants was detected by ELISA and intracellular VEGF was measured by FACScan analyzer.

Endocytosis inhibition assay. Metabolic inhibitor carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Sigma) and tyrosine kinase inhibitor genistein (Sigma) were used. Purified DCs were pre-treated with 50 μM CCCP or 20 μg/ml genistein at 37 °C for 30 min, and then treated with 5 ng/ml rhVEGF for 3 h. VEGF in supernatants was detected by ELISA and intracellular VEGF was measured by FACScan analyzer with mouse anti-VEGF (Abcam) followed by Alexa Fluor 488 goat anti-mouse IgG (Invitrogen).

Statistical analysis. Results are presented as means ± SD. Statistical differences between groups were compared using the two-sample *t*-test. $P < 0.05$ was considered significant.

Results and discussion

DC-dependent decrease of VEGF in the culture medium

It was found that the plasma levels of VEGF in tumor patients decreased during DC-based immunotherapy and the concentration of VEGF seemed to be very sensitive to the application of DC vaccines even there was not obvious clinical response (the shrink of tumor size). The decrease of VEGF was thought possibly resulting from enhanced immunologic clearing of VEGF from blood [17]. However, this phenomenon has not been further confirmed and the possible mechanism has not been clarified. In the current study, purified DCs (purification >85%) were $CD1a^+$ $HLA\text{-}DR^+$ (data not shown). DCs were incubated with exogenous rhVEGF (2.5 ng/ml) or TCM (containing large amount of VEGF secreted by tumor cells). Supernatants of DCs were collected at different time points and concentrations of VEGF were detected by ELISA. Our data showed that concentrations of VEGF decreased in the supernatants of DCs incubated with either rhVEGF or TCM in a time-dependent manner (Fig. 1A and B). VEGF in the DCs supernatants started to decrease from 3 h incubation and was undetectable at 24 h. However, the levels of VEGF kept unchanged in the absence of DCs. Furthermore, there was no detectable VEGF in the supernatants of DC cultured in the absence of rhVEGF or TCM (Fig. 1A and B). These results suggested that the significant decrease of VEGF was DC-dependent and not due to the protein degradation.

To determine whether DCs affect other cytokines in addition to VEGF, cells were incubated with TCM which contained HGF (11,000 pg/ml) and TGF-β (549.6 pg/ml) for 24 h and concentrations of the cytokines in supernatants with or without DCs were assessed by ELISA. The level of HGF and TGF-β in the supernatant of DCs did not change significantly (Fig. 1C), suggesting that the DC-mediated removal of VEGF was selective and specific.

We also evaluated the effect of rhVEGF or TCM on the induction of DC maturation. Neither rhVEGF (2.5 ng/ml) nor TCM changed the expression of CD80, CD83, and CD86 significantly (Fig. 1D), suggesting that VEGF treatment did not affect the cell maturation. These results were consistent with the findings by Laxmanan et al. [21], although it has been shown that VEGF inhibits maturation of immature DCs [22,23]. Gabrilovich et al. reported that DCs from $CD34^+$ precursors alter the maturation when incubated with 100 ng/ml VEGF for 2 h *in vitro* [22], suggesting that DCs prepared from different population might respond to VEGF differently. It has also been reported that incubation with 50% TCM from non-small cell lung cancer resulted an early maturation of monocyte-derived DCs [24]. It may be due to the TCM genertd from different tumors and the variation of VEGF concentrations.

Increase of intracellular VEGF in DCs treated with rhVEGF

To determine whether DCs were able to take up VEGF, DCs were incubated with rhVEGF (2.5 ng/ml) for 3 h. After washing, cells were lysed and the cellular level of VEGF was determined. As shown in Fig. 2A, the VEGF concentration in DCs treated with exogenous rhVEGF (437 ± 41 pg/ml) was significantly higher than in control DCs (151 ± 18 pg/ml) ($P < 0.01$). These data suggested that DCs could take up or bind exogenous rhVEGF.

To further confirm the finding, rhVEGF was labeled with Cy3 red fluorescent dye (VEGF-Cy3). After incubation with VEGF-Cy3 for 3 h, DCs were then permeabilized, and the internalization of VEGF-Cy3 by DCs was measured by flow cytometry. A FITC conjugated anti-VEGF antibody was used to mark both the endogenetic and exogenous VEGF. VEGF-Cy3 positive DCs were 0.1%, 61.0% after incubated with VEGF-Cy3 for 0 and 3 h, respectively (Fig. 2B). Furthermore, after DCs were incubated with VEGF-Cy3 for 3 h, they were labeled with an anti-DC-SIGN mAb followed by anti-mouse IgG-FITC. Double staining detected by immunofluorescent microscope showed that the VEGF-Cy3 positive cells were also DC-SIGN positive (Fig. 2C), suggesting VEGF only interacts with DCs but not with contaminated lymphocytes. However, the signal detected by FACS and microscope may include the internalized VEGF and surface bound VEGF. Therefore, we next examined the capacity of DCs to internalize VEGF by confocal microcopy. The data clearly showed that intracellular but not surface VEGF-Cy3 (red) was detected in DCs whose surfaces were labeled with DC-SIGN (green) while there was no detectable signal in DCs incubated with BSA-Cy3 (Fig. 2D). Taken together, these results demonstrated that VEGF could be specifically taken by DCs.

As shown in Fig. 2A and B, it is possible that DCs could synthesize VEGF by themselves. In order to exclude the possibility that the increased intracellular VEGF protein in DCs treated with

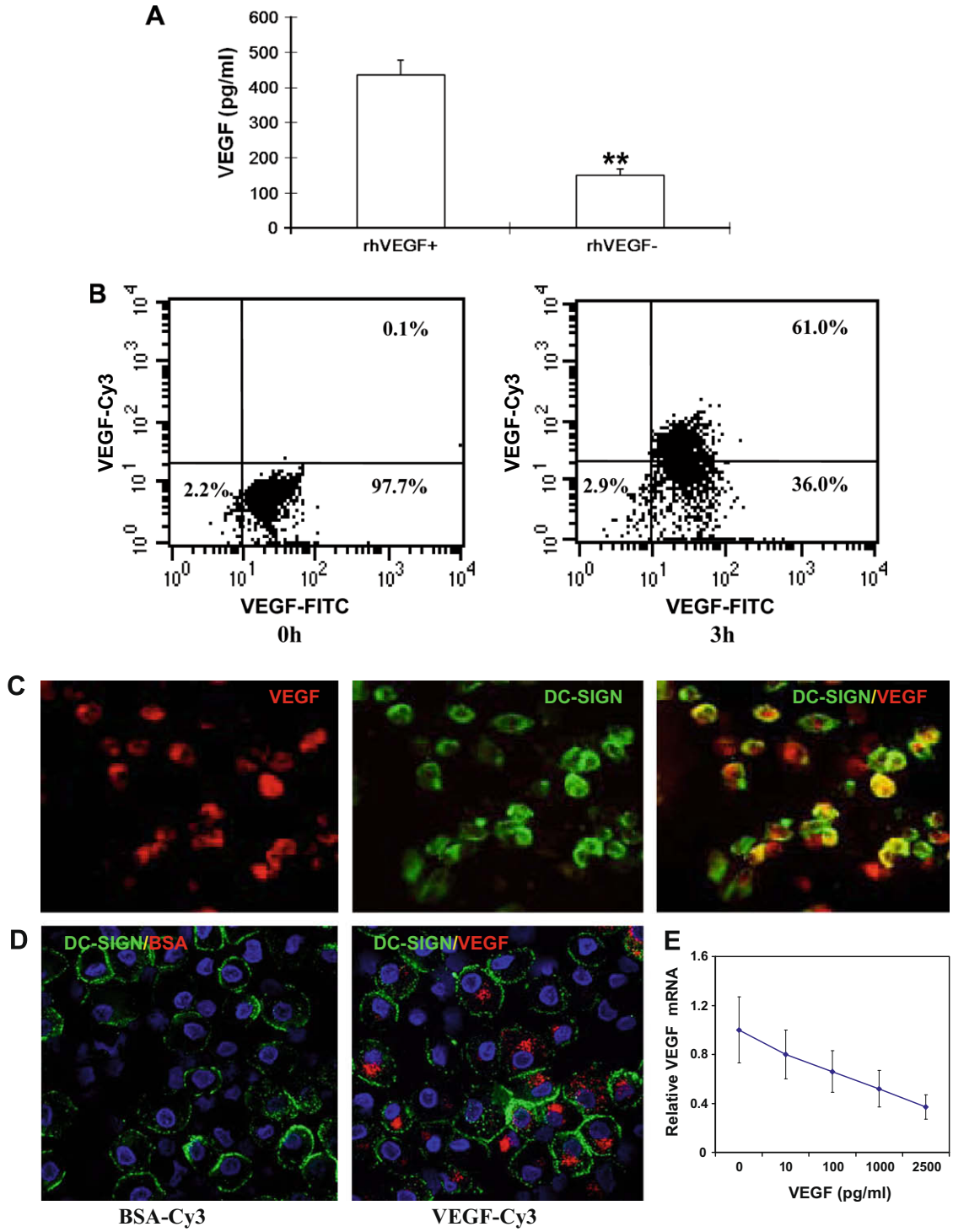

Fig. 2. Intracellular VEGF in DCs incubated with rhVEGF. (A) DCs incubated with (rhVEGF+) or without rhVEGF (rhVEGF−) and then DCs were lysed and the intracellular VEGF was detected by ELISA. The data were expressed as means ± SD. $^{**}P < 0.01$. (B) DCs incubated with VEG-Cy3 were detected by flow cytometry. (C,D) DCs incubated with VEGF-Cy3 were labeled with anti-DC-SIGN antibody, and then investigated by fluorescent microscopy (C) or confocal microscopy (D). VEGF-Cy3 was shown in red and DC-SIGN was shown in green (C,D). (E) DCs were co-cultured with rhVEGF for 3 h and mRNA was assessed by real time RT-PCR. The data were expressed as means ± SD and similar results were obtained from three independent experiments.

rhVEGF was not due to the *de novo* synthesis of VEGF by DCs, qRT-PCR was performed to detect VEGF mRNA level in DCs incubated with different concentrations of rhVEGF. The expression of VEGF mRNA was observed in DCs, inversely correlated with the levels of exogenous rhVEGF (Fig. 2E). These data further confirmed that VEGF could be efficiently endocytosed by DCs and exogenous VEGF did not stimulate *de novo* synthesis of VEGF in DCs.

Inhibition of VEGF uptake by blocking VEGF receptor-1 (Flt-1)

The biological activities of VEGF are mediated by its interaction with two tyrosine kinase receptors, Flt-1 and KDR [6,7]. KDR is responsible for VEGF-stimulated endothelial cell proliferation and migration, and microvascular permeability [25,26]. By contrast, Flt-1 undergoes weak tyrosine autophosphorylation in response

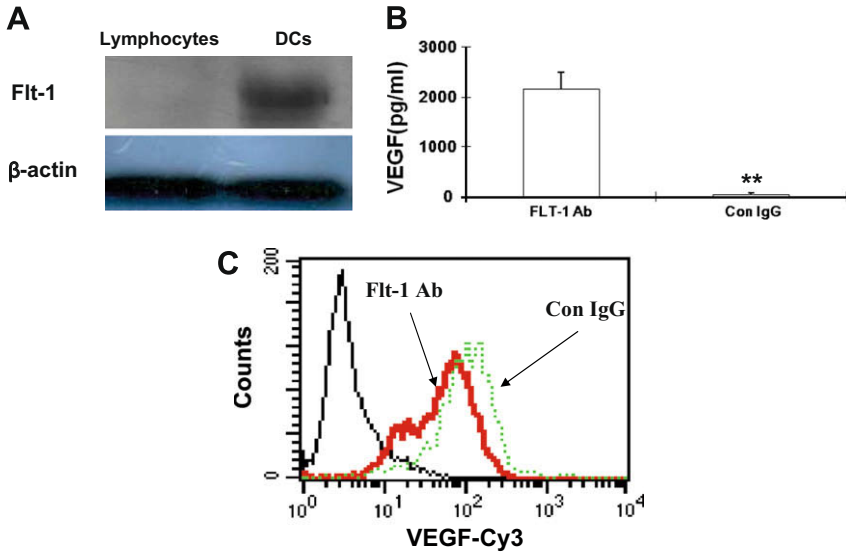

Fig. 3. Effect of blocking Flt-1 on DC uptake of VEGF. (A) Flt-1 expression on DCs or lymphocytes detected by Western blot analysis. (B) Purified DCs were pre-treated with anti-Flt-1 antibody or control IgG at 37 °C for 1 h, and then treated with rhVEGF (2.5 ng/ml) for 3 h. VEGF in supernatants was detected by ELISA. The data were expressed as means ± SD. $^{**}P < 0.01$. (C) DCs were pre-treated with anti-Flt-1 antibody or control IgG at 37 °C for 1 h, and then treated with VEGF-Cy3 for 3 h. Intracellular VEGF-Cy3 in DCs was assessed by flow cytometry. Results were representative of three experiments.

to VEGF compared to KDR [6] and might negatively regulate the activity of VEGF on endothelial cells by preventing VEGF binds to KDR [9]. Flt-1 was also found to be expressed on DCs [12] and may mediate the inhibitory role of VEGF on DCs [21]. Our data showed that DCs expressed Flt-1 (Fig. 3A) which was consistent with previous research [12], we therefore investigated whether blocking Flt-1-VEGF binding with anti-Flt-1 neutralizing antibody would affect DCs' action on VEGF uptake. DCs were pre-treated with Flt-1 neutralizing antibody (1 μg/ml) for 1 h and then incubated with rhVEGF or VEGF-Cy3 for 3 h. Capacity of DCs taking up VEGF was inhibited by anti-Flt-1 antibody (Fig. 3B and C). VEGF level in the supernatants of DCs treated with Flt-1 antibody (2162 ± 334 pg/ml) was significantly higher than treated with control antibody (48 ± 28 pg/ml) ($P < 0.01$) (Fig. 3B). In addition, intracellular VEGF-Cy3 in DCs treated with Flt-1 antibody is less than that of control antibody treatment (Fig. 3C), suggesting that the

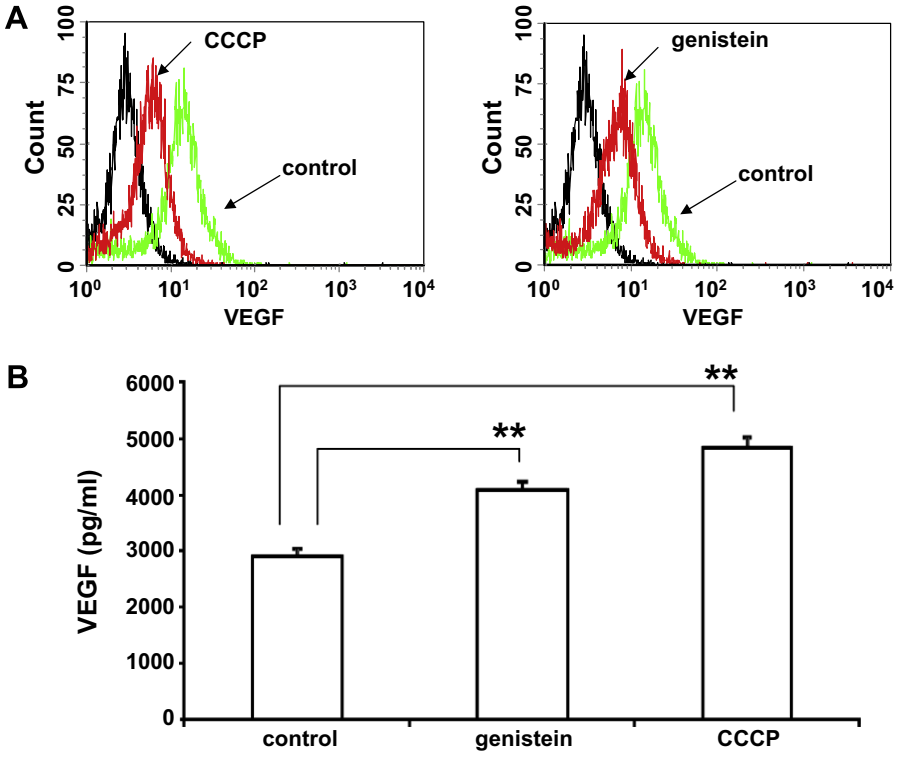

Fig. 4. Effect of classic inhibitors of endocytosis on DC uptake of VEGF. Purified DCs were incubated with CCCP (50 μM) or genistein (20 μg/ml) for 30 min, and then treated with rhVEGF (5 ng/ml) for 3 h. Intracellular VEGF and VEGF in supernatants were detected by flow cytometry (A) and ELISA (B). Results were representative of two experiments. $^{**}P < 0.01$.

capacity of DCs taking up VEGF may be VEGF receptor-1 dependent.

Inhibition of VEGF uptake by classic inhibitors of endocytosis

Metabolic inhibitors were used to demonstrate the energy-dependent internalization such as endocytosis [27]. CCCP uncouples oxidative phosphorylation [28] and has been used to investigate the process of endocytosis [29,30]. Tyrosine kinase inhibitor genestein is reported to inhibit internalization of a variety of ligands [31–33]. Genestein inhibited Flt-1 mediated VEGF- stimulated NO release by human trophoblast and endothelial cells [34], To investigate if VEGF internalization via Flt-1 is energy-dependent and if tyrosine kinase activity is required in this process, we pre-incubated cells with CCCP or genestein before rhVEGF. The results showed that either CCCP or genestein inhibited the uptake of VEGF by DCs. The intracellular VEGF was decreased in DCs treated with CCCP or genestein, while the VEGF in supernatants was higher than control group (Fig. 4A and B). The cell viability determined by Trypan blue was over 90% in two treatment groups (data not shown), suggesting the high level VEGF in the supernatant was not due to the cell death. These data demonstrated that endocytosis of VEGF by DCs is energy-dependent and tyrosine kinase activity is required.

Taken together, the present findings are important because they demonstrate a novel function of DC in clearance of VEGF. This study provides evidence explaining why plasma levels of VEGF in tumor patients decrease during DC-based immunotherapy, and allows a better understanding of the DC-VEGF interactions in anti-tumor immunity.

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