Vascular endothelial growth factor inhibits dendritic cell maturation

O fator de crescimento endotelial vascular inibe o processo de maturação das células dendríticas

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ABSTRACT

Objective: In this paper we investigated the effects of vascular endothelial growth factor on dendritic cells differentiation and maturation from monocytic precursors. Methods: CD14+/CD34- progenitor cells were obtained from umbilical cord blood, purified by magnetic cell sorting, and cultivated with IL-4 and GM-CSF, in the presence or absence of vascular endothelial growth factor. Maturation of dendritic cells was induced after six days of culture by 24 h/treatment with lipopolysaccharide (LPS). Results: Expression of marker proteins for immature (CD14 and DC-SIGN) and mature (CD83) dendritic cells was detected by fluorescence microscopy and flow cytometry using monoclonal antibodies (mAb). Under proper differentiating conditions, treatment with vascular endothelial growth factor did not change the expression of immature DC markers. Following maturation with LPS, increased amount of CD14+ and DC-SIGN+ cells and decreased CD83+ cell population were detected in vascular endothelial growth factor-treated cultures. Conclusions: Our data suggest that vascular endothelial growth factor does not affect the differentiation of CD14+/CD34- progenitor cells into immature dendritic cells, but it reduces the efficiency of dendritic cells maturation in vitro. This vascular endothelial growth factor-mediated effect on dendritic cells function may influence anti-tumor immune responses.

Keywords: Vascular endothelial growth factor A; Dendritic cells; Immune system

RESUMO

Objetivo: Investigar o efeito do fator de crescimento vascular endotelial na diferenciação e maturação das células dendríticas derivadas de precursores monociticos. Métodos: Células progenitoras CD14+/CD34- foram obtidas de sangue de cordão umbilical, purificadas por imunofaínha com esferas magnéticas e cultivadas com IL-4 e GM-CSF, na presença ou ausência de fator de crescimento vascular endotelial. A maturação das células dendríticas foi induzida após seis dias de cultura, mediante tratamento com lipopolissacaríde por 24 h. Resultados: A expressão de proteínas de membrana características de células dendríticas imaturas (CD14 e DC-SIGN) e de DCs maduras (CD83) foi detectada por microscopia de fluorescência e citometria de fluxo usando anticorpo monoclonal. Nas condições de cultivo testadas, o tratamento com fator de crescimento vascular endotelial não alterou a proporção de células dendríticas imaturas. Entretanto, após a maturação com LPS detectou-se um aumento da população de células CD14+ e DC-SIGN+ e concomitante diminuição da população de células CD83+ em culturas tratadas com fator de crescimento vascular endotelial. Conclusões: Estes resultados sugerem que o fator de crescimento vascular endotelial não afeta a diferenciação de células progenitoras CD14+/CD34- em células dendríticas imaturas, mas reduz a eficiência de maturação destas células in vitro. Este efeito mediado pelo fator de crescimento vascular endotelial pode influenciar a função de células dendríticas na resposta imune antitumoral.

Descritores: Fator A de crescimento do endotélio vascular; Células dendríticas; Sistema imune

INTRODUCTION

The immune system has the potential to naturally eliminate neoplastic cells, as evidenced by occasional spontaneous remission in renal-cancer and melanomas(1).
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Such capacity of the immune system to identify and destroy tumor cells is known as immunological surveillance(2). The induction of immunity against tumors can be initiated by the effectors of innate immunity and further developed by cells of adaptive immunity, with dendritic cells (DCs) playing a central regulatory role. DCs are potent antigen-presenting cells that modulate both immune response and induction of immune tolerance(3).

Numerous steps are involved in the immunological surveillance, including recognition of tumor molecules by DC precursors, direct and IFN-g-mediated killing by NK/NK T cells activated by DCs, capture and cross presentation of released-tumor-associated antigens (TAAs) by immune DCs, selection and activation of TAA-specific T cells as well as nonspecific effectors including macrophages and eosinophils, and homing of TAA-specific T cells to the tumor site.

However, tumor cells may evade the patient's tumor-specific immunity due to alterations at each of these steps(4). One possible mechanism is the suppression of DC function by molecules such as IL-6, IL-10, M-CSF and vascular endothelial growth factor, which are largely released by tumor cells(5). Since mature DCs are responsible for antigen presentation and further stimulation of T cells, abnormal DC differentiation and/ or maturation could lead to tumor immunotolerance.

In this study, we focused on the effects of tumor-associated molecules during the progress of DC maturation under controlled in vitro conditions.

**OBJECTIVE**

To investigate whether VEGF, an angiogenic growth factor commonly produced by tumor cells, affects myeloid DC differentiation and maturation processes in vitro.

**METHODS**

Isolation of CD14+/CD34- precursor cells Umbilical Cord Blood (UCB) was obtained from volunteer donors after informed written consent (CEP-IEPAE No. 105/02). Blood was collected from the umbilical vein, using Citrate Phosphate Dextran Adenine (CPDA1) as anticoagulant agent(6). Mononuclear cells were purified by density gradient centrifugation with Ficoll-PaqueTM Plus (GE Healthcare), according to a modified method previously published(7). The CD14+/CD34- cell population was purified after removing CD34+ cells with anti-CD34 mAb-coupled magnetic beads (Miltenyi Biotec) according to the manufacturer’s instructions. The CD34- fraction was then used to purify CD14+ cells using anti-CD14 mAb-coupled magnetic beads.

**Culture and Differentiation**

Enriched CD14+/CD34- cells were cultivated in 24 well plates with X-vivo 15 media (BioWhittaker) supplemented with 20ng/ml of IL-4 and 50ng/ml of GM-CSF, in the presence or absence of 20ng/ml rhVEGF(8) (added on day 0) for DC differentiation. After six days in culture, maturation of DCs was stimulated with LPS for 24hs(9). Immature and mature DCs were characterized by microscopy and by flow cytometry.

**Confocal Microscopy**

Following DC maturation, cells were harvested, transferred to glass slides and fixed with 4% paraformaldehyde. Glass slides were incubated with CD83 monoclonal antibody conjugated with phycoerythrin at appropriate dilution. Images were obtained with a Zeiss LSM510 laser confocal microscope.

**Flow Cytometry**

Harvested cells were stained with commercial monoclonal antibodies (Becton Dickinson, San Jose, CA). To monitor the expression of surface markers(10), the following monoclonal antibodies were used: CD14 (FITC), DC-SIGN/CD209 (PE), CD80 (PE), CD83 (PE), CD86 (PE) and CD45 (PerCP Cy-5.5) with isotype control IgG2a (FITC), IgG1 (PE), IgG2b (PE), IgG1 (PerCP Cy-5.5).

After incubation with antibodies at 4oC for 30 minutes, cells were washed and fixed with 1% paraformaldehyde. Fluorescent cellular events were acquired on the Epics XL/MCL flow cytometer (Coulter) and analyzed with System II software.

**RESULTS**

Enrichment of CD14+/CD34- cells after magnetic cell sorting was confirmed by flow cytometry, reaching a purity of about 80% (figure 1).

Differentiation of CD14+/CD34- progenitor cells into immature DCs was achieved after stimulation with IL-4 and GM-CSF for 6 days. Immature DCs are characterized by decreased expression of CD14, increased expression of DC-SIGN(11) and moderate expression of CD83. This expression pattern of CD14, DC-SIGN, and CD83 markers during differentiation of DCs was confirmed in our study and it did not alter with regard to the presence of VEGF in the culture media (figures 2 A-C). Similarly, VEGF did not alter the expression pattern of co-stimulatory molecules CD80 and CD86 on immature DCs (data not shown).
However, as shown in figures 2A-C, VEGF affected the expression of DCs surface markers following maturation with LPS on day+7. Maturation of immature DCs occurred after 24h-treatment with LPS, as evidenced by a distinct morphological appearance (figures 3 and 4), a slight increase in CD14 expression, lower DC-SIGN expression and a marked increase in CD83 expression (figure 2B and 2C). In the VEGF-treated cultures, the amount of DC-SIGN+ cells remained high (figure 5) and the increment in CD83+ cell population was not as accentuated as the one observed in cultures lacking VEGF (figure 6).
DISCUSSION

DCs derived from CD14+/CD34- population are known as myeloid DCs and the proper maturation of these cells is very important in immune response regulation. The process of DCs maturation can be elicited by different external stimuli such as microbial products and pro-inflammatory mediators, acting on an immature, antigen-capturing cell. Upon recognition of such external signals, mature DCs are responsible for antigen presentation and stimulation of T cells, playing critical roles in both innate and adaptive immune responses.

Functionally mature myeloid DCs can induce potent TAA-specific immunity in vivo. On the other hand, immature or partially differentiated myeloid DC induces either suppressive Treg cells or T-cell unresponsiveness\(^{(5)}\). Therefore, any condition affecting this immature to mature transition states may affect DCs function.

Although DCs are important in the patients immune surveillance against cancer, their function is impaired and their population decreased in a cancer-patients.

Figure 4. Mature dendritic cells detected by immunocytochemistry for CD83 expression - Scale bar (20 µm) - Confocal Microscopy.

Figure 5. Detection of DC-SIGN (CD209) expression on dendritic cells by flow cytometry.

Figure 6. Detection of CD83 expression on dendritic cells by flow cytometry.
In fact, myeloid DCs are rarely found within the tumor environment, probably due to deficient recruitment, differentiation or maturation\(^5\).

In this work, we tested whether molecules produced by tumor cells, such as VEGF, affect DCs function in vitro. VEGF is a 34 to 43-kDa protein known to bind specifically to endothelial cells and to stimulate endothelial cell growth in vitro and angiogenesis in vivo\(^12\). It is produced by almost all tumor cells\(^13\) and it can be detected in serum of cancer patients\(^14\). Furthermore, CD14+/CD34- progenitor cells are known to express receptors for VEGF\(^15\).

Previous studies reported effects of VEGF at very early stages of DC differentiation from CD34+ progenitor cells\(^16\). In our model, addition of exogenous VEGF into the culture media did not alter the expression pattern of immature DC markers, suggesting no effects on DC differentiation process. However, after stimulation with a maturation factor, cells treated with VEGF poorly expressed CD83, a marker of mature DCs, while keeping expression of the immature DCs markers CD14 and DC-SIGN at high levels. Altogether, these results suggest that the maturation process of DCs following stimulation with LPS is impaired in the presence of VEGF.

CONCLUSIONS

Under the conditions tested, VEGF did not affect differentiation of DCs from CD14+/CD34- precursors but inhibited LPS-induced maturation of these myeloid DCs. The present findings are in agreement with other studies supporting the notion that VEGF plays a role in the immune tolerance against tumor cells, and contribute to the understanding of the mechanisms involved in this process.

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