

Dendritic Cell Subsets Differentially Regulate Angiogenesis in Human Ovarian Cancer

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Abstract

Angiogenesis is essential for both primary and metastatic tumor growth. Tumor blood vessel formation is complex and regulated by many factors. Ovarian carcinomas have a poor prognosis, often associated with multifocal intraperitoneal dissemination accompanied by intense neovascularization. To examine tumor angiogenesis in the tumor microenvironment, we studied malignant ascites of patients with untreated ovarian carcinoma. We observed high numbers of plasmacytoid dendritic cells (PDCs) and significant stromal-derived factor (CXCL-12/SDF)-1 in their malignant ascites, attracting PDCs into the tumor environment. We now show that tumor-associated PDCs induced angiogenesis *in vivo* through production of tumor necrosis factor α and interleukin 8. By contrast, myeloid dendritic cells (MDCs) were absent from malignant ascites. MDCs derived *in vitro* suppressed angiogenesis *in vivo* through production of interleukin 12. Thus, the tumor may attract PDCs to augment angiogenesis while excluding MDCs to prevent angiogenesis inhibition, demonstrating a novel mechanism for modulating tumor neovascularization. Because dendritic cells (DCs) have long been known to affect tumor immunity, our data also implicate DCs in regulation of tumor neoangiogenesis, suggesting a novel role of DCs in tumor pathology.

Introduction

Angiogenesis is essential for both primary and metastatic tumor growth. Work to date suggests that vascular endothelial growth factor (VEGF) plays a central role in tumor angiogenesis. However, blood vessel formation is complex and regulated by many factors. For example, β_3 and β_5 integrins were thought to support angiogenesis based on *in vitro* work. However, recent *in vivo* experiments failed to show support for these molecules in angiogenesis *in vivo* (1, 2). Furthermore, the proangiogenic molecule basic fibroblast growth factor was found to be positively related to the prolonged survival of cancer patients (3). Importantly, early human clinical cancer treatment trials with antiangiogenic molecules have only demonstrated modest benefits (4–6). More strikingly, recent reports (7, 8) suggest that angiogenesis inhibitors (or antagonists) alone, by depriving tumors of oxygen, could have an unintended effect: promotion of tumor metastasis. These results reflect our growing understanding of the complexity of the tumor angiogenic process and metastasis process. Dendritic cells (DCs) prime naïve T cells and thereby activate antigen-specific immunity. The two principal human DC subtypes are MDCs (DC1) and plasmacytoid dendritic cells [PDCs (DC2; Ref. 9)]. MDCs expressing tumor antigens have been used in human clinical trials to

induce significant clinical responses against some tumors (10). Although much work has focused on the relevance of DCs to tumor immunity, there are no reports regarding how DCs influence tumor angiogenesis. Ovarian carcinomas have a poor prognosis, often associated with multifocal intraperitoneal dissemination accompanied by intense neovascularization. Because immune factors are known to modulate blood vessel formation in some settings (11), we hypothesized that specific DC subsets might differentially affect tumor neovascularization.

Materials and Methods

Human Subjects. We studied patients with International Federation of Gynecology and Obstetrics stage III or IV ovarian epithelial carcinomas. All patients gave written, informed consent. The study was approved by the local institutional review board. No patients received prior specific cancer treatments.

Plasmacytoid Dendritic Cells. We collected peripheral blood mononuclear cells and ovarian tumor ascites aseptically, harvested cells by centrifugation over a Ficoll-Hypaque density gradient (Amersham), and cryopreserved them at -86°C until use. CD3-, CD14-, CD16-, CD19-, and CD56-expressing cells were depleted using paramagnetic beads (Miltenyi, Auburn, CA), and blood PDCs or tumor ascites PDCs were sorted by flow cytometry gating on CD4⁺CD123⁺CD11c⁻ cells. Cell populations were $\geq 99\%$ pure by flow cytometry.

Myeloid Dendritic Cells. MDCs were differentiated from CD14⁺ tumor ascites cells with granulocyte macrophage colony-stimulating factor plus interleukin (IL)-4 as described previously (12, 13).

Activation of Dendritic Cells and Detection of Dendritic Cell-Derived Cytokines. Tumor-associated PDCs or MDCs were activated for 24 h with CD40 ligand (CD40L) stimulation (200 ng/ml; Immunex) or without stimulation. In some cases, the culture plates were precoated with growth factor-reduced Matrigel (BD Bioscience, Bedford, MA). Dendritic cells were collected for *in vivo* Matrigel assay. Culture supernatants were collected for detecting cytokines with commercial enzyme-linked immunosorbent assay kits (all from R&D Systems, Minneapolis, MN).

***In vivo* Matrigel Assay.** NOD.SCID mice (6–8 weeks old; The Jackson Laboratory, Bar Harbor, ME) were inoculated with growth factor-reduced Matrigel Matrix (BD Bioscience) bearing fresh or CD40L-activated tumor-associated PDCs or MDCs and/or the indicated cytokines and/or mouse anti-human antibody. Recombinant human VEGF, fibroblast growth factor (FGF), tumor necrosis factor (TNF)- α , IL-8 (all at 10 ng/ml) were from R&D Systems. Mouse antihuman TNF- α (clone 1825; IgG1), mouse antihuman-IL-8 antibody (clone 6217; IgG1), and mouse antihuman IL-12 antibody [clone 24910; IgG1 (500 ng/ml each)] were from R&D Systems. After 12 days, we isolated the Matrigel plugs. Matrigel plugs were subjected to immunohistochemistry with anti-von Willebrand factor antibody (polyclonal antibody; 1:10 dilution; DAKO, Carpinteria, CA). Microvessel density was analyzed (14) and quantified with ImagePro Plus software (Media Cybernetics, Silver Spring, MD) and expressed as a mean percentage of microvessel surface area by confocal Leica microscope. Hemoglobin (Hb) content in Matrigel plugs was detected with a commercial kit (Sigma, St. Louis, MO).

Received 4/9/04; revised 6/12/04; accepted 7/7/04.

Grant support: National Cancer Institute Grants CA092562 and CA100227, Department of Defense Grant OC020173, Louisiana Board of Regents Grant I26A, the Concern Foundation (W. Zou), and Grant RR00164 (X. Alvarez and A. Lackner).

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Results

Tumor-Associated Plasmacytoid Dendritic Cells Induce Angiogenesis *In vivo*. To study the role of tumor-associated PDCs in angiogenesis, we purified tumor-associated PDCs from malignant ascites as we described previously (15). We tested the *in vivo* angiogenic effects of freshly isolated primary tumor-associated PDCs in Matrigel (16, 17). We observed significant neovascularization in

Matrigel plugs bearing 10^6 primary tumor PDCs ($P < 0.0001$, compared with PBS). Activated tumor PDCs (10^5 or 10^6) also induced *in vivo* neovascularization ($P < 0.0001$, compared with PBS). Interestingly, 10^6 activated tumor PDCs were significantly more efficient in inducing neoangiogenesis than 10^6 primary tumor PDCs ($P < 0.0001$), indicating the enhanced angiogenic capacity of activated tumor PDCs. Furthermore, 10^6 activated tumor PDCs induced

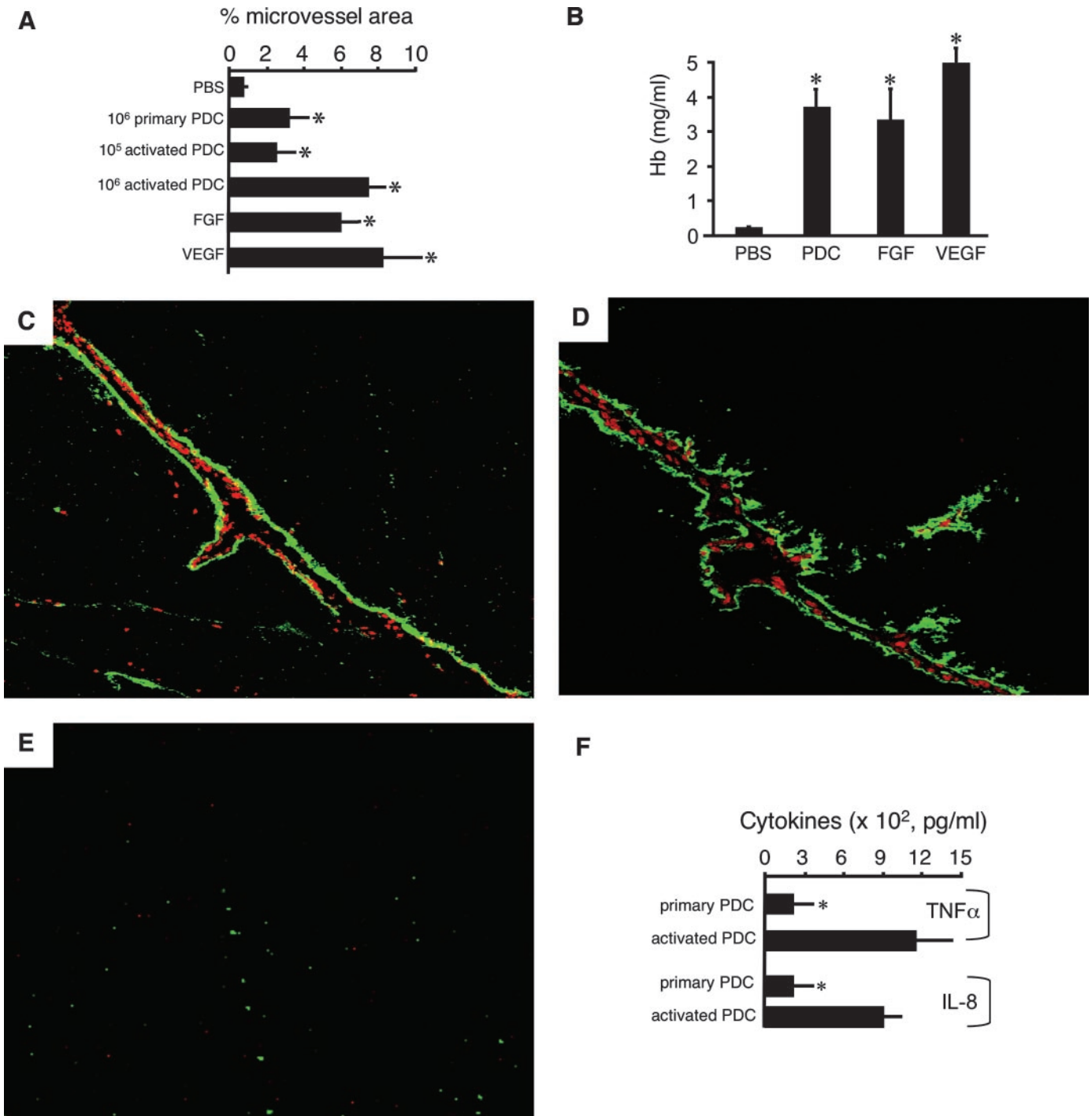


Fig. 1. Tumor PDCs induced angiogenesis *in vivo*. NOD.SCID mice were inoculated with Matrigel plugs bearing tumor-associated PDCs and/or the indicated reagents. **A**, day 12 Matrigel plugs were subjected to immunohistochemistry with anti-von Willebrand factor antibody. Microvessel density was analyzed and expressed as a mean percentage of microvessel surface area (*, $P < 0.0001$ for all, compared with PBS). **B**, Hb content in Matrigel plugs was detected (*, $P < 0.0001$ for all, compared with PBS). **C–E**, histological analysis showed vascular channel formation and tortuous neovessels in Matrigel plugs with (**C**) tumor-associated PDCs, (**D**) FGF, and (**E**) PBS. Green, von Willebrand factor; red, Topro. **F**, tumor-associated PDCs produced IL-8 and TNF- α (*, $P < 0.001$ for all, primary versus activated PDCs). **G**, tumor-associated PDC-derived IL-8 and TNF- α induced angiogenesis *in vivo* (*, $P < 0.01$ for all, compared with PDCs alone). **B**, **C**, **F**, and **G**, 10^6 PDCs. **A–E** and **G**, 7–10 mice/group.

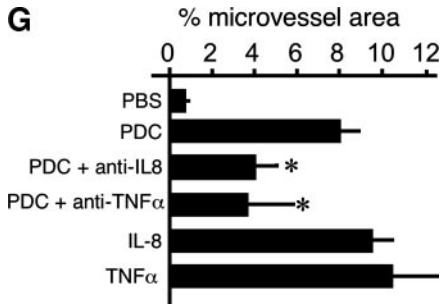


Fig. 1 Continued

more neoangiogenesis than 10^5 activated tumor PDCs ($P < 0.0001$), indicating a dose-dependent angiogenic induction. As positive controls, the angiogenic cytokines VEGF and FGF also induced strong angiogenesis *in vivo* ($P < 0.0001$, compared with PBS; Fig. 1A).

In our preliminary experiment, Spearman correlation coefficients were computed to assess the relationship between microvessel surfaces and Hb content in Matrigel plugs bearing different concentrations of VEGF (1–50 ng/ml). We showed that the percentage of microvessel surfaces highly correlated with Hb content in Matrigel plugs (Ref. 18; Spearman's $\rho = 0.32$; $P = 0.0097$). Therefore, as an alternative and confirmatory technique, we detected high levels of Hb content in Matrigel plugs containing 10^6 activated tumor PDCs, FGF, and VEGF ($P < 0.0001$ for all, compared with PBS; Fig. 1B). Histological analysis showed vascular channel formation and tortuous neovessels in Matrigel plugs bearing tumor PDCs (Fig. 1C) or FGF (Fig. 1D), but not PBS (Fig. 1E). These data demonstrate that tumor PDCs directly induced angiogenesis *in vivo*.

Tumor-Associated Plasmacytoid Dendritic Cells Produce Tumor Necrosis Factor α and Interleukin 8. We further explored the potential angiogenic mechanisms of tumor-associated PDCs. Strikingly, tumor-associated PDCs spontaneously produced high levels of the angiogenic cytokines TNF- α and IL-8 (Fig. 1F). Consistent with the induced *in vivo* neoangiogenesis, activated tumor PDCs (10^6 /ml) produced significantly more TNF- α and IL-8 ($n = 6$; $P < 0.001$ for primary PDCs *versus* activated PDCs; Fig. 1F). Tumor PDCs produced undetectable VEGF and IL-12 (data not shown). Furthermore, when tumor PDCs were placed in the plates precoated with growth factor-reduced Matrigel, we detected the identical amount of TNF- α and IL-8, suggesting that the *in vivo*-used growth factor-reduced Matrigel has no direct effects on PDC-derived cytokine production.

Tumor-Associated Plasmacytoid Dendritic Cell-Derived Tumor Necrosis Factor α and Interleukin 8 Are Angiogenic. We hypothesized that tumor PDC-derived TNF- α or IL-8 mediated PDC-driven angiogenesis. Consistent with this hypothesis, anti-TNF- α or anti-IL-8 antibody significantly decreased activated tumor PDC (10^6)-mediated angiogenesis *in vivo* ($P < 0.01$, compared with PDCs alone; Fig. 1G). In confirmation, recombinant TNF- α and IL-8 induced significant angiogenesis *in vivo* ($P < 0.0001$ for both, compared with PBS; Fig. 1G). Thus, tumor PDCs induce angiogenesis at least in part through TNF- α and IL-8 production *in vivo*.

Tumor-Associated Myeloid Dendritic Cells Inhibit Angiogenesis *In vivo*. We observed no significant numbers of MDCs in malignant ascites in patients with ovarian cancers (15). To evaluate the potential angiogenic role of tumor-associated MDCs *in vivo*, we differentiated tumor-associated MDCs from malignant ascites macrophages as we described previously (12, 13, 15). Tumor-associated MDCs did not induce angiogenesis *in vivo*, even after activation with CD40L. Recombinant human VEGF induced significant angiogenesis *in vivo* ($P < 0.0001$, compared with PBS; Fig. 2A). Interestingly,

VEGF mediated-angiogenesis was significantly reduced by 10^6 or 5×10^6 CD40L-activated tumor MDCs ($P < 0.01$, compared with VEGF alone; Fig. 2A), indicating that MDCs suppress angiogenesis *in vivo*. Tumor-associated MDCs (5×10^6) were more efficient in suppressing VEGF-mediated angiogenesis than 10^6 tumor-associated MDCs, indicating a dose-dependent angiogenic reduction ($P < 0.05$, 5×10^6 *versus* 10^6 MDCs; Fig. 2A). The Hb content (Fig. 2B) was also consistent with the percentage of microvessel quantification (Fig. 2A).

Additionally, we detected high levels of VEGF by enzyme-linked immunosorbent assay in malignant ascites (1440 ± 739 pg/ml; $n = 12$) and primary ovarian carcinoma tumor cell cultures (340 ± 139 pg/ml VEGF produced by 10^6 tumor cells/ml in 48 h). Thus, tumor-associated MDCs suppressed angiogenesis induced by tumor-derived VEGF.

Tumor-Associated Myeloid Dendritic Cell-Derived Interleukin 12 Suppresses Angiogenesis *In vivo*. We further studied the angiogenic suppressive factor derived from MDCs. Anti-IL-12 antibody blocked the suppressive effects of MDCs on angiogenesis ($P < 0.01$, 10^6 MDCs + VEGF *versus* 10^6 MDCs + VEGF + anti-IL-12), suggesting that MDC-derived IL-12 was the angiogenic suppressive factor (Fig. 2). In support of this, recombinant IL-12 inhibited VEGF-mediated angiogenesis ($P < 0.01$, VEGF *versus* VEGF + IL-12; Fig. 2). The data further support that MDCs are able directly to suppress angiogenesis *in vivo* through IL-12 production. In confirmation, we showed that after CD40L activation, tumor-associated MDCs produced significant IL-12 (1830 ± 250 pg/ml; $n = 8$). We observed the identical amount of IL-12 produced by MDCs cultured with the plates precoated with growth factor-reduced Matrigel. It indicates that the growth factor-reduced Matrigel has no detectable effects on MDC cytokine production.

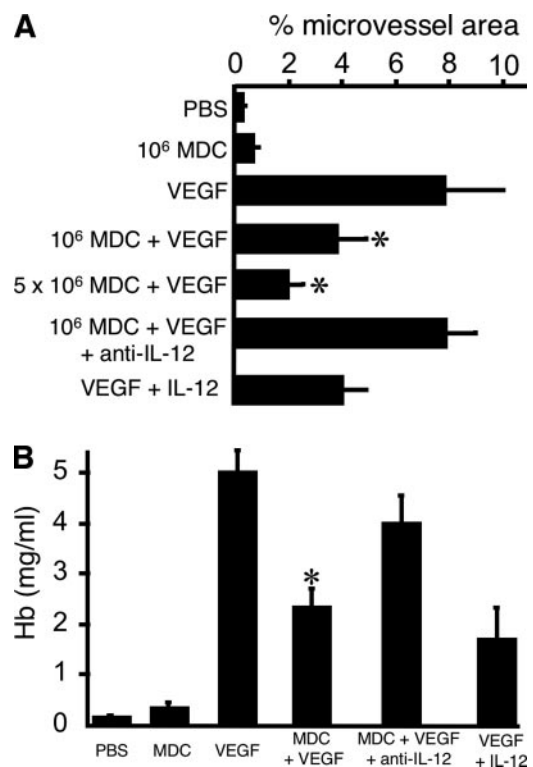


Fig. 2. Tumor MDCs suppress angiogenesis *in vivo*. NOD.SCID mice were inoculated with Matrigel plugs bearing activated tumor-associated MDCs plus the indicated reagents. Day 12 Matrigel plugs were removed to study neovascularization as described in the Fig. 1 legend. A, MDCs suppress VEGF-induced angiogenesis through IL-12. B, microvessel surface data are correlated with the Hb contents in Matrigel plugs. A total of 10^6 MDCs were used in B. A and B, 7–10 mice/group. *, $P < 0.001$, MDCs + VEGF *versus* VEGF.

Discussion

Tumor angiogenesis is essential for the growth of primary and metastatic tumors. The angiogenic process requires the coordinated activities of multiple factors and cell types (4, 11). Tumor angiogenesis occurs when the effect of angiogenic stimulatory factors outweighs that of inhibitory factors (11). Although vascular endothelial cells and their released angiogenic molecules have been studied extensively, the potential angiogenic roles of immune cells, particularly those of dendritic cells, in the tumor microenvironment have not been well defined.

There are substantial numbers of functional PDCs (but not MDCs) in tumor ascites in patients with ovarian carcinomas (15). MDCs produce significant amounts of IL-12 and induce high levels of T-cell interferon (IFN)- γ (15). IL-12 and IFN- γ are potent angiogenic inhibitory cytokines (11). We hypothesize that MDCs may suppress tumor angiogenesis *in vivo* through these cytokines. Lack of MDCs in the tumor microenvironment may be orchestrated by the tumor to minimize the angiogenesis-inhibiting effects of these MDCs. Consistent with our hypothesis, we showed here that MDCs significantly suppressed angiogenesis *in vivo*. MDC-derived IL-12 is a critical factor inhibiting tumor angiogenesis. These data may help explain the beneficial effects of MDCs in reducing tumor burden even when they do not bear tumor-associated antigens.

Cells resembling PDCs were observed in histological sections of lymphoid tumors, granulomas, and multicentric Castleman's disease (19–21). Apart from our recent report (15), however, functional PDCs have never been recovered from pathological tissues for study. There are no reports regarding whether PDCs have effects on tumor angiogenesis. After virus infection, PDCs produce a large amount of type I IFN [IFN- α , IFN- β , and IFN- ω (15, 22–24)]. IFN- α and IFN- β are potent tumor angiogenic inhibitory cytokines. Numerous functional PDCs accumulate in tumor ascites; however, type I IFNs are undetectable in tumor ascites (15). In contrast to MDCs, T-cell signal CD40L stimulates little or undetectable IL-12 production by human PDCs (25, 26). We recently demonstrated that PDCs, but not MDCs, accumulate in the tumor environment and that tumor PDCs inhibit antitumor immunity (15). We now show that tumor PDCs produce high levels of the angiogenic cytokines TNF- α and IL-8 and induce potent neovascularization *in vivo*. Thus, tumors manipulate DC distribution and alter DC function to support tumor angiogenesis. MDCs suppress tumor angiogenesis. PDCs enhance tumor angiogenesis.

In summary, our data demonstrate a novel role for DCs in human cancer. The DC system is relevant to tumor angiogenesis: MDCs inhibit tumor angiogenesis; and tumor-associated PDCs enhance tumor angiogenesis. Maximal vascularization of tumors may thus require the simultaneous accumulation of PDCs and the absence of MDCs, as observed in ovarian tumors. Blocking PDC-mediated neovascularization in tumors may be a novel strategy to treat human cancers.

Acknowledgments

We thank Dominique Emilie, Roy Weiner, and Jules Puschett for their constant support.

References

1. Reynolds LE, Wyder L, Lively JC, et al. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. *Nat Med* 2002;8:27–34.
2. Carmeliet P. Integrin indecision. *Nat Med* 2002;8:14–6.
3. Obermair A, Speiser P, Reisenberger K, et al. Influence of intratumoral basic fibroblast growth factor concentration on survival in ovarian cancer patients. *Cancer Lett* 1998;130:69–76.
4. Ellis LM, Liu W, Ahmad SA, et al. Overview of angiogenesis: biologic implications for antiangiogenic therapy. *Semin Oncol* 2001;28:94–104.
5. Ellis LM, Liu W, Fan F, et al. Role of angiogenesis inhibitors in cancer treatment. *Oncology (Huntingt)* 2001;15:39–46.
6. Jung YD, Ahmad SA, Akagi Y, et al. Role of the tumor microenvironment in mediating response to anti-angiogenic therapy. *Cancer Metastasis Rev* 2000;19:147–57.
7. Staller P, Sulitkova J, Lisztwan J, et al. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. *Nature (Lond)* 2003;425:307–11.
8. Schioppa T, Uranchimeg B, Saccani A, et al. Regulation of the chemokine receptor CXCR4 by hypoxia. *J Exp Med* 2003;198:1391–402.
9. Colonna M, Krug A, Cella M. Interferon-producing cells: on the front line in immune responses against pathogens. *Curr Opin Immunol* 2002;14:373–9.
10. Cerundolo V, Hermans IF, Salio M. Dendritic cells: a journey from laboratory to clinic. *Nat Immunol* 2004;5:7–10.
11. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature (Lond)* 2000;407:249–57.
12. Curiel TJ, Wei S, Dong H, et al. Blockade of B7–H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* 2003;9:562–7.
13. Zou W, Borvak J, Marches F, et al. Macrophage-derived dendritic cells have strong Th1-polarizing potential mediated by beta-chemokines rather than IL-12. *J Immunol* 2000;165:4388–96.
14. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis: correlation in invasive breast carcinoma. *N Engl J Med* 1991;324:1–8.
15. Zou W, Machelon V, Coulomb-L'Hermin A, et al. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nat Med* 2001;7:1339–46.
16. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 2001;7:1194–201.
17. Montrucchio G, Lupia E, Battaglia E, et al. Tumor necrosis factor alpha-induced angiogenesis depends on in situ platelet-activating factor biosynthesis. *J Exp Med* 1994;180:377–82.
18. Carmeliet P, Moons L, Luttun A, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 2001;7:575–83.
19. Facchetti F, De Wolf-Peeters C, De Vos R, et al. Plasmacytoid monocytes (so-called plasmacytoid T cells) in granulomatous lymphadenitis. *Hum Pathol* 1989;20:588–93.
20. Facchetti F, De Wolf-Peeters C, Van den Oord JJ, Desmet VJ. Plasmacytoid monocytes (so-called plasmacytoid T cells) in Hodgkin's disease. *J Pathol* 1989;158:57–65.
21. Facchetti F, De Wolf-Peeters C, Van den Oord JJ, De Vos R, Desmet VJ. Plasmacytoid monocytes (so-called plasmacytoid T-cells) in Kikuchi's lymphadenitis. An immunohistologic study. *Am J Clin Pathol* 1989;92:42–50.
22. Zou W, Borvak J, Wei S, et al. Reciprocal regulation of plasmacytoid dendritic cells and monocytes during viral infection. *Eur J Immunol* 2001;31:3833–9.
23. Cella M, Jarrossay D, Facchetti F, et al. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* 1999;5:919–23.
24. Siegal FP, Kadowaki N, Shodell M, et al. The nature of the principal type I interferon-producing cells in human blood. *Science (Wash DC)* 1999;284:1835–7.
25. Krug A, Towarowski A, Britsch S, et al. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur J Immunol* 2001;31:3026–37.
26. Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. *J Exp Med* 2000;192:219–26.