Recruitment of CD11b⁺Ly6C⁺ monocytes in non-small cell lung cancer xenografts challenged by anti-VEGF antibody

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Abstract. A series of antibodies against vascular endothelial growth factor (VEGF) have been developed for the treatment of various types of cancer, including non-small cell lung cancer (NSCLC) in recent years. However, tumors frequently demonstrate resistance to these strategies of VEGF inhibition. Efforts to better understand the mechanism underlying the acquired resistance to anti-VEGF antibodies are warranted. In the present study, in order to develop a xenograft model of acquired resistance to anti-VEGF antibody, xenografts of human adenocarcinoma A549 cells were generated through the successive inoculation of tumor tissue explants into first (F1), second (F2) and third (F3) generations of mice treated with the anti-VEGF antibody B20. Tumor growth rate and vessel-forming ability, assessed via cluster of differentiation (CD) 31 staining, were significantly lower in the F1, F2 and F3 groups compared with in the F0 control group (P<0.01), suggesting that drug resistance was not successfully acquired. The percentages of CD11b⁺ myeloid-derived suppressor cells and lymphocyte antigen 6C (Ly6C⁺) subsets were significantly smaller in F1, F2 and F3 groups compared with in F0 (P<0.01). However, the ratio of Ly6C⁺ to CD11b⁺ cells was significantly higher in the F3 group compared with in F0 and F1 groups (P<0.01), indicating increasing recruitment of the Ly6C⁺ subset with successive challenges with the anti-VEGF antibody.

In conclusion, the recruitment of CD11b⁺Ly6C⁺ monocytes increased with successive generations of NSCLC-xenografted mice challenged by B20, an anti-VEGF agent.

Introduction

Cancer is one of the leading causes of mortality worldwide, and lung cancer is one of the leading causes of cancer-associated mortality with a 5-year survival rate of ~16% (1). Representing >85% of lung cancer cases, non-small cell lung cancer (NSCLC) is the most common type (2). Of patients with NSCLC, >2/3 are initially diagnosed at an advanced stage, at which point palliative chemotherapy is the primary option. Although 30-40% of patients may respond to cytotoxic chemotherapy, the majority eventually suffer from disease progression (3). The survival rate of patients with advanced lung adenocarcinoma improved in the early 2000’s, most probably due to the emergence of inhibitors of epidermal growth factor receptor, anaplastic lymphoma kinase and vascular endothelial growth factor (VEGF) (4).

Angiogenesis, a process mainly mediated by VEGF, has been demonstrated to be crucial for tumor growth, invasion and metastasis (5); although small tumors are able to obtain nutrients and oxygen through diffusion, the formation of new vasculature is critical for tumor expansion and metastasis (6). The associations between various measures of tumor aggressiveness and increases in intratumoral microvessel density illustrate the essential role of angiogenesis in tumor growth and metastasis (7).

The role of VEGF in angiogenesis has been well established (8-10). A range of evidence has suggested that VEGF and its receptors exhibit altered activity in various human cancer types (11). In general terms, VEGF expression correlates negatively with clinical outcome (12-14).

In 1971, it was proposed by Judah Folkman (15) that inhibiting angiogenesis may be an effective approach to anticancer therapy. More than 30 years after this original hypothesis, the first proof of anti-angiogenic therapy in NSCLC came with the approval of bevacizumab, a monoclonal antibody directed against human VEGF (16). Since then, a series of antibody-based agents against VEGF have been developed, the majority of which are undergoing clinical trials (16,17), including trials of patients with NSCLC (18). Despite promising clinical effects, resistance to these agents has been reported (19). Investigations into the...
mechanisms underlying resistance to anti-VEGF antibodies are warranted in order for the successful advancement of these promising therapeutic agents.

Myeloid-derived suppressor cells (MDSCs) are a group of myeloid cells comprising precursors of macrophages, granulocytes, dendritic cells and myeloid cells at earlier stages of differentiation (20-23). In mice, these cells are broadly defined by their dual expression of Gr-1 and cluster of differentiation (CD) 11b. Evidence has demonstrated that tumor-infiltrating MDSCs are present in the peripheral blood of patients with different types of cancer, including lung, breast, and head and neck cancer (24,25). These MDSCs have been suggested to contribute to the development of resistance to several forms of treatment, including anti-angiogenic agents that target VEGF receptor (VEGFR) signaling (26-32).

The myeloid differentiation antigen Gr-1 consists of two epitopes, recognized by anti-lymphocyte antigen (Ly) 6G and anti-Ly6C antibodies, which divide CD11b+Gr-1− MDSCs into Ly6G+ granulocytes and Ly6C+ monocytes (33). These two subpopulations may have different functions in infectious diseases and cancer (34-36). Ly6C+ monocytes have been reported to function as transient accessory cells to enhance angiogenesis and remodeling of existing small vessels into larger conduits upon their onset ‘education’ by VEGF (37). There are two well-established polarized phenotypes of tumor-associated macrophages (TAMs): Classically activated macrophages (M1) and alternatively activated macrophages (M2) (38,39). It is generally accepted that M2 macrophages function in the moderation of inflammatory responses, promoting angiogenesis and contributing to tissue remodeling, all of which have been suggested to promote tumor progression (40-42). Ly6C+ inflammatory monocytes can also differentiate into M2 macrophages in autoimmune encephalomyelitis (43) or promote M2 macrophage polarization in acute tissue injury (44).

To better understand the mechanisms underlying resistance to anti-VEGF antibodies, an ideal model of anti-VEGF is required. The current study aimed to develop a model of acquired resistance to B20, a monoclonal antibody against VEGF, in nude mice following chronic exposure to B20. Subsequently, the migration of CD11b+Ly6C+ monocytes into the tumor tissue was detected. These investigations may provide the basis for strategies to improve the efficacy of anti-VEGF antibody treatments.

Materials and methods

Preparation of monoclonal antibody B20. The monoclonal antibody B20 is the mouse equivalent of bevacizumab (Avastin®), which is able to bind to mouse and human VEGF (45). B20 antibody was obtained from Genentech, Inc. (San Francisco, CA, USA) and administered intraperitoneally (150 mg/mouse) every 3 days.

Cell culture and reagents. The human NSCLC cell line A549 was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 4 mol/l glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. The cell line was cultured at 37°C in 5% CO2.
anti-mouse IgG (cat. no., A23410) and DyLight 488 AffiniPure goat anti-mouse IgG (cat. no., A23210; both 1:1,000 dilution; Abbkine Scientific Co., Ltd., Wuhan, China) were used as secondary antibodies. Immunofluorescence images were captured using a Nikon TE-2000E laser confocal microscope (magnification, x200, x400; Nikon Corporation, Tokyo, Japan). For analysis, images at x200 magnification of non-necrotic and viable tumor regions were obtained. At least 4 sections per tumor and 3 animals per group were analyzed. Image-Pro Plus software (MediaCybernetics, Inc., Rockville, MD, USA) was used to determine area densities or co-localization, as described previously (47).

Table I. CD31 and CD11b ratios in non-small cell lung cancer xenografts.

| Group   | CD31 ratio          | CD11b ratio        |
|---------|---------------------|--------------------|
| F0      | 0.18±0.01          | 0.16±0.02          |
| F1      | 0.11±0.01          | 0.10±0.01          |
| F2      | 0.07±0.02          | 0.09±0.03          |
| F3      | 0.05±0.01          | 0.06±0.01          |
| F4      | 0.16±0.02          | 0.15±0.03          |

\*P<0.01 vs. F1, F2 and F3 groups. All data are presented as the mean ± standard deviation. CD, cluster of differentiation; F0, mice injected with A549 cells only; F1, mice injected with A549 cells and treated with B20 twice weekly; F2, mice transplanted with F1 tumor explant and treated with B20 twice weekly; F3, mice transplanted with F2 tumor explant and treated with B20 twice weekly; F4, mice transplanted with F3 tumor explant with no treatment.

**Figure 1. Tumor growth curves of non-small cell lung cancer xenografts.** In the F1-F3 groups, the tumor sizes were similar, indicating the continuous inhibitory effects of anti-VEGF antibody on tumor growth. The tumor growth rate was significantly lower in the F3 group compared with in the F0 (control) group (P<0.01), suggesting that resistance to the B20 antibody was not successfully acquired. The tumor growth rate in the F4 group was similar to that in F0, indicating the withdrawal of inhibition by the anti-VEGF antibody. **P<0.01 vs. F0 vs. F1/F2/F3; \*P<0.05, F4 vs. F1/F2; n=3-6. VEGF, vascular endothelial growth factor; F0, mice injected with A549 cells only; F1, mice injected with A549 cells and treated with B20 twice weekly; F2, mice transplanted with F1 tumor explant and treated with B20 twice weekly; F3, mice transplanted with F2 tumor explant and treated with B20 twice weekly; F4, mice transplanted with F3 tumor explant with no treatment.

**Statistical analysis.** The statistical significance of each set of experimental results was assessed using an analysis of variance or a Student’s t-test. All statistical analyses were performed using SPSS v17.0 (SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Tumor growth curves.** To determine whether resistance to the anti-VEGF antibody was acquired in a xenograft model, A549 cells were injected into the mice and the antibody administered generation by generation. Tumor growth curves were produced for each group and the tumor growth rates between different groups were compared (Fig. 1). At the endpoint of observation (day 21), the tumor volume was significantly larger in the F0 group compared with in F1, F2, and F3 groups (all P<0.01), suggesting continuous inhibition of tumor growth by the anti-VEGF B20 antibody. Although the antibody reduced the growth of xenograft tumors, the tumors grew progressively in F1, F2 and F3 groups during the course of the experiment; complete inhibition of tumor growth was not observed. In the F4 group, the tumor volume was significantly larger compared with that in the F1 and F2 groups (P<0.05), and was almost the same as in F0, reflecting the removal of anti-VEGF antibody-mediated inhibition. Overall, these findings indicated that the NSCLC xenograft model of acquired resistance to anti-VEGF antibody had not been successfully established.

**Detection of CD31 staining.** The ratio of endothelial cells (CD31-positive, red) to all cells in the field of vision (DAPI-positive, blue) was calculated to evaluate the vessel density and degree of angiogenesis. Immunostaining revealed that CD31 expression was higher in the F0 (control) and F4 groups compared with that in the F1-F3 groups (Fig. 2). CD31 expression progressively reduced from F1 (Fig. 2B) to F2 (Fig. 2C) to F3 (Fig. 2D), indicating the decreasing degree of angiogenesis and the inhibitory effects of the anti-VEGF antibody. The ratio of CD31/nucleus continuously decreased from the F1 to F2 to F3 groups, and was significantly lower in each of these three groups compared with in the F0 and F4 groups (all P<0.01; Table I). These results suggested that the B20 anti-VEGF antibody effectively inhibited blood vessel formation in the F1, F2 and F3 groups. Withdrawal of the drug from F4 group partially reversed the suppression of angiogenesis, but the lower vessel density in the F3 group compared with that in the F0 control indicated unsuccessful establishment of the anti-VEGF resistance model.

**Assessment of the migration of CD11b+ myeloid cells.** The ratio of CD11b+ myeloid cells (green) to cells in the field of vision (blue) was calculated. The blood vessels were simultaneously marked by CD31. Confocal microscopy demonstrated that the number of CD11b+ myeloid cells was markedly higher in the F0 (control) and F4 groups compared with in the other groups (Fig. 3), and continuously decreased from F1 (Fig. 3B) to F2 (Fig. 3C) to F3 (Fig. 3D), suggesting the inhibition of MDSC migration by the anti-VEGF antibody. The ratio of CD11b/nucleus (CD11b ratio) progressively reduced from the F1 to F2 to F3 groups, and the CD11b ratio...
Figure 2. Immunostaining of CD31 (red) in non-small cell lung cancer xenografts. Blue fluorescence indicates DAPI (nuclear) staining. (A) The F0 (control) group exhibited high vessel density. (B) F1, (C) F2 and (D) F3 groups exhibited lower expression of CD31 compared with that in the F0 group. (E) The F4 group demonstrated similar vessel density to that in F0, and higher expression of CD31 compared with that in F1, F2 and F3. CD31, cluster of differentiation 31; F0, mice injected with A549 cells only; F1, mice injected with A549 cells and treated with B20 twice weekly; F2, mice transplanted with F1 tumor explant and treated with B20 twice weekly; F3, mice transplanted with F2 tumor explant and treated with B20 twice weekly; F4, mice transplanted with F3 tumor explant with no treatment.

Figure 3. Immunostaining of CD11b+ myeloid-derived suppressor cells (green) and CD31 (red) in non-small cell lung cancer xenografts. Blue fluorescence indicates DAPI (nuclear) staining. (A) The F0 (control) group possessed a large percentage of CD11b+ cells. (B) F1, (C) F2 and (D) F3 groups exhibited a lower percentage of CD11b+ compared with that in F0. (E) The percentage of CD11b+ cells in the F4 group was similar to that in F0 and higher compared with that in F1, F2 and F3. CD, cluster of differentiation; F0 mice, injected with A549 cells only; F1 mice, injected with A549 cells and treated with B20 twice weekly; F2 mice, transplanted with F1 tumor explant and treated with B20 twice weekly; F3 mice, transplanted with F2 tumor explant and treated with B20 twice weekly; F4 mice, transplanted with F3 tumor explant with no treatment.
was significantly lower in each of these three B20-treated groups compared with in the F0 and F4 groups (all P<0.01; Table I). The changes observed in the number of CD11b+ cells were consistent with those of CD31+ endothelial cells. These results indicated that anti-VEGF antibody effectively decreased the recruitment of MDSCs to the tumor tissue, thus inhibiting the vessel formation in F1, F2, and F3 groups. The lower presence of CD11b+ myeloid cells in the F3 compared with the F0 group demonstrated that drug resistance was not successfully induced.

**Table II. Ly6C and Ly6C/CD11b ratios in non-small cell lung cancer xenografts.**

| Group | CD31 ratio | Ly6C ratio | Ly6C/CD11b |
|-------|------------|------------|------------|
| F0    | 0.18±0.03a | 0.06±0.01a | 0.38±0.05a |
| F1    | 0.10±0.02  | 0.05±0.01  | 0.44±0.10  |
| F2    | 0.06±0.02  | 0.04±0.01  | 0.51±0.13  |
| F3    | 0.04±0.01  | 0.04±0.00  | 0.68±0.11b |
| F4    | 0.17±0.02a | 0.06±0.01a | 0.40±0.10  |

P<0.01 vs. F1, F2 and F3 groups; aP<0.01 vs. F0, F1 and F4 groups.

**Figure 4.** Immunostaining of Ly6C+ cell subsets (green) and CD31 (red) in non-small cell lung cancer xenografts. Blue fluorescence indicates DAPI (nuclear) staining. (A) The F0 (control) group had a large percentage of Ly6C+ cells. (B) F1, (C) F2 and (D) F3 groups displayed a lower percentage of Ly6C+ compared with that in F0. (E) The percentage of Ly6C+ in the F4 group was similar to that in F0 and higher compared with that in F1, F2 and F3. Ly6C, lymphocyte antigen 6C; CD, cluster of differentiation; F0, mice injected with A549 cells only; F1, mice injected with A549 cells and treated with B20 twice weekly; F2 mice, transplanted with F1 tumor explant and treated with B20 twice weekly; F3 mice, transplanted with F2 tumor explant and treated with B20 twice weekly; F4 mice, transplanted with F3 tumor explant with no treatment.

**Assessment of Ly6C+ subset of CD11b+ MDSCs.** The anti-Ly6C antibody was used to mark the Ly6C+ subset of CD11b+ MDSCs, which may serve an essential role in promoting angiogenesis and tumor progression. The ratio of Ly6C+ cells (green) to all cells in the field of vision (blue) was calculated. The blood vessels were simultaneously marked by staining of CD31. Under a confocal microscope, the largest numbers of Ly6C+ monocytes were present in the F0 (control) and F4 groups (Fig. 4), and the number of Ly6C+ monocytes decreased from F1 (Fig. 4B) to F2 (Fig. 4C) to F3 (Fig. 4D), indicating inhibitory effects of the anti-VEGF antibody on Ly6C+ monocyte migration and angiogenesis. Consistent with the trend for CD11b+ cells, the percentage of Ly6C+ monocytes in the field of vision (Ly6C ratio) continuously decreased from the F1 to F2 to F3 groups, and was significantly lower in each of these three groups compared with in the F0 and F4 groups (P<0.01; Table II). These data indicated that anti-VEGF antibody repressed the migration of MDSCs, including the Ly6C+ subpopulation, to the tumor tissue, and that drug resistance had not been acquired. To further determine the migration tendency of Ly6C+CD11b+ monocytes, the ratio of Ly6C+ monocytes to CD11b+ MDSCs (Ly6C/CD11b) was calculated. Notably, it was identified that the Ly6C/CD11b ratio was highest in the F3 group and significantly higher in F3 compared with in F0, F1, and F4 groups (all P<0.01) (Table II). These findings suggested that the migration tendency of the Ly6C+ subset relatively increased with the increasing mouse generation compared with...
the other subsets in the whole CD11b+ population. Thus, if the generations of nude mice or the tumor growth period (between inoculation and harvesting) is increased, it is possible that the xenograft model of acquired resistance to anti-VEGF antibody may be successfully established.

Discussion

Molecular inhibition of VEGF/VEGFR signaling is currently being investigated as a promising cancer treatment strategy. For lung cancer, one study has demonstrated that angiogenesis inhibitors are superior to non-angiogenesis inhibitors with regard to objective response, disease control, progression-free survival and overall survival rates in patients with advanced NSCLC (18). The advantages of anti-angiogenesis therapy have mostly been demonstrated with antibody-based agents (48). Monoclonal antibodies against VEGF, such as bevacizumab, have demonstrated efficacy when used alone or in combination with chemotherapy (49). However, modest effects have been reported and drug resistance has been widely observed in preclinical and clinical trials (17,50,51). Thus, efforts to better understand the mechanisms underlying acquired resistance to anti-VEGF antibodies and potential strategies to overcome resistance are warranted.

In the present study, the aim was to establish an NSCLC xenograft model of acquired resistance to anti-VEGF antibody in nude mice, in order to provide a tool for mechanism investigation and future research, and then to detect the expression of CD31 and the migration of CD11b+Ly6C+ monocytes. In the xenotransplantation experiments, tumor growth rate in the last generation of drug administration (F3) was lower compared with that in the control (F0). Following immunostaining, the expression of CD31 was revealed to be lower in the F3 group compared with that in the F0 group. These data indicated that the drug resistance model had not been successfully established.

It was hypothesized that these negative data may result from the changing microenvironment in distinct generations of mice. It has been recognized that the development of resistance to anti-angiogenesis agents is largely associated with the tumor microenvironment, including stromal cells, extracellular matrix-components, TAMs, and autocrine and paracrine signaling factors (52). Tumor cells communicate with components of their microenvironment via a complex network of growth factors, cytokines and chemokines. TAMs support lung cancer progression by inducing cancer cell motility and metastasis, and angiogenesis (52,53). As a result, changing the microenvironment of the tumor leads to updated recruitment of accessory cells and molecules, thus decreasing the tumor growth rate in xenografts. Therefore, elongation of the period between tumor inoculation and harvesting may compensate for the change in microenvironment. In the present study, the mice were sacrificed on day 29 post-inoculation in F1 group, on day 21 in F2 group, day 25 in F3 group, and day 25 in F4 group. However, in a similar study, Gyanchandani et al (54) generated a head and neck squamous cell carcinoma xenograft model of acquired resistance to bevacizumab, in which the time for a generation was ≥56 days. In the study by Curtarello et al (55), mice were maintained for ≥45 days after tumor inoculation to induce resistance to bevacizumab in ovarian and breast cancer cells. In the present study, the Ly6C/CD11b ratio increased from the F1 to the F3 group. A larger number of generations may promote the successful acquisition of resistance to anti-VEGF antibody by tumor cells. The short time and low generation numbers of the present study were its limitations.

However, the ratio of Ly6C/CD11b was higher in the F3 group compared with that in the other groups, suggesting an enhanced migration tendency of the Ly6C+ subset. This subset is a population of cells that are able to polarize into M2 macrophages and serve a role in promoting angiogenesis (38,39). The primary functions of M2 macrophages are limitation of the immune response and promotion of tumor invasion, growth and metastasis via the secretion of inhibitory cytokines and the prevention of T cells from exerting antitumor effects (56). Although Ly6C+ and Ly6G+ MDSC numbers are equally increased in tumor-bearing mice (36), the Ly6C+ subset has a greater tendency to polarize into M2 macrophages following proper stimulation. In contrast to these reports, Ly6C+ monocytes are preferentially recruited to inflamed tissues in a C-C motif chemokine receptor-2-dependent manner and generate inflammatory macrophages, such as M1 macrophages, as described in myocardial infarction (57), muscle injury (58), and bacterial infection (59). Ly6C+ monocytes digest damaged tissue, whereas Ly6C+ monocytes promote healing via myofibroblast accumulation, angiogenesis and deposition of collagen (57). It appears that Ly6C+ monocytes cooperate with M1 macrophages in inflammatory functions, whereas Ly6C+ monocytes work together with M2 macrophages to achieve angiogenic functions (60). Notably, Ly6C+ monocytes can give rise to Ly6C+ monocytes under steady-state conditions (61-63). Therefore, regardless of whether M2 macrophages derive from Ly6C+ or Ly6C+ monocytes, increased recruitment of Ly6C+ monocytes indicates enhanced angiogenesis.

Although Shojaei et al (30) did not provide definitive evidence of macrophage involvement in tumor refractoriness following anti-VEGF therapy, it was revealed that tumor relapse is affected by the heterogeneous CD11b+Gr-1+ MDSCs; the combination of anti-VEGF and anti-Gr-1 antibodies given to tumor-bearing mice was more effective in preventing angiogenesis and slowing tumor growth compared with either antibody alone. Since the Gr-1 antibody recognizes Ly6C, a receptor expressed on inflammatory monocytes, and Ly6G, it can be inferred that monocytes/macrophages may be partially responsible for refractoriness following anti-angiogenic therapy. Notably, resistance to conventional chemotherapies did not involve CD11b+Gr-1+ MDSCs in these models, suggesting that myeloid cells specifically initiate refractoriness to anti-angiogenic therapies (30).

Other studies have demonstrated upregulation of VEGF expression in macrophages following radiotherapy in patients, suggesting that increased levels of TAM-derived pro-angiogenic factors can stimulate the formation of a new blood supply to radio-resistant tumor cells (64). In agreement with these data, Ahn et al (65) revealed the important contribution of matrix metallopeptidase 9-expressing CD11b+ myeloid cells to tumor revascularization and recovery following radiation. Taken together, these findings indicate that inhibiting monocyte recruitment to tumors or neutralizing the factors that they produce in tumors, in combination with conventional therapeutic agents, may have considerable therapeutic potential.
In conclusion, in the current study, the increased migration tendency of CD11b+Ly6C+ myeloid cells suggests the potential for successful resistance acquisition and implies a possible contribution of these cells to tumor refractoriness. Increasing the number of generations or the time for post-inoculation tumor growth may generate an NSCLC model of acquired resistance to the anti-VEGF antibody. The role of CD11b+Ly6C+ monocytes in angiogenesis and their association with M2 macrophages may have implications for improving the efficacy of anti-VEGF therapies.

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