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Alternatively Activated RAW264.7 Macrophages Enhance Tumor Lymphangiogenesis in Mouse Lung Adenocarcinoma

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ABSTRACT

Tumor-associated macrophages (TAMs) have been implicated in promoting tumor progression and invasion. The onset and maintenance of tumor angiogenesis and lymphangiogenesis also seem to be partly driven by a group of polarized alternatively activated macrophages (aaMphi) in lung adenocarcinoma. Here, the aaMphi and classically activated macrophages (caMphi) were obtained using RAW264.7 cells via IL-4 and IFN-γ + LPS treatment, respectively. Co-inoculation of aaMphi with Lewis lung carcinoma (LLC) cells promoted tumor growth, increased lymph node metastasis, and reduced the survival in C57BL/6 mice bearing LLC. Furthermore, the effects of the activated macrophages on the lymphangiogenesis-related properties of lymphatic endothelial cells (LECs) were investigated in vitro. When LECs were cultured in macrophages conditioned medium or in a co-culture system of macrophages and LECs, aaMphi significantly promoted proliferation, migration, and tube-like formation of LECs. We identified high VEGF-C expression in aaMphi and low expression in caMphi as well as unactivated macrophages by ELISA and Western blotting. In LECs, co-culture with aaMphi resulted in a significant increase of mRNA levels of specific lymphatic marker VEGF receptor-3 and the homeobox gene Prox-1, as well as lymphangiogenic factor VEGF-C rather than VEGF-D by quantitative RT-PCR. Furthermore, enhanced LECs migration and capillary formation by co-culture with aaMphi were significantly inhibited by rVEGF receptor-3/Fc chimera. In conclusion, these data show that aaMphi play a critical role in tumor-induced lymphangiogenesis through up-regulating VEGF-C and increasing lymphangiogenesis-related behavior of LECs, which may contribute to lymphatic invasion in lung adenocarcinoma. J. Cell. Biochem. 107: 134–143, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: ALTERNATIVE ACTIVATION; LECS; LYMPHANGIOGENESIS; TUMOR-ASSOCIATED MACROPHAGES; VEGF-C

Previously, activated macrophages were viewed as a group of cells that secreted a wide range of inflammatory mediators and killed intracellular pathogens. Recent studies show that there appears to be at least three different subpopulations of activated macrophages, possessing different physiologies, and performing distinct immunological functions [Mosser, 2003; Edwards et al., 2006]. The first and most well described is classically activated macrophages (caMphi), which are activated by double signals, interferon (IFN)-γ, and tumor necrosis factor-α (TNF-α) or lipopolysaccharide (LPS). They can secrete nitrogen monoxidum (NO), interleukin (IL)-12 and TNF-α, and function as effector cells in T-helper (Th)1 cellular immune responses. The second type is alternatively activated macrophages (aaMphi) [Stein et al., 1992]. Exposure to IL-4, -13, vitamin D3, glucocorticoids or transforming growth factor-β (TGF-β) decreases the antigen-presenting capability of the macrophages, and up-regulates the expression of macrophage mannose receptors (MMR, also known as CD206) and scavenger receptors. The aaMphi can produce factors that suppress T-cells proliferation and activity, but fail to produce NO and IL-12. As a result, they are relatively poor at killing intracellular pathogens but adapt to scavenging debris, and play an important role in stroma formation and tissue repair through producing several components involved in the synthesis of extracellular matrix [Gratchev et al., 2001]. More recently, it seems that aaMphi are involved in tumor

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growth, angiogenesis, and immunosuppression [Gordon, 2003; Gratchev et al., 2006]. The third type of cells is the type-2-activated macrophages, which possess anti-inflammatory effects and preferentially induce Th2-type humoral-immune responses to antigen [Anderson and Mosser, 2002]. Thus, the above three populations of cells may form their own regulatory network to prevent a well-intentioned immune response from progressing to immunopathology.

Although macrophages within different tissues have an ability to guard against microbial infections and regulate normal cell turnover and tissue remodeling, they also form a major component of the inflammatory infiltrate seen in both primary and secondary tumors, where they also exhibit a distinct phenotype similar to aaMphi and are termed tumor-associated macrophages (TAMs) [Mantovani et al., 2002; Sica et al., 2007]. In recent years, accumulating evidences have shown that TAMs influence diverse processes such as angiogenesis, lymphangiogenesis, tumor cell proliferation, and metastasis during tumor progression [Lewis and Pollard, 2006; Mantovani et al., 2006; Porta et al., 2007; Allavena et al., 2008]. The protumoral role of TAMs is further supported by clinical studies that found a correlation between the high macrophage number in tumor tissue and poor patient prognosis [Takanami et al., 1998]. Lymphangiogenesis, whose elementary process includes the proliferation, migration, and tube-like structure formation of lymphatic endothelial cells (LECs), is considered as the initial step and necessary event of lymphatic and regional lymph node metastasis [Alitalo et al., 2005; Stacker et al., 2007]. Many studies have provided support for the contribution of vascular endothelial growth factor (VEGF)-C and VEGF-D, and their respecting receptors, including vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3, in lymphatic development as well as lymphangiogenesis in malignancy [Mandriota et al., 2001; Stacker et al., 2001]. Schoppmann et al. reports that the peritumoral inflammatory reaction and VEGF-C expressing TAMs increase tumor lymphangiogenesis and lymphovascular invasion in cervical cancer [Schoppmann et al., 2002] and invasive breast cancer [Schoppmann et al., 2006].

Here, in vivo, Lewis lung carcinoma (LLC) mice were used to investigate the effect of activated macrophages including aaMphi and caMphi on tumor growth, lymphangiogenesis, lymph node, and distant metastasis. Furthermore, using isolated murine LECs as an in vitro model, we analyzed whether aaMphi have a potent effect on the lymphangiogenesis-related properties of LECs, including proliferation, migration, and tube-like formation, and further investigated the possible mechanisms.

MATERIALS AND METHODS

ANIMALS

Specific pathogen-free female C57BL/6 mice (4–6 weeks old) and female Balb/c mice (6 weeks old) were purchased from the Institute of Experimental Animal of Third Military Medical University (Chongqing, China). All animals had free access to standard laboratory mouse food and water. This study was conducted in accordance with the national and regional guidelines for the care and use of laboratory animals.

CELL LINES

Mouse RAW264.7 macrophages and LLC cells were purchased from American Type Culture Collection (Manassas, VA), and maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen).

Isolation and culture of LECs were performed as described previously [Wang et al., 2007]. Briefly, female Balb/c mice were intraperitonealy injected with emulsified incomplete Freund's adjuvant (Sigma–Aldrich, St Louis, MO) to induce lymphangiomas. At 30 days of induction, tumors in the peritoneal cavity were removed and mechanically disrupted, and LECs were isolated and resuspended in Endothelial cell basal medium-2 (EBM-2; Cambrex BioScience, Wokingham, UK) supplemented with 20% FBS and 50 ng/ml endothelial cell growth supplement (Cambrex BioScience) at 37°C in a humidified atmosphere of 5% CO_2. LECs were used in appropriate experiments or cultivated till the fourth passage.

ACTIVATION AND CHARACTERIZATION OF RAW264.7 MACROPHAGES

The aaMphi and caMphi were prepared by stimulating RAW264.7 macrophages with 10 IU/ml mouse recombinant IL-4 (Cytolab Ltd, Rehovot, Israel) or 100 IU/ml IFN-γ (Cytolab Ltd) + 10 ng/ml LPS (Sigma–Aldrich) overnight, respectively [Edwards et al., 2006]. Untreated RAW264.7 cells were used as control. For identification of aaMphi and caMphi, total RNA were isolated with the RNAeasy kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. The cDNA was synthesized with SuperScript III polymerase (Invitrogen) and PCR for the mouse iNOS and MMR mRNA was performed with the primers for β-actin, 5′-CCC TGT ATG CCT CTG GTC-3′ (sense) and 5′-GTC TTT ACG GAT GTC AAC G-3′ (antisense); iNOS: 5′-CAC GGA CGA GAC GGA TAG-3′ (sense) and 5′-GGG AGG AGC TGA TGG AGT-3′ (antisense), and MMR (C-type 1), 5′-CTC GTG GAT CTC CGT GAC AC-3′ (sense) and 5′-GCA AAT GGA GCC GTC TGT GC-3′ (antisense).

IMPLANTATION OF TUMORS AND METASTASIS ANALYSIS

LLC cells (1×10^6) mixed with or without various macrophages (3×10^6) in 0.1 ml Hanks' Balanced Salt Solution were injected subcutaneouly into the dorsal side of the ear of female C57BL/6 mice as described previously [Bobek et al., 2004; Orimo et al., 2005]. Mice were randomly chosen and assigned to 4 groups (10 mice per group) based on the difference of macrophages co-inoculated with LLC cells: control group (LLC cells alone), caMphi group (caMphi + LLC cells), aaMphi group (aaMphi + LLC cells), and RAW264.7 group (RAW264.7 cells + LLC cells). After 1-week of implantation, tumor volumes were measured with a caliper twice a week and calculated by the formula: volume $= 0.52\ ab^2$, where a = long diameter and b = short diameter. Mice were anesthetized with ethyl ether and sacrificed 28 days after co-inoculation of LLC cells with macrophages. The primary tumors in these mice were harvested and weighted, and the presence of metastases (in lymph nodes and lung) was determined grossly and microscopically.

IMMUNOHISTOCHEMISTRY AND LMVD

Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) was used as an immunohistochemical marker for endothelial cells

[Mouta et al., 2001]. The sections were deparaffinized in xylene, and rehydrated through graded alcohol and deionized water. For antigen retrieval, the sections were heated in microwave oven in 0.3% citrate buffer (pH 6.0) for 4×5 min and washed with phosphate-buffered saline (PBS) for 4×5 min. Endogenous peroxidase were inactivated by 30 min incubation in methanol containing 3% H_2O_2, followed by PBS wash for 3×5 min. To block non-specific biding sites, the sections were further treated with normal horse serum for 15 min. The slides were immunostained with a rabbit polyclonal antibody against LYVE-1 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Slides were then washed three times in PBS and exposed to a biotinylated secondary antibody (Zhongshan, China) for 20 min, followed by treatment with streptavidin peroxidase (Zhongshan, China). For color development, the slides were stained with 3,3′diaminobenzidine (DAB, Sigma–Aldrich), then were counterstained with hematoxylin and eosin (H&E). A reddish brown precipitate in the cytoplasm of LECs indicated a positive reaction. After scanning the immunostained sections at low magnification (100×), three areas of transplantation tumors with the greatest number of distinctly highlighted lymphatic/vascular foci (hot spots) were selected by two observers at the same time. The two observers then independently evaluated the slides for microvessel counting using 200× magnification (0.17 mm^2 field) without knowledge of mice status and stains used. Single immunoreactive endothelial cells, or endothelial cell clusters separate from other microvessels, were counted as a vessel according to previous procedures [Schoppmann et al., 2002]. The mean number of lymphatic microvessel density (LMVD) was counted and used in the statistical analysis.

SURVIVAL ASSAY

In another animal experiment, 40 mice were also randomly chosen and assigned to 4 treatment group (10 mice per group): control group, caMphi group, aaMphi group, and RAW264.7 group. After LLC tumor was transplanted as described above, mice were monitored for survival in 2 months.

CELL GROWTH

A transwell system with a porous polycarbonate membrane filters (0.4 μm pore size, Millipore, Bedford, MA) and 24-well plastic culture plates were used for macrophages-LECs co-culture. The LECs were seeded into the lower chamber of the transwell apparatus. After allowing 24 h to reach confluence, the LECs were washed with PBS three time, and DMEM and EBM-2 was added. The upper chamber was placed into the lower chamber and seeded with aaMphi, caMphi or RAW264.7 cells (5×10^5/well in DMEM). After 48 h of co-culture, the total viable cell number of LECs was counted by Trypan blue dye exclusion.

MIGRATION ASSAY

For migration assays, 24-well culture inserts with a porous polycarbonate membrane (8.0 μm pore size, Millipore) were coated with 10 μg/ml fibronectin (BD Biosciences Discovery Labware, Bedford, MA) on their inside surfaces and dried at room temperature for 1 h. The aaMphi, caMphi or RAW264.7 cells were incubated in serum-free DMEM for 24 h, after which culture supernatants were collected as conditioned medium. The LECs (5×10^4 cells/insert) were placed in the upper chamber then cultured with conditioned medium (aaMphi or caMphi RAW264.7 media and EBM-2 [1:1]). Control media were DMEM and EBM-2 (1:1). After incubation of 48 h, non-migrated cells in the upper chamber were scraped off, and the migrated cells on the bottom part of the filter were identified through fixing in 70% methanol for 10 min and staining with hematoxylin. In each individual experiment, cells that migrated through the filters were counted under the microscope at 200× magnification in 12 randomly selected fields. The data were expressed as a percentage of control. Each experiment was performed in triplicate or quadruplicate. To detect the effect of rVEGFR-3/Fc chimera on migratory responses of LECs cultured in conditioned medium from aaMphi, LECs were treated with rVEGFR-3/Fc chimera (1 μg/ml, R&D Systems, Abingdon, UK) as described previously [Ochi et al., 2007], then were incubated in conditioned medium.

TUBE-LIKE STRUCTURE FORMATION ASSAY

LECs (6×10^4 cells/well) were seeded into a 24-well plate that had been pre-coated with 100 μl Matrigel (10 mg/ml, Clontech, Palo Alto, CA) and cultured for 24 h. The aaMphi, caMphi or RAW264.7 cells were seeded into transwell chambers consisting of polycarbonate membranes (0.4 μm pore size, Millipore) and allowed to adhere overnight. Transwell inserts were then placed into the LECs culture system. The LECs were co-cultured with macrophages for a total of 2 days. Cells that cultured in basal medium were used as control. Formation of tube-like structures was monitored by microscopic observation at 100× magnification and over 12 different fields of each well were photographed to measure the length of tube-like structures as described previously [Wang et al., 2007]. In the same way, effects of rVEGFR-3/Fc chimera (1 μg/ml) on LECs tube-like formation in the presence of aaMphi were also assessed.

ELISA ASSAY

The aaMphi, caMphi or RAW264.7 cells (2×10^6 cells/well) were incubated in serum-free DMEM for 24 h. Medium was removed, and cells were cultured for another 24 h. Culture media were collected and centrifuged at 1,200 rpm for 5 min at 4°C. The levels of VEGF-C secretion were measured using mouse enzyme-linked immunosorbent assay (ELISA) kits (Uscnlife, Wuhan, China). Absorbance at 450 nm was determined using a microplate reader (Bio-Rad, Hercules, CA). All experiments were performed on at least three separate occasions.

WESTERN BLOTTING ANALYSIS

Cell extracts were prepared and Western blotting were performed according to previous procedures [Wang et al., 2008]. Briefly, after the various treatments as described above, cells were washed with cold phosphate-buffered saline, pelleted, and lysed in radioimmunoprecipitation assay buffer (Sigma–Aldrich) supplemented with protease inhibitors. Protein concentration was determined with Bradford reagent (Bio-Rad), and equal amounts of protein were run on a 10% SDS–PAGE gel and blotted onto polyvinylidene fluoride membranes. After blocking (5% bovine serum albumin and 0.5%

Tween-20) and washing with phosphate-buffered saline, the polyvinylidene fluoride membranes were incubated overnight with rabbit anti-VEGF-C (1: 200; Boster Biotechnology) as the primary antibodies. The secondary antibody used was horseradish-peroxidase-conjugated anti-IgG (Boster Biotechnology). Membranes were then developed using enhanced chemiluminescence reagents (Amersham Biosciences).

QUANTITATIVE RT-PCR

LECs were co-cultured with activated macrophages or RAW264.7 cells for 48 h, then total RNA derived from LECs was isolated and subjected to quantitative RT-PCR analysis in an ABI 7300 Prism Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously [Wang et al., 2007]. Primers (Invitrogen, Shanghai, China) used for RT-PCR were VEGF-C, 5′-GGG GAA GGA GTT TGG AGC-3′ (sense) and 5′-CTG CCT GAC ACT GTG GTA AT-3′ (antisense); VEGF-D, 5′-CAT ACG GGT TGT AAG TGC-3′ (sense) and 5′-GGT TCC TGG AGG TAA GAG T-3′ (antisense), VEGFR-3, 5′-TGG ACA GCT GGA CGG AGT TT-3′ (sense) and 5′-CTG GCA GAG GAG TTT ACG CA-3′ (antisense), and Prox-1, 5′-TGG CTT ATC CAT TTC AGA GTC-3′(sense) and 5′-CTT CAC GTC CGA GAA GTA GG-3′ (antisense). The relative expression levels of the target genes (VEGF-C, VEGF-D, VEGFR-3, and Prox-1) against that of the β-actin was defined as $-\Delta CT = -(CT_{Target} - CT_{\beta\text{-actin}})$. The target mRNA/β-actin mRNA ratio was calculated as $2^{-\Delta CT}$ according to the manufacture's specification.

STATISTICAL ANALYSIS

The quantitative data were expressed as the mean ± SD from triplicate samples of at least three independent experiments, and qualitative variables as percentages. Statistical differences between the means were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test or Student's *t*-test. Survival rates were graphed using Kaplan–Meier method and compared among different groups by log-rank statistical analysis. A value of $P < 0.05$ was considered as a statistically significant difference. All statistical analyses were performed with SPSS 13.0 (SPSS, Inc., Chicago, IL).

RESULTS

ACTIVATION AND PHENOTYPE IDENTIFICATION OF RAW264.7 MACROPHAGES

Mouse RAW264.7 cells were stimulated with IL-4 and IFN-γ + LPS respectively to obtain aaMphi and caMphi. After 24 h of stimulation, the total RNA was isolated and different phenotype markers for the activated populations of macrophages including iNOS and MMR were analyzed by RT-PCR (Fig. 1). These results indicate that stimulation of RAW264.7 cells with IL-4 or IFN-γ + LPS is a successful method to acquire aaMphi and caMphi that possess specific activated phenotypes of macrophages.

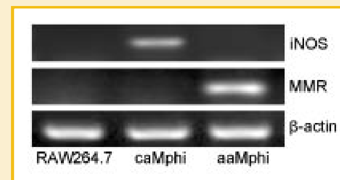

Fig. 1. Characterization of aaMphi and caMphi in activated macrophages. The aaMphi and caMphi groups were prepared by stimulating mouse RAW264.7 macrophages with 10 IU/ml IL-4 and 100 IU/ml IFN-γ + l0 ng/ml Ultra-Pure LPS overnight, respectively. Total RNA was isolated and the mRNA levels of activated makers including MMR and iNOS, were determined as detected by RT-PCR. β-actin mRNA was shown as a control for RNA loading.

EFFECT OF ACTIVATED MACROPHAGES ON GROWTH OF LLC

After 7 days of injection with LLC cells-macrophages in C57BL/6 mice, tumors were found subcutaneously in the dorsal side of the ear in both aaMphi group and RAW264.7 macrophages group. However, in control group and caMphi group, transplantation tumors were firstly found at 10 and 15 days, respectively. At 14 days of inoculation, there was no significant difference in tumor volumes between aaMphi group and RAW264.7 group. At 15, 16, and 21 days of transplantation, the mice in aaMphi group, RAW264.7 group, and control group began to die, respectively. The mice were sacrificed at 28 days of inoculation, and tumors were harvested for weights. As shown in Figure 2A and Table I, the weights and volumes of LLC transplantation tumors in aaMphi group and RAW264.7 macrophages group, but not caMphi group, were bigger than those in control group. There was no significant difference in tumor weights between aaMphi group and RAW264.7 group. These data indicate that aaMphi and initial macrophages, but not caMphi, promote the rate of LLC tumor growth.

EFFECT OF ACTIVATED MACROPHAGES ON LYMPHANGIOGENESIS, LYMPH NODE, AND DISTANT METASTASIS

We next analyzed whether activated macrophages affect lymph node metastasis and distant metastasis. All mice were biopsied for detecting lymph nodes and distant metastasis by H&E staining. As shown in Table I, compared with the control group, the aaMphi group, and RAW264.7 group had higher metastatic lymph node numbers. And, no obvious lymph node metastasis was examined in caMphi group. In addition, the metastatic nodes in lungs, but not in liver and brain, were found in all mice. The numbers of metastatic nodes in lungs in aaMphi group as well as RAW264.7 group were higher than that in control group.

Furthermore, immunohistochemistry results showed that LYVE-1-positive lymphatics occurred mostly in peritumoral stroma of implanted LLC tumors in aaMphi group and RAW264.7 group, while was almost absent in caMphi group and blank control (Fig. 2B). In all the four groups, intratumor LYVE-1 staining was completely negative (data not shown), which is in line with previous report that the functional lymphatics in the tumor margin alone are sufficient for lymphatic metastasis in hepatocellular carcinomas [Mouta et al., 2001]. So, the numbers of lymphatic vessels were calculated only in the area surrounding the tumor. There was a higher LMVD in aaMphi group or RAW264.7 group, as compared with that in control group (Table I). These results suggest that aaMphi possess a potent ability to promote tumor metastasis through increasing lymphangiogenesis in mice.

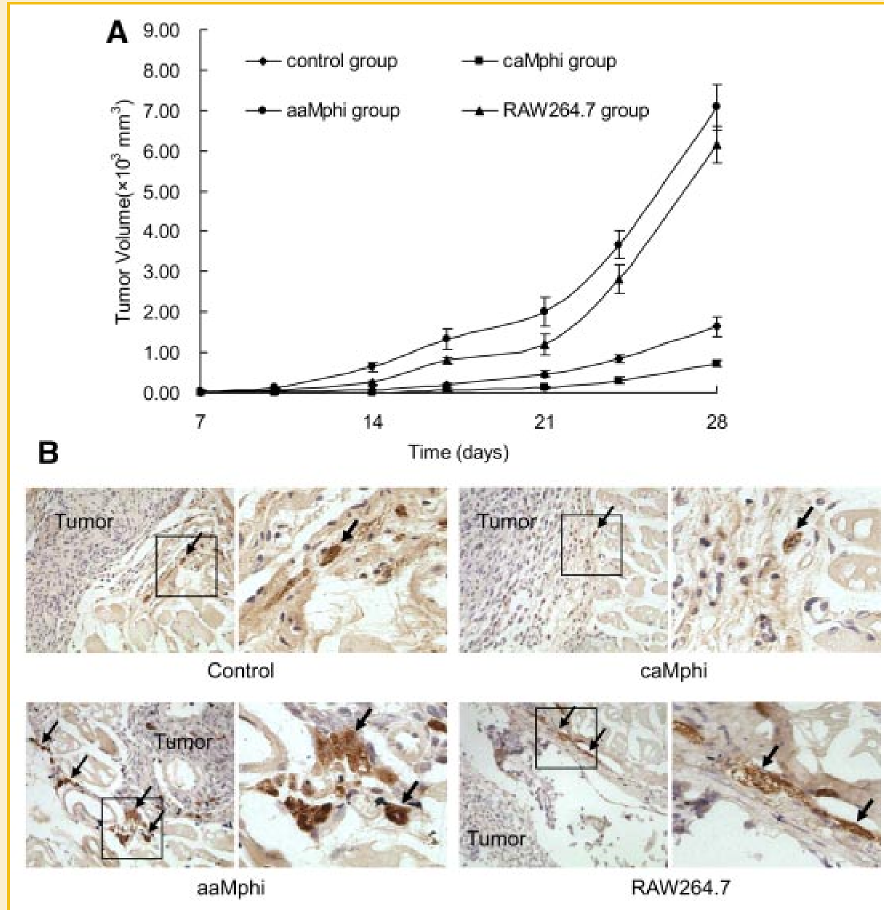

Fig. 2. Dynamic changes of LLC tumors growth and tumor-induced lymphangiogenesis. A: LLC cells (1×10^6) mixed with different activated macrophages (3×10^6) in 0.1 ml Hanks' Balanced Salt Solution were injected subcutaneouly into the dorsal side of the ear of female C57BL/6 mice. Mice were randomly assigned to 4 groups (10 mice per group): control group, caMphi group, aaMphi group, and RAW264.7 group. After 1-week of injection, tumor volumes were measured with a caliper twice a week. The data shown represents three experiments. Data is displayed as the mean ± SD. B: Expression of LYVE-1 was used to count LMVD in LLC tumors by immunochemistry method. LYVE-1-positive lymphatics (black arrow) were found mostly in peritumoral stroma of implanted LLC tumors in the indicated groups. Left, lower magnification; right, the boxed areas in higher magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

EFFECT OF ACTIVATED MACROPHAGES ON SURVIVAL OF TUMOR-BEARING MICE

As shown in Figure 3, tumor-bearing mice in control group began to die on the 21st day, and all of mice died after 55 days of co-inoculation. In contrast, 60% of mice in caMphi group still remained alive at 60 days. All of mice in aaMphi group and RAW264.7 group died at 40 and 42 days of implantation, respectively. There is a lower cumulative survival in aaMphi and RAW264.7 group than that in control group, suggesting that aaMphi and initial macrophages can reduce the survival in mice bearing LLC.

EFFECT OF ACTIVATED MACROPHAGES ON PROLIFERATION, MIGRATION, AND TUBE-LIKE STRUCTURE FORMATION OF LECS

The growth, migration, and tube-like formation of LECs have been found to be linked to lymphangiogenesis by activating VEGFR-3

TABLE I. Tumor Weight, Lymph Node and Pulmonary Metastasis, and Tumor LMVD in Mice

Groups	Tumors weight (g)	Lymph node metastasis	Pulmonary metastatic nodes	LMVD
Control	2.19 ± 0.34	3.50 ± 0.97	16.40 ± 2.34	7.49 ± 3.12
caMphi	1.07 ± 0.22*	0.60 ± 0.84**	5.80 ± 1.15**	3.73 ± 2.15
aaMphi	5.85 ± 0.98**	10.80 ± 1.32**	32.50 ± 4.91**	16.80 ± 2.64**
RAW264.7	5.49 ± 1.04**	7.30 ± 0.95**	29.30 ± 4.42**	13.50 ± 2.07*

*$P < 0.05$.
**$P < 0.01$, as compared with control group. The values were shown as the mean ± SD.

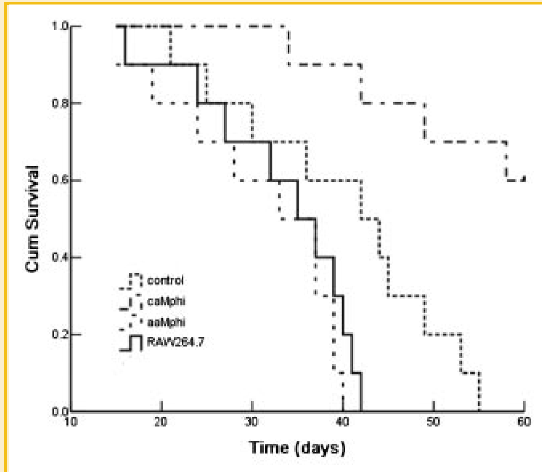

Fig. 3. Mice survival analysis. LLC cells co-inoculated with macrophages with different activated phenotypes in C57BL/6 mice. Within 60 days of inoculation, mice survival was analyzed in control group, caMphi group, aaMphi group, and RAW264.7 group.

tyrosine kinase signals [Alitalo et al., 2005]. We next examined the effect of activated macrophages on the proliferation of murine LECs that were obtained from intraperitoneal lymphangiomas induced with IFA. A macrophages-LECs co-culture system was established using the transwell cell culture chambers. As shown in Figure 4A, LECs in aaMphi group had a high proliferation rate than other three groups including caMphi, RAW264.7, and control group within 7 days of co-culture. After 24 h of culture in conditioned medium, the number of LECs permeating septum in aaMphi group was highest among the four groups (Fig. 4B,D), and aaMphi increased cell migration to more than 4-fold. Furthermore, after 24 h of macrophages-LECs co-culture, LECs established a complex tube system. The network of tube-like structures in aaMphi group was more extensive than that in caMphi, RAW264.7, and control group, and there was no significant difference among the three groups (Fig. 4C,E).

EXPRESSIONS OF VEGF-C IN AAMPHI, VEGFR-3, PROX-1, VEGF-C, AND VEGF-D IN LECS

The cellular and secreted VEGF-C protein by activated or unactivated macrophages was examined by Western blotting and ELISA, respectively. The aaMphi had an increased VEGF-C protein

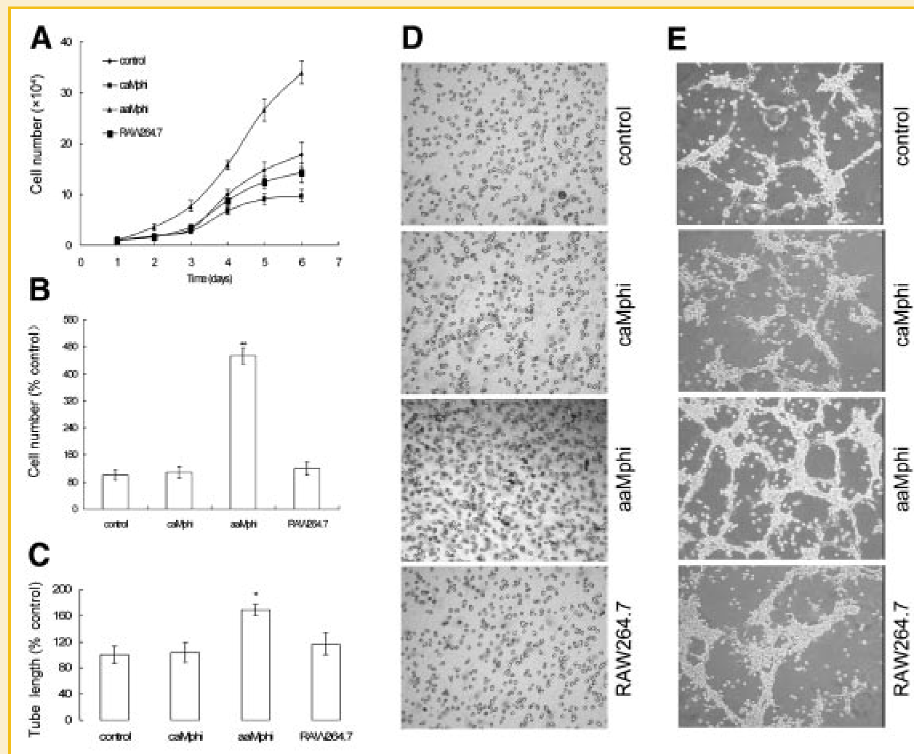

Fig. 4. Effect of activated macrophages on proliferation, migration, and tube-like formation of LECs. A: After 48 h of macrophages-LECs co-culture, the cell number and viability of LECs were determined by the Trypan blue exclusion method. B,D: LECs were incubated in macrophage-conditioned medium using a 24-well transwell chamber, and the invasive LECs on the outside surface of the upper chambers were stained by hematoxylin stain (200×). C,E: LECs were seeded in a 24-well plate pre-coated with Matrigel and formed capillary-like structures in the presence or absence of macrophages (aaMphi, caMphi, or RAW264.7). The network of tube-like structures was determined at 2 days (200×). The results shown are the average of three independent experiments. Data is displayed as the mean ± SD and significance was determined with a $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to control.

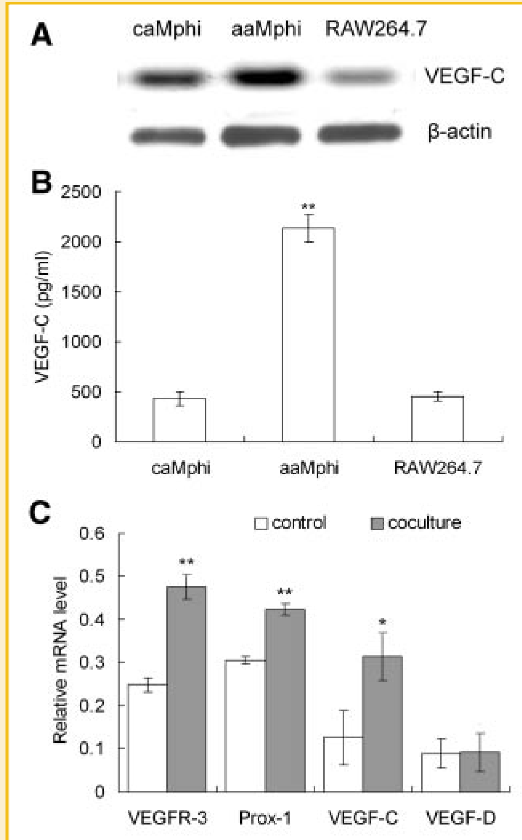

Fig. 5. VEGF-C expression in macrophages and VEGFR-3, Prox-1, VEGF-C, and VEGF-D expressions in LECs. A,B: Detection of cellular and secreted levels of VEGF-C protein from cultured caMphi, aaMphi or RAW264.7 cells by Western blotting and ELISA, respectively. Values are expressed as the mean ± SD. $^{**}P < 0.01$, as compared to RAW264.7. C: The aaMphi were co-cultured with LECs for 48 h, and total RNA was isolated from LECs. The mRNA expression of VEGF-C, VEGF-D, VEGFR-3 and Prox-1 were confirmed by quantitative RT-PCR assay. β-actin was detected as an internal control. The results presented are representative of at least three experiments. Data is displayed as the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$, as compared to control group.

level compared with caMphi or RAW264.7 cells (Fig. 5A). Levels of secreted VEGF-C were also significantly elevated in supernatant for aaMphi (2,135 ± 134 pg/ml), and were higher than those for caMphi and unactivated RAW264.7 cells (Fig. 5B). Furthermore, in the aaMphi-LECs co-culture system, the mRNA expressions of the lymphatic vessel growth factors, VEGF-C and VEGF-D, and their receptor VEGFR-3 and a key homeobox transcription factor Prox-1 in LECs, were determined by quantitative RT-PCR assay. As shown in Figure 5C, co-culture of these two cell lines led to an increased expression of VEGFR-3 and Prox-1 mRNA in LECs. Interestingly, there was also a significant up-regulation of VEGF-C mRNA, but not VEGF-D mRNA in LECs when aaMphi were co-cultured with LECs.

EFFECT OF rVEGFR-3/Fc ON LECS MIGRATION AND CAPILLARY FORMATION

We next analyze whether rVEGFR-3/Fc chimera influences LECs migration and capillary formation when aaMphi were co-cultured

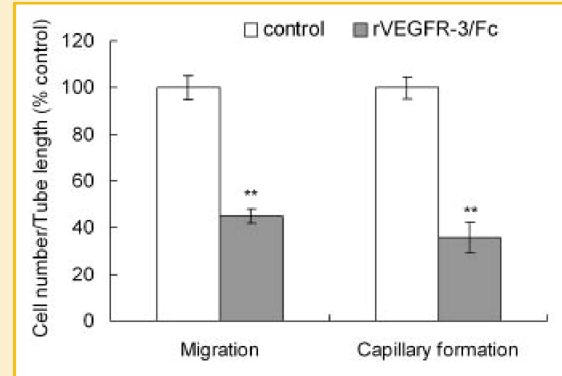

Fig. 6. Effect of rVEGFR-3/Fc on LECs migration and capillary formation. LECs were cultured in aaMphi conditioned medium for 48 h, at presence or absence of rVEGFR-3/Fc chimera (1 μg/ml). The migratory ability of LECs was assessed as described above (left). To determine the effect of rVEGFR-3/Fc on LECs capillary formation, LECs suspensions were seeded in a 48-well plate previously coated with Matrigel, and co-cultured with aaMphi at present or absence of rVEGFR-3/Fc chimera (1 μg/ml) for 48 h (right). Data represent the mean ± SD. $^{**}P < 0.01$ as compared to control.

with LECs. As shown in Figure 6, a statistically significant reduction of LECs migration by 55% was observed in rVEGFR-3/Fc-treated group compared with the control group. Addition of rVEGFR-3/Fc chimer also significantly suppressed the tube-like formation of LECs by 66% as compared with the control group.

DISCUSSION

As a major component of the leukocyte infiltrate of many solid tumors, TAMs are found to play a critical role in carcinogenesis, tumor growth, invasion, and metastasis, and induce tumor angiogenesis and lymphangiogenesis in different types of cancers such as non-small cell lung cancer (NSCLC) [Chen et al., 2005], breast cancer [Vicioso et al., 2006], malignant melanoma [Varney et al., 2005], and colorectal cancer [Bailey et al., 2007]. The effects of TAMs are related to a number of secreted proinflammatory cytokines, growth factors, chemotatic factors, and proteinases. Chen et al. [2005] reported that the density of TAMs did positively correlate with microvessel counts and negatively correlate with patient relapse-free survival in NSCLC specimens. After co-culture with macrophages, lung cancer cells possess higher invasive potentials and matrix-degrading activities through up-regulating IL-6, -8, and MMP-9. In human cervical cancer, TAMs produce large amounts of VEGF-C and VEGF-D, and its density is correlated with peritumoral inflammatory stroma reaction, peritumoral lymphangiogenesis, and lymph node metastasis [Schoppmann et al., 2002]. However, the activated phenotypes of TAMs, and the relationship between the subpopulation of macrophages and tumor-induced lymphangiogenesis are still poor understood.

Recently, Mantovani et al. found that [Mantovani et al., 2002, 2004a,b; Martinez et al., 2008]. TAMs might be polarized towards an aaMphi phenotype under stimulation of Th2 cytokines such as IL-4, -6, and -13, and express higher levels of MMR and IL-10 but produce

fewer NO and IL-12. Activated TAMs can inhibit T cells proliferation, and participate in circuits that regulate cell growth, adaptive immunity, angiogenesis, and stroma formation. In ovarian tumors, a large proportion of CD68 positive macrophages in cancer tissues and ascites were also positive for MMR expression and scavenger receptors, which indicate that aaMphi occur in tumors [Hagemann et al., 2006]. In consistent with these results, TAMs in lung adenocarcinoma is identified to have an activated phenotype of aaMphi (data not shown).

In order to investigate the role of aaMphi in lung adenocarcinoma growth and tumor-induced lymphangiogenesis, we treated RAW264.7 macrophages with IL-4 or IFN-γ + LPS, respectively, according to previously described procedures [Edwards et al., 2006]. The resulted cells were identified as aaMphi and caMphi. Using these activated or unactivated macrophages, we established a LLC transplantation model through co-inoculation with macrophages and LLC cells. We found that not only aaMphi but also unstimulated macrophages could enhance tumor growth, and promote lymphangiogenesis and lymph node metastasis in LLC tumors. Several reports have showed that co-inoculation of athymic nude mice with PC-3 prostate cancer cells and IL-4 stimulated U937 promonocytic cells enhanced tumor growth and microvascular density within the tumor bed, which indicates that IL-4 stimulated macrophages have a tumor-promoting phenotype and promote tumor progress and metastasis via up-regulating several cytokines and chemokines (such as IL-8, VEGF-A, CCL2, and CCL5) [Pollard 2004; Craig et al., 2008]. However, no differences between mice inoculated with LLC lines and aaMphi and RAW264.7 macrophages in relation to tumor weight, presence of lymph node metastasis, number of pulmonary metastasis, and LMVD are not clearly understood. Craig et al. [2008] found that the presence of macrophages without IL-4 stimulation also increases tumor microvascular density in vivo and enhances the rate of tumor growth. Recently, Hagemann et al. [2006] reported that ovarian cancer cells can polarize co-cultured macrophages toward a tumor-associated phenotype and results in TAMs formation similar to that found in ovarian tumors. Macrophages respond to microenvironmental signals and represent a spectrum of different phenotypes. Activation by stimulation with IL-4, -13, and -10 drives macrophages toward the alternative phenotype (Martinez et al., 2008). In this article, unactivated RAW264.7 cells but not aaMphi cells in the co-inoculated lung cancer microenvironment could have a potential to switch a phenotype that resembles the alternatively activated state of TAMs. The RAW264.7 macrophages might be polarized towards an aaMphi phenotype under stimulation of Th2 cytokines including IL-4, -6, and -13. We believe that this switch involved a dynamic "chemical conversation" between the tumor cells and macrophages.

Similar to angiogenesis, lymphangiogenesis consists of a complex multi-step process involving endothelial proliferation, migration, and tube-like formation, which may be triggered by lymphangiogenic factors such as VEGF-C and VEGF-D through binding to their receptors. Studies show that macrophages support lymphangiogenesis in two different ways, either by transdifferentiation and directly incorporating into the endothelial layer or by stimulating division of pre-existent local LECs [Kerjaschki, 2005; Maruyama et al., 2005]. Using macrophages-LECs co-culture, we found that aaMphi, but not caMphi and unactivated RAW 264.7 macrophages, increased the ability of LECs to proliferate, migrate, and form tube-like structures. Significant amounts of VEGF-C protein were also detected in the supernatant of aaMphi, but not in the supernatant of caMphi and RAW264.7 supernatant. These results seem to be not consistent with in vivo findings where in a co-inoculation model there was not difference in the lymph node metastasis and lung metastatic nodes between in mice inoculated with aaMphi and RAW264.7 cells. Some reports have demonstrated that proinflammatory mediators from tumor microenvironment promote the expression and secretion of VEGF-C and expression of its receptor VEGFR-3 via various autocrine and paracrine regulation mechanisms [Tang et al., 2005; Soumaoro et al., 2006]. In the other hand, the close correlation of inflammation, angiogenesis, lymphangiogenesis, and cancer progression and metastasis is now highlighted. Inflammatory reaction and macrophages infiltration in tumors can activate the angiogenic and lymphangiogenic switch [Schoppmann et al., 2006; Watari et al., 2008]. Integrin $\alpha 1\beta 4$ has been found to express highly on tumor lymphatic endothelium and proved to correlate positively to lymphangiogenesis and tumor metastasis [Avraamides et al., 2008]. Based on these reports, it is reasonable to speculate that regional inflammation is present in implanted tumors, and additional pathway regulates lymphangiogenic factors including endogenous VEGF-A, VEGF-C, VEGF-D, and their receptors or other lymphangiogenesis-related factors in tumor microenvironment. Further studies to clarify the effect of lung cancer cells, macrophages, fibroblasts, and stromal cells in tumor-induced lymphangiogenesis will be undertaken.

Furthermore, increased levels of VEGFR-3 and Prox-1 mRNA in LECs were examined when aaMphi were co-cultured with LECs. Enhanced migration and capillary formation was significantly suppressed by rVEGFR-3/c chimeric protein that functions as a competitor by binding to its receptor. Therefore, we propose that secreted VEGF-C by aaMphi exerts an important role in LECs migration and tube-like formation occurring as part of tumor-associated lymphangiogenesis. In addition, unique molecular markers specific for LECs, including VEGFR-3 and Prox-1, is also up-regulated in LECs, suggesting the presence of other possible mechanism by which co-culture of aaMphi changes the lymphangiogenesis-related properties. Interestingly, VEGF-C mRNA levels, but not VEGF-D levels are much higher than in non-co-cultured LECs. We propose that induction of VEGF-C expression in co-cultured LECs is modulated by proinflammatory cytokines such as IL-1 and TNF-α derived from macrophages because these cytokines are significantly increased after co-culture with lung cancer cells and macrophages [Yao et al., 2005], and are found to stimulate VEGF-C production by transcriptional activation in endothelial cells [Ristimäki et al., 1998]. On the other hand, VEGF-C may promote local tumor growth through paracrine signaling to stromal cells expressing VEGFR-3 and support the entry of cancer cells into functional peritumoral lymphatics [Schneider et al., 2006; Schoppmann et al., 2006]. Therefore, although we would rather focus on the role of VEGF-C derived from aaMphi in this study, the further direction need to approach from paracrine and autocrine interaction between VEGF-C and its receptor in tumor [Ochi et al., 2007].

CONCLUSION

A great large number of TAMs in tumor microenvironment play a crucial role in tumor genesis, growth, invasion, and metastasis. These TAMs possess an alternatively activated phenotype in tumors. Macrophages are forced to change their phenotypes available for tumor development to escape from the killing of macrophages. A subgroup of aaMphi is an ''accomplice'' in tumor progression by the means of producing inflammatory factors, chemotatic factors, angiogenic and lymphangiogenic factors, which implies that aaMphi has an important potential to become a new target for tumor therapy [Colombo and Mantovani, 2005; Luo et al., 2006]. Recently, a combination of CpG oligonucleotides and anti-IL-10R is sufficient to convert TAMs phenotype from aaMphi to caMphi, and tumors can be regressed in vivo by intratumoral injection of TAMs-activating chemokines such as CCL16 or CCL20, followed by treatment with CpG and anti-IL-10R [Guiducci et al., 2005]. In addition, Zoledronic acid, an aminobisphosphonate clinically approved for treatment of symptomatic skeletal events, Abrus agglutinin, a plant lectin, and autologous Hsp70, has been shown to shift the balance of TAMs from a tumor promoting to a tumor-eliminating phenotype [Ghosh and Maiti, 2007; Kumar et al., 2007]. Altering macrophages phenotypes and functions via administration of biological response modifiers may have a therapeutic benefit for chronic diseases involving lymphatic vessel abnormal development or disorder-induced lymphoedema and excessive pathological lymphangiogenesis in inflammation and cancer.

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