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Bevacizumab potentiates chemotherapeutic effect on T-leukemia/lymphoma cells by direct action on tumor endothelial cells

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ABSTRACT

Vascular endothelial growth factor-A, an angiogenesis stimulator expressed on both tumor endothelial and malignant T cells, is involved in tumor progression in T-leukemia/lymphoma. Here, we assessed the impact of therapeutic vascular endothelial growth factor-A blockade on tumor-endothelial cell interaction and on tumor progression. In a murine xenograft T-leukemia/lymphoma model, combined bevacizumab (monoclonal antibody against vascular endothelial growth factor-A) with doxorubicin, compared with doxorubicin alone, significantly delayed tumor growth and induced prevalence of tumor cell apoptosis over mitosis. More importantly, the combined treatment induced endothelial cell swelling, microvessel occlusions, and tumor necrosis. *In vitro*, co-culture of endothelial cells with T-leukemia/lymphoma cells showed that doxorubicin induced expression of intracellular cell adhesion molecule-1, provided endothelial and malignant T cells were in direct contact. This was abrogated by bevacizumab treatment

with doxorubicin. Taken together, bevacizumab enhances the chemotherapeutic effect on T-leukemia/lymphoma cells. Directly targeting tumor endothelial cells might be a promising therapeutic strategy to counteract tumor progression in T-cell malignancies.

Key words: T-cell leukemia/lymphoma, bevacizumab, and endothelial cells.

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Introduction

Over the past decade, biologically targeted therapies have significantly improved the outcome of patients with hematologic malignancies. Rituximab (Rituxan; IDEC-C2B8), a recombinant humanized monoclonal antibody against CD20, successfully treats B-cell lymphoma and has recently been applied in CD20-positive pre-B leukemia.^{1,2} For T-cell leukemia/lymphoma, which are relatively resistant to conventional chemotherapy and have a poor prognosis,³ bio-therapeutic targets are being investigated.

Angiogenesis is linked to clinically aggressive tumor subtypes and advanced-stage disease.⁴ Vascular endothelial growth factor-A (VEGF-A) is a main stimulator of tumor-initiated angiogenesis.⁵ Increased VEGF-A levels in blood and tissue are associated with adverse prognosis in leukemia and lymphoma patients.^{6,7} In angioimmunoblastic T-cell lymphoma (AITL), we have shown that VEGF-A was over-expressed on endothelial cells, and that this overexpression

was related to tumor infiltration and poor survival. This demonstrated that endothelial cells, like lymphoma cells, contribute to tumor progression in AITL.⁸

Tumor progression is a complex process that requires tumor-endothelial cell interaction.⁹ Cell adhesion molecules, such as intracellular cell adhesion molecule-1 (ICAM-1) and its ligand leukocyte function-associated antigen-1 (LFA-1), are major mediators of tumor-endothelial crosstalk.¹⁰ ICAM-1 is over-expressed on lymphoma cells,¹¹ and its blockade significantly inhibits lymphoma cell aggregation *in vitro* and lymphoma growth *in vivo*.^{11,12}

Bevacizumab (Avastin™), a recombinant humanized monoclonal antibody directed against VEGF-A, can reduce tumor angiogenesis and inhibit tumor growth, either alone or in combination with chemotherapy.⁵ It has recently been introduced in hematology and its therapeutic effect has been demonstrated in acute myeloid leukemia and AITL.^{13,14} Here we studied *in vivo* and *in vitro* the modalities of its therapeutic efficiency on T-leukemia/lymphoma.

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Design and Methods

Cells and reagents

T-leukemia/lymphoma cell lines Jurkat, HUT78, and endothelial cell line EAhy.926 (American Tissue Culture Collection, Bethesda, MD, USA) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Bevacizumab was obtained commercially (Hoffmann-la Roche, Basel, Switzerland).

Murine model

Four-week old nude mice (Shanghai Laboratory Animal Center, China), maintained in pathogen-free conditions, were subcutaneously injected with 4×10⁷ Jurkat cells (Day 0). Treatments were performed on Days 21, 24, 28 and 31 in 3 groups of 10 mice. The untreated group only received RPMI-1640, the doxorubicin group received intraperitoneal injection of doxorubicin (6mg/kg), and the combined treatment group received intraperitoneal injection of doxorubicin (6mg/kg) and bevacizumab (10mg/kg). All animal procedures were approved by the Investigational Review Board of Shanghai Jiao Tong University School of Medicine. Tumor volume was calculated by the formula: 0.5×a×b² in centimeters, where 'a' is the length and 'b' is the width.

For each group, mice were sacrificed at two time points: Day 27 and Day 35. Subcutaneous tumors were dissected and cut into three parts. These were: (i) immediately snap frozen; (ii) formaldehyde-fixed and paraffin-embedded; (iii) glutaraldehyde-fixed and epoxy-resin-embedded for ultrastructural study.

Pathological analysis

Cell mitosis and apoptosis

Pathological study focused on cell proliferation and cell death on both tumor and endothelial cells. Mitotic and apoptotic cells were identified by electron microscopy (Hitachi-7560, Tokyo, Japan), or using Ki67 antibody (MIB-1, Abcam, Cambridge, UK) and TUNEL assay³ on tumor sections. Cells were counted on 5 consecutive microscopic fields by two pathologists who had not been informed of treatment modalities of the mice. A ProvisAX70 microscope (Olympus, Tokyo, Japan), with wide-field eyepiece number 26.5 was used. At x400 magnification, this wide-field eyepiece provided a field size of 0.344mm². Results were expressed as the mean number of cells/field at x400 magnification.

Tumor microvessel

Tumor microvessels were analyzed ultrastructurally, focusing on tumor-endothelial cell relationship. Microvessel density was assessed on 5 microscopic fields at x400 magnification on tumor sections immunostained with rat anti-mouse CD31 antibody (MEC13.3, BDpharmingen, Franklin Lakes, NJ, USA).

Tumor necrosis

Necrosis area was assessed on hematoxylin and eosin stained tumor sections. Whole slides were scanned and analyzed with a virtual slide system (Olympus). The ratio between tumor necrosis area and whole tumor area gave the relative necrosis area

ICAM-1 expression

ICAM-1 expression of human tumor cells and mouse endothelial cells was detected by immunostaining with goat anti-human ICAM-1 antibody (polyclonal, R&D Systems, Minneapolis, MN, USA) and goat anti-mouse ICAM-1 antibody (polyclonal, R&D Systems) on 5-μm frozen sections, respectively.

Enzyme-linked immunosorbent assay (ELISA)

5×10⁵ endothelial cells were cultured in 6-well plates and grown

in confluence; 1×10⁶ T-leukemia/lymphoma cells were added after 24 h. The T-leukemia/lymphoma and endothelial cells were: (i) cultured in the same vial; (ii) in Millicell Hanging Cell Culture System (Millipore Corporation, Billerica, MA, USA) with 1-μm pore filter only allowing fluid exchange. Cell co-cultures were treated with doxorubicin (200 ng/mL), or doxorubicin (200 ng/mL) combined with bevacizumab (100 μg/mL), or untreated. ELISA was performed in triplicate using ICAM-1 Kit (R&D Systems) on 100 μL supernatant, according to the manufacturer's instructions.

Flow cytometry

5×10⁵ endothelial cells and 1×10⁶ T-leukemia/lymphoma cells were co-cultured and treated as described above. Cells were dissociated with EDTA, incubated with Alexa 488-conjugated mouse anti-human ICAM-1 (84H10, Chemicon, Temecula, CA, USA) and allophycocyanin-conjugated mouse anti-human CD3 antibodies (BW264/56, Miltenyi Biotec, Auburn, CA, USA). Flow cytometry was used to measure fluorescent intensity (BD, Franklin, NJ, USA).

Confocal microscopy

5×10⁵ endothelial cells and 1×10⁶ T-leukemia/lymphoma cells co-cultured in the same vials were immunolabeled using mouse anti-human ICAM-1 (84H10, Chemicon) and rabbit anti-human LFA-1 (polyclonal, Abcam, Cambridge, UK) as primary antibodies, and FITC-conjugated anti-mouse IgG or TexasRed-conjugated anti-rabbit IgG as secondary antibodies. Nuclei were counterstained with DAPI. Z-stacked images were captured by confocal microscope (Zeiss-LSM-5, Welwyn Garden City, UK) and analyzed by IMARIS software (Bitplane, Zurich, Switzerland).

Cell adherence assay

Co-cultured endothelial cells and T-leukemia/lymphoma cells were treated as described above. Each well was then filled with culture medium, covered with parafilm, and inverted for 30 min to remove unattached cells. Remaining adherent lymphoma cells were counted on five microscopic fields per well, and observed on inverted phase-contrast microscope (Nikon-TE-300, Japan). All assays were performed in triplicate.

Statistical analyses

Results were expressed as the mean±SD. Statistical significance of differences between treatment groups was measured by two-tailed t-test. *P*<0.05 was considered to be significant. All statistical analyses were performed on SAS8.2 (SAS-Institute-Inc., Cary, NC, USA).

Results

Bevacizumab targeted tumor cells and inhibited tumor growth

To assess the therapeutic efficiency of bevacizumab *in vivo*, a xenograft murine model was established. Jurkat and HUT-78 cells were both tested; only Jurkat cells developed a tumor at the site of subcutaneous injections. Compared with doxorubicin alone, combined bevacizumab and doxorubicin significantly reduced tumor size, at Day 27 and Day 35 (*P*=0.0446 and *P*=0.0030, respectively, Figure 1A).

To find how bevacizumab enhanced doxorubicin effect on T-leukemia/lymphoma cells, mitotic and apoptotic cells were assessed. In the untreated group, mitotic and apoptotic cells were respectively 35.3±5.1 and 5.2±1.6 at Day 27, with few changes at Day 35 (mitotic cells: 31.9±6.8 and apoptotic cells: 6.4±2.7). With doxorubicin,

mitotic cell numbers were significantly reduced (Day 27: 22.5 ± 4.6 and Day 35: 20.40 ± 4.13 , $P < 0.0001$), and apoptotic cell numbers significantly increased (Day 27: 14.2 ± 2.3 and Day 35: 21.4 ± 5.2 , $P < 0.0001$). Compared to doxorubicin alone, combined bevacizumab with doxorubicin induced two important changes at Day 27: (i) a significant increase in apoptotic cells (21.9 ± 3.9 , $P < 0.0001$); (ii) a prevalence of apoptosis over mitosis. This was linked to a significant increase of apoptosis, since mitosis cells were similar between the two groups (Figure 1B).

Bevacizumab targeted endothelial cells and induced tumor necrosis

Compared with the untreated group, the microvessel density remained unchanged in the doxorubicin-treated group, but was significantly reduced, at Day 27 and Day 35, when bevacizumab was associated with doxorubicin ($P = 0.0218$ and $P = 0.0004$, respectively, Figure 2A).

Ultrastructural study at Day 27 showed that endothelial cells were flat with normal nuclei in the untreated group and in the doxorubicin-treated group. A striking feature in the combined treatment group was the swelling of endothelial cells in all microvessels studied. This reduced the lumen area of microvessels. Complete occlusion of some microvessels was observed with fibrin clot alone or

fibrin plus tumor cells, some of them apoptotic. At Day 35, endothelial cells remained swelling, and relative tumor necrosis area was significantly increased in the combined treatment group swollen, when compared with the doxorubicin group ($P = 0.0103$, Figure 2B and C).

Bevacizumab interrupted tumor-endothelial cell interaction through ICAM-1 downregulation

Immunostainings of xenograft tumor sections showed that the numbers of human tumor cells and of murine microvessels expressing ICAM-1 were significantly increased with doxorubicin, compared with the untreated group. Combined bevacizumab with doxorubicin consequently reduced these numbers (Online Supplementary Figure S1A).

Since such an effect of bevacizumab had not been previously reported, we further analyzed ICAM-1 expression *in vitro*. When Jurkat and HUT-78 cells were co-cultured with endothelial cells in the same vial, doxorubicin treatment induced a significant ICAM-1 overexpression, which was inhibited by addition of bevacizumab. This effect is absent when using a 1- μm pore filter allowing cytokine exchange, but not cytoplasmic contact, to separate tumor and endothelial cells (Online Supplementary Figure S1B, left panel). Therefore, ICAM-1 reduction by

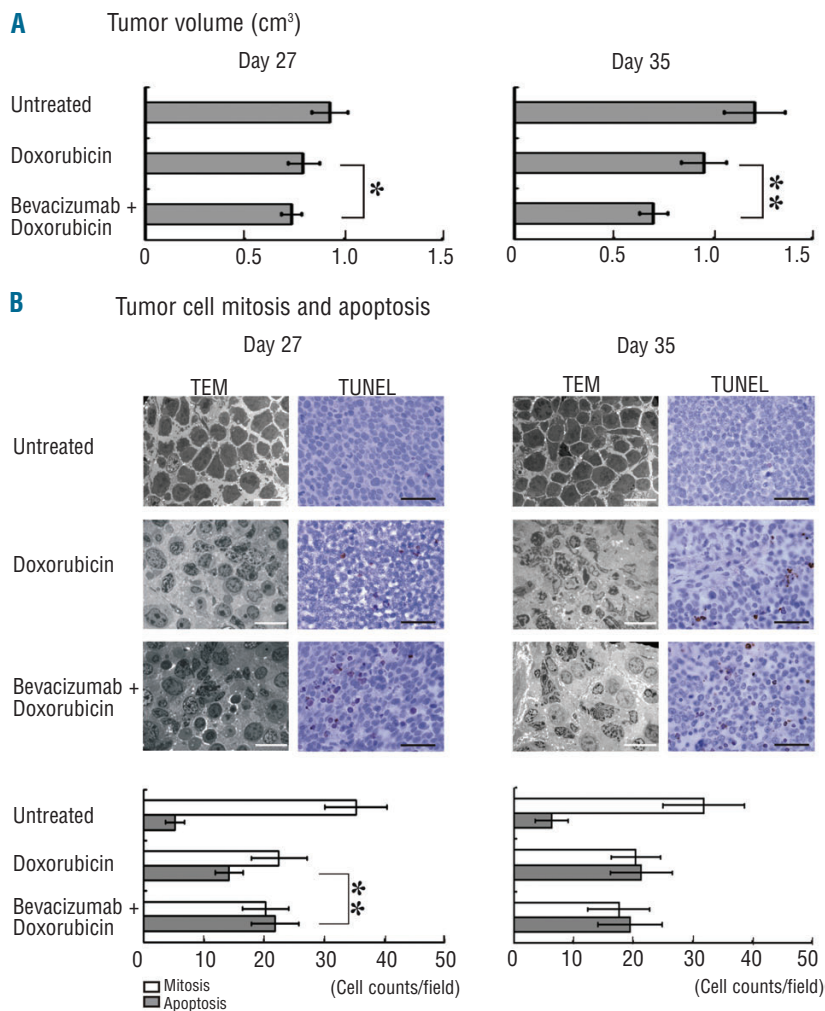


Figure 1. Bevacizumab targeted tumor cells and inhibited tumor growth. (A) Combined bevacizumab and doxorubicin significantly reduced tumor size at Day 27 and Day 35, compared with doxorubicin alone. Untreated mice only received intraperitoneal injection of RPMI1640. * $P < 0.05$, ** $P < 0.01$. (B) Transmission electron microscopy (TEM, Hitachi H7650, bar=10 μm) and terminal deoxytransferase-catalyzed DNA nick-end labeling (TUNEL, bar=50 μm) analyses showed that combined bevacizumab with doxorubicin induced a prevalence of apoptosis over mitosis in tumor cells as early as Day 27. □ Mitosis, ■ Apoptosis, ** $P < 0.01$.

bevacizumab was only observed when direct cytoplasmic contact occurred.

Moreover, flow cytometry analysis was carried out to detect the cell surface ICAM-1 of T-leukemia/lymphoma cells (CD3⁺) and endothelial cells (CD3⁻). Compared with the untreated group, membrane ICAM-1 of endothelial cells significantly increased after doxorubicin treatment ($P < 0.05$), which was also abrogated by addition of bevacizumab. The ICAM-1 of T-leukemia/lymphoma cells had a similar trend (*Online Supplementary Figure S1B, right panel*).

In adhesion assays, combined bevacizumab with doxorubicin significantly reduced T-leukemia/lymphoma cell adhesion to the endothelial monolayer when compared to doxorubicin alone (*Online Supplementary Figure S1C*).

On a confocal microscope, untreated cells were individualized, expressing LFA-1 but not ICAM-1. Doxorubicin induced: (i) close association of endothelial with tumor cells; (ii) strong ICAM-1 expression on endothelial cells. In particular, ICAM-1 and LFA-1 were co-expressed in areas where endothelial and tumor cells were in close contact. Addition of bevacizumab abrogated ICAM-1 expression by endothelial cells, and their close association with tumor cells (*Online Supplementary Figure S1D*).

Discussion

Addition of bevacizumab to doxorubicin, in our xenograft murine model, significantly delayed tumor growth. First, bevacizumab could induce lymphoma cell apoptosis, as observed in another xenograft solid tumor model.¹⁵ More importantly, it has a direct effect on tumor vessels. Systematic ultrastructural analysis of tumor sections revealed that bevacizumab induced endothelial cell swelling. This striking morphological change diminished the area of microvessel lumen and could explain the decreased tumor blood flow observed with computed tomography perfusion scan in the bevacizumab-treated tumor.¹⁶ In our model, bevacizumab and doxorubicin also induced microvessel occlusions, contributed to reduced microvessel density, and increased tumor necrosis area. This agrees with clinical data showing that: (i) anti-angiogenesis reduces the risk of chemotherapy resistance by targeting endothelial cells rather than tumor cells;¹⁷ (ii) combining bevacizumab with chemotherapy results in greater anti-tumor effects than either treatment alone.^{17,18}

Tumor invasion requires interaction between invading tumor cells and tumor microvessels.¹⁹ ICAM-1 plays an important role in cell adhesion.¹⁰ In our model, doxoru-

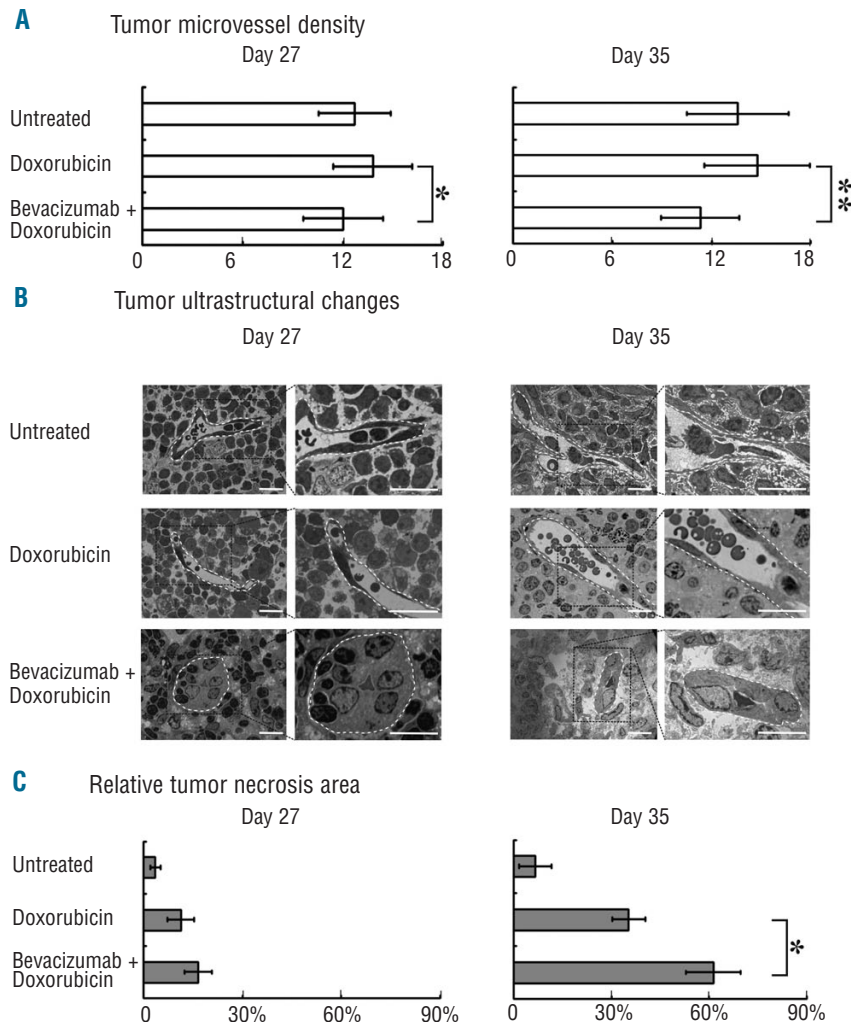


Figure 2. Bevacizumab targeted endothelial cells and induced tumor necrosis. (A) Combined bevacizumab with doxorubicin significantly reduced tumor microvessel density at Day 27 and Day 35. * $P < 0.05$, ** $P < 0.01$. (B) Ultrastructural analysis showed at low magnification, numerous apoptotic cells in combined treatment group at Day 27 and in doxorubicin group at Day 35. At high magnification, microvessels in untreated mice had flat endothelium and circulating intact tumor cells in their lumen. Microvessels in the doxorubicin group had flat endothelial cells and apoptotic bodies in their lumen at Day 35. With the combined treatment, endothelial cells were swollen with voluminous nucleus and cytoplasm, reducing the lumen. Complete occlusion of some microvessel lumen was observed with fibrin clot alone or fibrin plus tumor cells. Microvessels were indicated by white broken lines, bar=20 μm. (C) Compared with doxorubicin alone, combined bevacizumab with doxorubicin significantly increased the relative tumor necrosis area at Day 35. * $P < 0.05$.

bicin significantly enhanced ICAM-1 expression on both human tumor cells and murine endothelial cells. In tumor-endothelial cell co-cultures, we showed that doxorubicin significantly enhanced ICAM-1 expression only when direct cytoplasmic contact occurred, mimicking the situation *in vivo*. The need for cytoplasmic contact between tumor and endothelial cells for upregulation of ICAM-1 expression has also been demonstrated in breast cancer cell lines.²⁰ Using flow cytometry and confocal analyses, we further confirmed that doxorubicin favored: (i) adhesion of multiple T-lymphoma/leukemia cells to endothelial cells; (ii) ICAM-1 upregulation in endothelial cells; (iii) concomitant expression of LFA-1 and ICAM-1 in the adhesive tumor and endothelial cells, respectively. The fact that the addition of bevacizumab, an anti-VEGF antibody, abrogated this ICAM-1 expression underlines the role of VEGF in doxorubicin-induced ICAM-1 expression in T-leukemia/lymphoma. The inter-relationship between doxorubicin and VEGF has also been shown in breast, lung, and melanoma cancer, the addition of doxorubicin increasing VEGF expression.²¹ As far as we know, our study is the first to show that anti-VEGF therapy could inhibit doxorubicin-induced ICAM-1 expression, leading to interruption of the ICAM-LFA-1 ligation and a decrease in T-leukemia/lymphoma-endothelial adhesion. Very

recently, bevacizumab has been reported to block lymphocyte adhesion to the endothelium.²²

The ICAM-1/LFA-1 pathway also plays a key role in trans-endothelial migration and extravasation.¹⁰ Interrupting ICAM-1-LFA-1 ligation inhibits melanoma cell extravasation and metastases in a xenografted murine model.²³ By modulating tumor-endothelial interaction through the ICAM-1/LFA-1 pathway, bevacizumab could possibly interfere with the metastatic potential of T-leukemia/lymphoma.

Taken together, bevacizumab enhanced the chemotherapeutic effect in T-leukemia/lymphoma. Targeting tumor endothelial cells might be a promising therapeutic strategy to counteract tumor progression in T-cell malignancies.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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