Anti-angiogenic Properties of Bevacizumab Improve Respiratory System Inflammation in Ovalbumin-Induced Rat Model of Asthma

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INTRODUCTION

Respiratory disease, which is commonly associated with lung disease, includes a group of diseases that interferes with the function of the lungs by involving different parts of the respiratory tract. Respiratory disease can
It efficiently diminishes bronchial inflammation by reducing the rate of tumor growth, thereby improving quality of life and survival for patients suffering from cancer [15]. Bevacizumab binds to VEGF ligands and decreases tumor angiogenesis; it can be a cost-effective replacement [13, 14]. Bevacizumab binds to ranibizumab, but its economic usefulness remains to be an intriguing possibility [16].

**MATERIALS AND METHODS**

**Preparation of Animal Model and Study Groups**

Twenty-one male Wistar albino rats, 8 weeks old, weighing between 250 and 300 g, were purchased from Pasteur Institute, Tehran, Iran. The animals were kept under controlled conditions at 22 °C, with 45% humidity, 12-h light cycles, and free access to food, water, and libitum. The study procedures were performed according to the Ethical Committee of Azad University. The ethical code assigned for this study was IR.IAU.K.REC.1398.088. Ovalbumin grade V (OVA), aluminum hydroxide, and other compounds were purchased from Sigma Chemicals, St Louis, USA. All
animals were randomly divided into three groups (n = 7 in each group), including (1) control group, (2) OVA-treated group, and (3) OVA + Bmab (bevacizumab drug). Sensitization and challenge instructions relevant to working with rats were performed as previously described [31] (Fig. 1). Aluminum hydroxide solution (Aldrich, Milwaukee, WI) was diluted in saline buffer and incubated with OVA (grade V; Sigma, St. Louis, MO) overnight. On days 1 and 8, 1 mg of OVA and 200 mg of aluminum hydroxide (Aldrich, Milwaukee, WI) in 0.5 mL sterile phosphate-buffered saline (PBS) were intraperitoneally injected into rats in groups 2 and 3. The control group was only subject to intraperitoneal injection of saline on days 1 and 8. One week after the last injection, the rats (groups 2 and 3) were exposed to OVA inhalation for 30 min at 2-day intervals from days 15 to 25.

**Drug Treatment**

After preparation of asthma rat models through OVA exposure protocols, the OVA + Bmab group (group 3) were treated with 2.5 mg/kg bevacizumab drug (Avastin, Roche 25 mg/mL, Basel, Switzerland) diluted in 9% NaCl solution to a final concentration of 5 mg/mL [32]. Two days after the end of the study, the rats were euthanized with ketamine/xylazine and blood samples were collected, and their lung tissues were isolated for further examinations.

**Gene Expression Evaluation of IL-1β and TNF-α**

The quantitative expression of IL-1β and TNF-α genes was evaluated in lung tissues obtained from all three study groups of rats by Real-Time PCR System (Applied Biosystems, Warrington, UK). To this end, total RNA from lung tissues was extracted by TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s protocols, and subject to RNase-free DNase treatment. Then, RNA samples were transcribed by using a reverse transcriptase enzyme, and cDNAs were applied to qPCR. The primers designed for detecting IL-1β and TNF-α gene expression were represented in Table 1. The real-time PCR program employed in all processes was defined as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 two-step cycles comprising of 95 °C for 15 s and 60 °C for 60 s. Finally, the relative gene expression was examined through the 2−ΔΔCt method. Each assay was run in triplicate.

**Immunohistochemistry of IL-1B, TNF-α, and VEGF**

The lung tissues obtained from all three study groups of rats were steeped with buffered formalin in PBS, pH 7.2, and then infused in formalin at 4 °C overnight. After the fixation period, the lung tissues were embedded in paraffin. Six-micrometer sections of tissues

**Table 1** The Primers Used to Detect IL-1β and TNF-α Expression

| Gene   | Primer sequence (5′—3′) |
|--------|------------------------|
| IL-1β-F | TGTGACTGTTGGAGTAGATGA  |
| IL-1β-R | GTCTGTCTATGGGAGTAGAGA  |
| TNF-α-F | GAGATGGAATGGCAGAGGA    |
| TNF-α-R | GAGAAGATGAGTGAGTTGAGG   |
| GAP-R  | CATACTCAGCACGATCACC     |
| GAP-F  | AAGTTCAACGGCACAGTC      |

![Fig. 1](image) Effects of Bmab on OVA-induced asthmatic rats. The schematic protocol of the study. Bmab, Bevacizumab; OVA, ovalbumin.
were prepared, deparaffinized, and dehydrated. The samples were incubated in 50% formamide solution and 2× standard sodium citrate buffer at 65 °C for 2 h and then incubated twice in 100 mM of sodium borate (pH = 8.5). The samples were blocked with 0.4% Triton X-100 in PBS and 10% goat serum for 30 min. Next, the sections were incubated with monoclonal primary antibodies in PBS containing 0.05% Tween-20 and 1% BSA at 4 °C overnight. The five used primary antibodies were against IL-1β (rabbit anti-IL-1β; 1:100, Abcam, Cambridge, UK), TNF-α (rabbit anti-TNF-α; 1:100, Abcam, Cambridge, UK), and VEGF (Rabbit anti-VEGF; 1:100, Abcam, Cambridge, UK). Afterward, the samples were washed with PBS and exposed to FITC-conjugated secondary antibodies (goat anti-rabbit; 1:200, Abcam, Cambridge, UK) diluted in the PBS at 37 °C for 90 min in the dark. DAPI was used for nuclear staining. The samples were visualized under a light microscope (Olympus, Japan) at 400× magnification. FITC expression was measured by ImageJ software based on nuclear staining, indicating a positive reaction for the desired markers.

Periodic Acid–Schiff Staining and Goblet Cell Population

To detect the histopathological changes indicating inflammation such as the hyperplasia goblet cells in lung tissues affected by OVA treatment, the PAS staining was used on formalin-fixed tissue sections. In brief, the coverslips containing the hydrated tissue sections were immersed in 0.5% periodic acid solution for 2 min and then rinsed in tap water for 5 min. Next, the coverslips were placed in Schiff reagent for 30 min and again washed with tap water for 15 min. To differentiate the diagnosis, the tissues were stained with Harris hematoxylin solution (Sigma-Aldrich, Germany) for 6 min and washed with tap water for 5 min. Finally, the tissues were dehydrated and mounted using a mounting medium. The samples were examined under a light microscope (Olympus, Japan) at 100× and 400× magnification, and the PAS-positive goblet cells were counted measured by ImageJ software based on nuclear staining.

Differential Cell Counting in BALF

To evaluate the inflammatory cell population, the lung tissues were removed from the sacrificed animals and prepared for differential cell count as described by Yang et al. [33]. Briefly, left lungs were washed thrice with 1 ml saline, and the lavages were pooled (mean volume, 2.0±0.0 ml). The retrieved volume of BAL was centrifuged immediately at 3000 rpm for 10 min at 4 °C. Then, cell pellets were resuspended and washed twice in PBS. The differential cell count was performed with Wright-Giemsa staining by two operators blind to the groups. The results are expressed as the total cell number ×105.

Measurement of Serum OVA-Specific IgE

To analyze the level of OVA-specific IgE in serum, blood samples were collected from rats by cardiac puncture. Whole blood was centrifuged and the serum samples were collected. VA-specific IgE was determined by an enzyme-linked immunosorbent assay (ELISA) kit based on manufacturer protocol (R&D Systems, Inc., Minneapolis, MN, USA). Absorbance was measured at 450 nm using a plate reader (BioTek ELx800, USA).

Statistical Analysis

There were 7 animals (n = 7) per group, and specimens were taken from all 21 animals for each assay. All experiments, including real-time PCR, IHC, PAS, cell counting, and ELISA, were conducted at least 3 times. The analysis of the resulting data was performed using GraphPad Prism version 6.0. The results were represented as the mean ± standard deviation. Statistical differences were analyzed using one-way ANOVA and Tukey post hoc test. The p<0.05 was considered significant.

RESULTS

Goblet Cell Population

Goblet cell population decreased significantly in airways after treatment with bevacizumab compared to the asthma group (p < 0.05). Meanwhile, goblet cell hyperplasia was observed in rats treated with OVA in the asthmatic model (Fig. 2 A and B).

Gene Expression of Cytokines in Lung Tissue

The gene expression of IL-1β and TNF-α cytokines was evaluated in three animal groups including control, OVA − , and OVA + Bmab-treated by real-time PCR.
The results showed that IL-1β and TNF-α expression was increased in treated animals with OVA ($p = 0.004$, $p = 0.0127$; respectively), compared to the control group; while treatment with Bmab (OVA + Bmab) caused a decrease in IL-1β and TNF-α mRNA levels that all were significant in comparison to OVA group ($P < 0.05$) (Fig. 3).

**Protein Expression Level of Cytokines in Lung Tissue**

To detect probable alterations in protein expression of pro-inflammatory factors, including TNF-α and IL-1β in asthma animal models (OVA group) and drug-treated group (OVA + Bmab) compared to the control group, immunohistochemistry assay was applied on lung tissue of all three groups. As shown in Fig. 4, the expression of IL-1β protein increased significantly in the OVA group ($p = 0.02$) compared to the control group. Parallel to IL-1β mRNA level, the Bmab treatment group showed a significant decrease similar to the TNF-α protein level ($p < 0.0001$) (Fig. 5).

**The Expression of VEGF Protein**

To evaluate the expression of VEGF protein in lung tissues and microgravity, IHC was performed. An obvious increase in the expression of VEGF protein in the OVA group was observed compared to the control group (Fig. 6; $p < 0.011$). On the other hand, it was revealed that Bmab in the OVA group (OVA + Bmab group) reduced the expression of VEGF protein ($p = 0.01$) compared to the OVA group (Fig. 6).

![Fig. 2](image-url) Effects of bevacizumab on airway goblet cell hyperplasia by PAS staining of lung sections. A Goblet cells hyperplasia in OVA-induced rat model of asthma, magnification: 400×. B Quantitated PAS-positive cells are illustrated as a graph. Different signs show significant differences between experimental groups. $p < 0.01$ compared with control rats. PAS, periodic acid Schiff; OVA, ovalbumin; Bmab, bevacizumab.
As shown in Fig. 7, Bmab treatment could significantly reduce the number of macrophages, neutrophils, and lymphocytes in lung tissue compared to the asthma group ($p < 0.05$), while no significant difference in the number of eosinophils was observed among the OVA and OVA + Bmab.

**Serum OVA-Specific IgE**

A higher level of OVA-specific IgE was detected in the asthma group compared to the control ($p < 0.05$). However, the results in treatment groups revealed that Bmab significantly suppressed OVA-specific IgE secretion in the treated group ($P < 0.05$) (Fig. 8).

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**Counting of BALF Leukocytes**

As shown in Fig. 7, Bmab treatment could significantly reduce the number of macrophages, neutrophils, and lymphocytes in lung tissue compared to the asthma group ($p < 0.05$), while no significant difference in the number of eosinophils was observed among the OVA and OVA + Bmab.
**DISCUSSION**

In the present study, a new strategy was introduced to treat symptoms of asthma. Bevacizumab-treated animals displayed a decrease in goblet cell population and migration in airways of mice treated with OVA treatment, indicating symptoms of asthma. The asthmatic airway mucosa inflammation is usually associated with enhanced vascular permeability and plasma exudation. Vesicular-vascular organelles which are present largely in venular endothelium provide the major route of extravasation of macromolecules at sites of increased vascular permeability [34–37]. In the airways, the plasma exudation may readily pass through the inflamed mucosa and reach the airway lumen through the leaky epithelium. Leaked plasma proteins induce a thickened, engorged, and edematous airway wall, resulting in airway obstruction. The leaked plasma proteins can also traverse the epithelium and agglomerate in the airway lumen, leading to a reduction of mucus clearance and ciliary dysfunction [38]. Due to the interaction between VEGF and inflammation situation, our results showed that VEGF inhibition by bevacizumab might influence or possibly inhibit the inflammatory activity of inflammation cells in BALF.

Fig. 4 Effects of bevacizumab on OVA-induced lung inflammatory protein of TNF-α. Immunohistochemical staining was performed in rats’ lung sections. Data are expressed as means ± SE. Different signs represent statistically significant differences between the mean values (p<0.02) and similar signs are not significant. OVA, ovalbumin; Bnab, bevacizumab.
The density of leukocytes and especially of macrophages and neutrophils was lower in the OVA-bevacizumab treated group compared to the OVA group. Macrophages, in addition to stimulating VEGF, can induce epithelium of airway lumen proliferation and migration through the release of cytokines, proteases, and growth factors based on the results of Sunderkötter et al. in 1994 [39]. Therefore, experimental studies for potential new anti-angiogenic treatment strategies target the complement system [40], ICAM [41], macrophages, monocytes [42, 43], and TNF [44]. Tsuchihashi S et al. in 2006 investigated that anti-VEGF drugs might be expected to have

Fig. 5 Effects of bevacizumab on OVA-induced lung inflammatory protein of IL-1β. Immunohistochemical staining was performed in rats’ lung sections. Data are expressed as means ± SE. Different signs represent statistically significant differences between the mean values (p <0.0001) and similar signs are not significant. OVA, ovalbumin; Bmab, bevacizumab.
an anti-inflammatory effect [45]. VEGF acts as a survival factor for epithelium growth of airway lumen by inhibiting apoptosis. Alon et al. revealed that deprivation and/or inhibition of VEGF could induce vascular regression through epithelial cell apoptosis in the retina, especially in neovascular vessels [46]. Therefore, the usage of bevacizumab as an anti-angiogenesis drug was employed to prevent angiogenesis and reduce access to inflammatory cells in the lung tissue area. Microvascular leakage is an essential component of the inflammatory responses, and the majority of inflammatory mediators induce this vascular leakage in asthma [47–50]. On the basis of COVID-19-induced pulmonary pathological and vascular changes, it can be hypothesized that the anti-vascular endothelial growth factor (VEGF) drug bevacizumab plus standard care might be highly beneficial for patients with severe COVID-19 [51]. The results of the present study showed that VEGF expression decreased in the group receiving bevacizumab compared to that of the asthma group. VEGF by binding to VEGF triggers a signaling network in endothelial cells which causes angiogenesis [52]. Thus, VEGF is an endothelial marker and its reduction demonstrated that bevacizumab can reduce the rate of angiogenesis in lung tissue. In the

**Fig. 6** Effects of bevacizumab on OVA-induced lung inflammatory protein of VEGF. Immunohistochemical staining was performed in rats’ lung sections. Data are expressed as means±SE. Different signs represent statistically significant differences between the mean values (p<0.01), and similar signs are not significant. OVA, ovalbumin; Bmab, bevacizumab.
case of wound healing, VEGF expression, as one of the vital angiogenic stimulators, is significantly upregulated to accelerate wound healing by regulating and restoring blood flow to the injured area. As wound healing resolves, the expression of VEGF is downregulated, and most angiogenic capillaries regress, resulting in residual normal vascularity [53]. New evidence indicates that a number of endogenous angiogenic inhibitors are
present in the normal retina to balance the stimulatory effect of VEGF in the regulation of angiogenesis and vascular permeability [54]. It is suggested that endogenous angiogenic inhibitors can be utilized to balance the effect of angiogenic stimulators. TNF-α and IL-1β were known as major regulatory cytokines which can initiate the inflammatory. These processes are performed by switching on many downstream genes, inducing expression of signaling molecules and transcription factors, cytokines, chemokines, components of the coagulation system, cell surface receptors and adhesion molecules, cell proliferation-related genes, apoptosis-related genes, and other inflammatory response genes [30, 55, 56]. Our study showed that the expression of TNF-α and IL-1β increased in the induced rat model of asthma, but their expression decreased significantly in the asthmatic animal group receiving bevacizumab compared with the non-treated group; however, their expression is higher than the control group. Hence, bevacizumab can have good effects on inflammatory diseases such as asthma. Some studies have reported the increase of TNF-α and lymphotoxin alpha expression in mouse lungs after M. pulmonic infection [57–60], but in the viewpoint of vessel remodeling in the airways, no report has been published yet. Lung expression of TNF-α increases within a few hours of infection and then decreases to near baseline values [57–59], but TNF-α expression is stable for a period of time in the trachea. This difference may indicate a histological difference in the cell population in the trachea and lung, as well as a complex combination of parenchymal alveoli and intrapulmonary airways. Evaluation of the relationship between TNF-α expression and vascular regeneration or lymphatic angiogenesis in the trachea indicates an increase in TNF-α involvement in vascular regeneration. This therefore suggests that bevacizumab controls asthma by angiogenesis inhibition through the reduction of TNF-α and IL-1β expression. Decreased expression of chemoattractants and adhesive molecules may help reduce the loss of leukocytes and lymph node hypertrophy in infected mice treated with anti-TNF antibodies or TNF-R1 deficiency. Similarly, TNF-α causes the expression of VCAM-1, E-selectin, and ICAM-1 in endothelial cells, which mediates the adhesion and exit of leukocytes [61]. Among the logical approaches for airway diseases such as severe asthma and chronic obstructive pulmonary, blocking TNF-α signaling would seem to be a noticeable therapy [62]. Recent clinical studies of asthma treated with anti-TNF-α have improved lung function and quality of life and reduced intensified airway responses [63–65]. Therefore, this type of therapy may need to be administered over a long period of time. In the normal healthy body, the process of angiogenesis is dormant, and the angiogenesis switch is kept “off” with inhibitors that are dominant over stimulators. Since anti-angiogenesis therapy is a targeted therapy that aims specifically at the angiogenic stimulators and the angiogenic microvascular endothelial cells, anti-angiogenesis therapy usually produces only mild side effects and is less toxic to most healthy cells. Meanwhile, some investigations have shown that intra-vitreal injection of bevacizumab may induce a significant rise in IOP [14]. It is important to mention that since angiogenesis is vital in the wound healing process and reproduction, anti-angiogenic agents in long-term treatments can result in problems in case of bleeding, heart function, blood clotting, the immune system problems, and the reproductive system, which are still unknown and need to be studied [66, 67]

CONCLUSION

Based on the present investigation, angiogenesis plays an important role in the expression and release of inflammatory mediators in lung airways in asthma. Targeting VEGF through the application of bevacizumab would be a great therapeutic option to prevent the complication of asthma.

AUTHOR CONTRIBUTIONS

Seyed Mohammadreza Bolandi, Zohreh Abdolmaleki, and Mohammad-Ali Assarehzadegan searched the literature and designed the study. All the authors performed the experiments, analyzed the data, and wrote the manuscript. Seyed Mohammadreza Bolandi and Zohreh Abdolmaleki cheng revised the manuscript.

Declarations

Ethics Approval The study procedures were performed according to the Ethical Committee of Azad University. The ethical code assigned for this study was IR.IAU.K.REC.1398.088.

Conflict of Interest The authors declare no competing interests.
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