Allergology International. 2005;54:283-288

ORIGINAL ARTICLE

Tulobuterol, a β2-agonist, Attenuates Eosinophil Adhesion to Endothelial Cells

Takefumi Yamaguchi[1], Makoto Nagata[1], Hitoshi Miyazawa[1], Izumi Kikuchi[1], Shinya Kikuchi[1], Koichi Hagiwara[1] and Minoru Kanazawa[1]

ABSTRACT

Background: Transdermal patches containing tulobuterol, slow-releasing β2-agonist patches, are now widely used in the treatment of asthma in Japan. Unlike inhalational β2-agonists, tulobuterol patches act systemically and may modify the functional status of inflammatory cells in the peripheral circulation. The objective of this study is to examine the effect of tulobuterol on the adhesive interaction between blood eosinophils and endothelial cells.

Methods: Peripheral blood eosinophils and human umbilical vein endothelial cells (HUVEC) were pretreated with either tulobuterol or a control medium, and adhesion of eosinophils to HUVEC was examined using an eosinophil peroxidase assay.

Results: Spontaneous adhesion of eosinophils to resting HUVEC was not modified by tulobuterol. On the other hand, eosinophil adhesion to IL-4 + TNFα-stimulated-HUVEC was inhibited minimally but significantly by tulobuterol. Furthermore, both IL-5- and FMLP-activated adhesions of eosinophils to HUVEC were partially but significantly inhibited by tulobuterol.

Conclusions: Tulobuterol can decrease adhesion of blood eosinophils to endothelial cells. This finding suggests that tulobuterol patches have anti-inflammatory properties, and may therefore contribute to the treatment of airway inflammation in asthma.

KEY WORDS

cell adhesion, endothelial cells, eosinophils, tulobuterol, β2-agonist

INTRODUCTION

Eosinophils are the predominant inflammatory cells present in asthmatic airways. An initial step in accumulation of eosinophils in asthmatic airways is adhesion of peripheral blood eosinophils to endothelial cells. This process is known to involve interactions between α-4 or β-2 integrins of eosinophils and VCAM-1 or ICAM-1, which are immunoglobulin superfamily molecules expressed on endothelial cells.[1,2] The authors and Kaiser *et al.* reported that corticosteroids do not inhibit adhesion of eosinophils to endothelial cells.[3,4] On the other hand, the authors reported that theophylline does attenuate the adhesive interaction between eosinophils and endothelial cells.[5]

β2-agonists are another important class of drugs used in the treatment of asthma. The Global Initiative for Asthma has emphasized the significance of long-acting inhalational β2-agonists together with inhalational steroids in the treatment of moderate or severe asthma. In Japan, transdermal patches containing tulobuterol, which have been developed as a slow-releasing β2-agonist patch preparation, are now widely used in treatment of asthma.

Since the active ingredient of tulobuterol patches, unlike long-acting inhalational β2-agonists, is distributed systemically in the bloodstream before exerting its effect, this preparation is expected to affect inflammatory cells in the peripheral circulation and/or vascular endothelial cells. In the present study, we examined the effect of tulobuterol on the adhesive interac-

[1]Department of Respiratory Medicine, Saitama Medical School, Saitama, Japan.
Correspondence: Makoto Nagata M.D., Department of Respiratory Medicine, Saitama Medical School, 38– Morohongo, Moroyama-cho, Iruma-gun, Saitama 350–0495, Japan.
Email: favre4mn@saitama–med.ac.jp
Received 15 January 2004. Accepted for publication 10 September 2004.
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tion of peripheral blood eosinophils with vascular endothelial cells.

METHODS

REAGENTS

Percoll was obtained from Pharmacia (Uppsala, Sweden). Anti-CD16 antibody-coated magnetic beads were purchased from Miltenyl Biotec (Auburn, CA, USA). HuMedia-EG2 was obtained from Kurabo Industries, Ltd. (Osaka, Japan), and Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) were obtained from Invitrogen Corporation (Grand Island, NY, USA) and Biomedicals, Inc. (Aurora, Ohio, USA), respectively. Human umbilical vein endothelial cells (HUVEC) from healthy humans were obtained from Kurabo Industries, Ltd. (Osaka, Japan). Tulobuterol was provided by Abbott Japan Co., Ltd. Recombinant human interleukin (IL-) 4, IL-5 and TNF-α were purchased from R & D Systems Inc. (Minneapolis, MN, USA). All other reagents including formyl-methionyl-leucyl-phenylalanine (FMLP) were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

EOSINOPHIL SEPARATION

Eosinophils were isolated from peripheral blood specimens of normal volunteers with less than 5% eosinophils in the total leukocyte count. Subjects ranged in age from 20 to 37 years, with an even gender. Eosinophil isolation was performed using negative immunomagnetic bead selection as previously described.[6] Briefly, heparinized blood was diluted with HBSS without Ca^{2+} and centrifuged for 20 min at 700xg over 1.090 g/ml Percoll. Plasma, the mononuclear cell band, and Percoll were removed, and the red blood cells in the pellet were lysed by hypotonic shock. The resulting granulocytes were washed with 4℃ HBSS supplemented with 2% newborn calf serum NCS (HBSS/NCS), and then incubated with anti-CD16 antibody-coated magnetic beads for 40 min at 4℃. The cells were filtered through a steel wool column in a magnetic field (Miltenyl Biotec) to remove neutrophils bound to magnetic beads. CD16-negative eosinophils (>98% purity and >99% viability) were collected, washed and resuspended in HBSS supplemented with 5% fetal calf serum (FCS) (HBSS/FCS).

PREPARATION OF HUVEC

HUVEC (purchased from Clonetics, San Diego, CA, USA) were prepared as previously described.[7] Briefly, HUVEC were cultured on type IV collagen-coated tissue culture flasks until confluent, transferred to collagen-coated 96-well-tissue culture plates, and cultured for 24 h in 5% CO_2 at 37℃. After culture, the incubated mixture was decanted and HUVEC were washed three times with HBSS before use.

EOSINOPHIL ADHESION TO HUVEC

Eosinophil suspensions and monolayers of HUVEC were pre-cultured with either tulobuterol (1−100nM) or medium control for one hour and 24 hours, respectively. For the IL-4 + TNF-α−stimulated HUVEC, pretreatment was performed 1 hour prior to cytokine treatment. Eosinophil adhesion to HUVEC was assessed as residual eosinophil peroxidase (EPO) activity of adherent eosinophils as previously described.[7,8] Briefly, eosinophils (100 µl of 1×10^5 cells/ml in HBSS/FCS) were then placed onto HUVEC monolayers and incubated at 37℃ for 30 min in the presence or absence of an activator. After five washes with 37℃ HBSS, 100 µl HBSS/FCS was added to the reaction wells. As standards, 100 µl of serially diluted cell suspensions (1×10^3, 3×10^3, 1×10^4, 3×10^4, and 1×10^5 cells/ml) were added to empty wells. EPO substrate (1 mM o-phenylenediamine, 1 mM H_2O_2, and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to all of the wells. After a 30-min incubation interval at room temperature, 50 µl of 4 M H_2SO_4 was added to stop the reaction, and absorbance was measured at 490 nm. Percent eosinophil adhesion was calculated from the log dose-response curve.

DETERMINATION OF EXPRESSION OF ADHESION MOLECULES ON EOSINOPHILS

The expressions of adhesion molecules on eosinophils was examined by flow-cytometric analysis as previously described.[9] EOS were exposed to either 0.1 µM tulobuterol or buffer (HBSS/5%FCS) alone for 15 min at 37℃, and then stimulated with either 100 pM IL-5 or buffer for 15 min. The cells were washed three times and resuspended in 4℃ PBS supplemented with 2% BSA and 0.2% sodium azide (FACS buffer). Cells (1×10^5/100 µl) were incubated with FITC-conjugated mouse anti-human CD18 (clone : MHM23, IgG1, Dako A/S, Glostup, Denmark), FITC-conjugated mouse anti-human CD11a (clone : MHM 24, IgG1, Dako A/S), phycoerythrin (PE)-conjugated mouse anti-human C3bi (CD11b) (clone : 2LPM19c, IgG1, Dako A/S), PE-conjugated mouse anti-human VLA-4 (CD49d) (clone : 9F10, IgG1, BD PharMingen, San Diego, CA, USA), or PE-conjugated mouse anti-human CD29 (clone : MAR4, IgG1, BD PharMingen) on ice for 30 min. Similarly PE- and FITC- conjugated mouse IgG1 (Dako A/S) were used as isotype matched controls. Cells were then washed and resuspended in FACS buffer. Mean fluorescence was measured on at least 10,000 events using FACScan (Becton Dickinson). Relative mean fluorescence was determined by subtraction of values for the IgG1 isotype-matched control.

DETERMINATION OF VCAM-1 AND ICAM-1 EXPRESSION ON HUVEC

The expression of VCAM-1 and ICAM-1 was determined by a cell-ELISA developed as previously re-

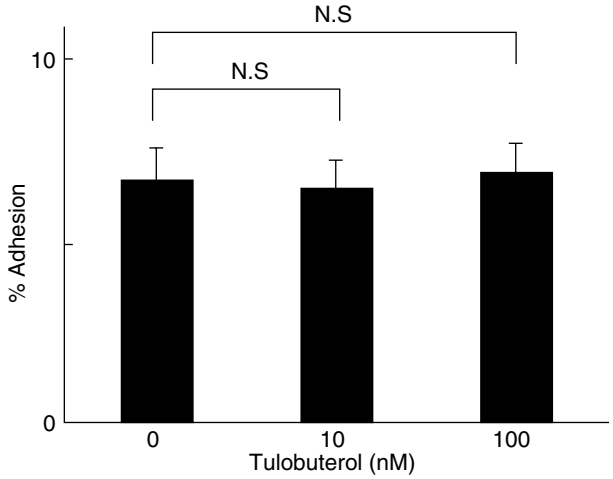

Fig. 1 The effect of tulobuterol on spontaneous adhesion of eosinophils to resting HUVEC. Values are the mean ± S.E.M. of ten experiments.

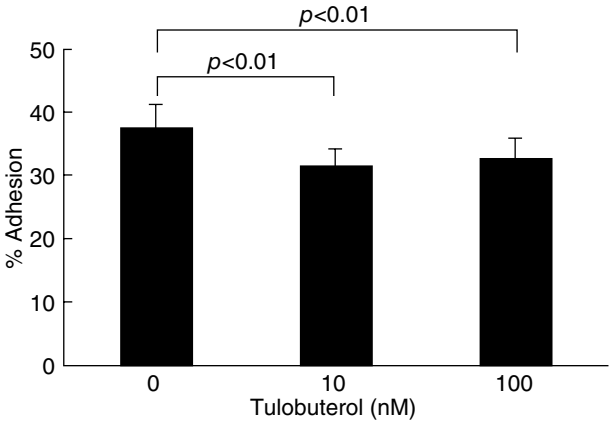

Fig. 2 The effect of tulobuterol on eosinophil adhesion to HUVEC stimulated with IL-4 + TNF-α (both at 100 pM, 24 hrs). Adhesion was significantly attenuated by 10 to 100 nM tulobuterol (p<0.01). Values are the mean ± S.E.M. of ten experiments.

ported.[7] Briefly, HUVEC monolayers were cultured in 96-well tissue culture plates in the presence or absence of 0.1 mM tulobuterol for 1 hr in 5% CO_2 at 37℃ and then stimulated with a combination of IL-4 and TNF-α (both at 100 pM, 24 hrs). Prior to evaluation, HUVEC were washed and incubated for 30 min at 37℃ with a blocking buffer (PBS containing 5% NCS and 3% nonfat dry milk). The primary antibodies (R & D Systems, Minneapolis, MN, USA), anti-ICAM-1 (clone BBIG-I1), anti-VCAM-1 (clone BBIG-V1) or the isotype control mouse IgG1, were added to the wells and incubation was continued for an additional 2 hrs at 37℃. The HUVEC were again washed three times with blocking buffer, and the secondary antibody (sheep antimouse IgG peroxidase conjugate) was added to the wells. Following 2 hrs incubation, the cells were washed three times with PBS. Detection of the peroxidase conjugate was performed in citrate urea buffer using OPD as a substrate, in a fashion similar to the eosinophil adhesion assay. The VCAM-1 or ICAM-1 concentration present on cells was expressed as absorbance at 490 nm and reported as the actual value minus absorbance from the IgG1 isotype control.

STATISTICS

Repeated-measures ANOVA and Scheffe constants were used for comparison in order to determine significance. p -values less than 0.05 were considered significant. Values shown are mean ± SEM.

RESULTS

Initial experiments were conducted to confirm whether tulobuterol modifies the spontaneous adhesion of eosinophils to resting HUVEC. The spontaneous adhesion of eosinophils to resting HUVEC was not modified by tulobuterol at concentrations up to 100 nM (Fig. 1).

When HUVEC were stimulated with IL-4 + TNF-α to upregulate the expression of adhesion molecules, the percentage of spontaneous adhesion of eosinophils to activated HUVEC was 37.4 ± 3.9% in the absence of tulobuterol, which was higher than that noted with resting HUVEC, and was decreased to 31.4 ± 2.6% and 32.6 ± 3.2% in the presence of tulobuterol at concentrations of 10 and 100 nM, respectively ($p < 0.01$, $n = 10$, Fig. 2).

Percentage adhesion of 100 pM IL-5-stimulated eosinophils to resting HUVEC was 16.6 ± 2.1% in the absence of tulobuterol, which was higher than the rate of spontaneous adhesion of eosinophils to resting HUVEC, and 15.8 ± 2 and 13.1 ± 1.7% in the presence of tulobuterol at concentrations of 10 and 100 nM, respectively(Fig. 3). The decrease at 100 nM was statistically significant ($p < 0.01$, $n = 9$).

When eosinophils were stimulated with FMLP, the percentage of eosinophils adhering to resting HUVEC was 16.3 ± 4.1% in the absence of tulobuterol and 13.7 ± 3.7% and 12.7 ± 3.2% in the presence of tulobuterol at concentrations of 10 and 100 nM (Fig. 4). The decrease at 100 nM was statistically significant ($p < 0.01$, $n = 9$).

EFFECT OF TULOBUTEROL ON THE EXPRESSION OF ADHESION MOLECULES

We performed experiments using flow cytometry to evaluate whether tulobuterol can modify surface expressions of CD11b, CD18 or VLA-4 on eosinophils. The results confirmed that tulobuterol does not modify the surface expression of CD11b or CD 18 on eosinophils in the presence of IL-5. (mean fluorescence index (M.F.I.) : CD11b, 577.4 ± 43.0 by control

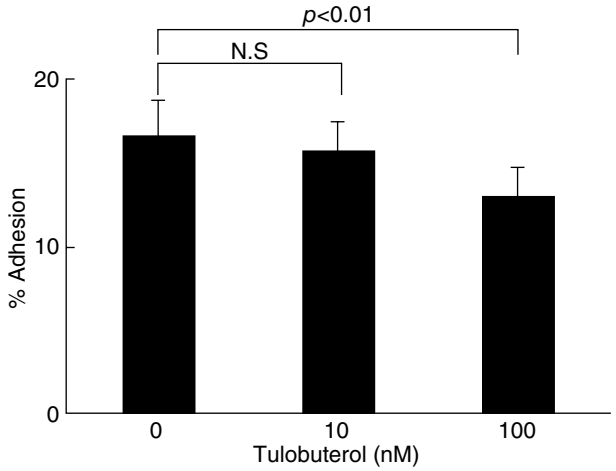

Fig. 3 The effect of tulobuterol on 100 pM IL-5-activated eosinophil adhesion to resting HUVEC. Administration was significantly attenuated by 100 nM tulobuterol ($p<0.01$). Values are the mean ± S.E.M. of nine experiments.

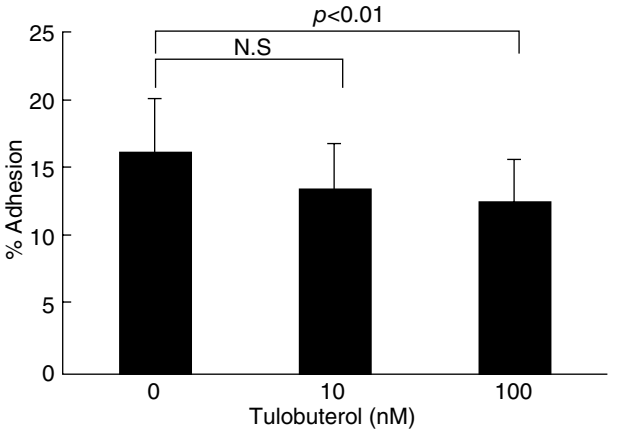

Fig. 4 The effect of tulobuterol on FMLP-activated eosinophil adhesion to resting HUVEC. Adhesion was significantly attenuated by 100 nM tulobuterol ($p<0.01$). Values are the mean ± S.E.M. of nine experiments.

vs. 592.4 ± 40.4 by tulobuterol, $p > 0.1$, $n = 5$, CD18, 1071.2 ± 62.6 by control *vs.* 1047.6 ± 50.6 by tulobuterol, $p > 0.1$, $n = 5$). Similarly, turobuterol dose not modify the expression of VLA-4 (CD49d) on eosinophils (35.7 ± 6.7 by control *vs.* 37.0 ± 8.5 by tulobuterol, $p > 0.1$, $n = 3$).

We also performed experiments employing a cell-ELISA to determine whether tulobuterol modulates the expression of ICAM-1 or VCAM-1 on endothelial cells. The results confirmed that tulobuterol does not modify the surface expression of adhesion molecules on endothelial cells stimulated by IL-4 + TNF-α (O. D. : ICAM-1, 0.36 ± 0.06 by control *vs.* 0.36 ± 0.06 by tulobuterol, $p > 0.1$, $n = 9$, VCAM-1, 0.18 ± 0.04 by control *vs.* 0.18 ± 0.04 by tulobuterol, $p > 0.1$, $n = 9$).

DISCUSSION

In the present study, tulobuterol significantly inhibited the spontaneous adhesion of peripheral blood eosinophils to vascular endothelial cells stimulated with IL-4 + TNFα. Tulobuterol also inhibited the adhesion of eosinophils stimulated with IL-5 or FMLP to resting vascular endothelial cells at concentrations nearly equivalent to those expected in patients using tulobuterol patches for clinical treatment.[10] These findings suggest that tulobuterol inhibits the adhesion of eosinophils to vascular endothelial cells by affecting either integrin molecules of eosinophils or adhesion molecules of vascular endothelial cells or both.

In this study, both eosinophils and vascular endothelial cells were treated with tulobuterol to investigate the significance of use of tulobuterol in the clinical setting. Both eosinophils and vascular endothelial cells express β2-receptors.[11,12] It has been reported that β2-agonists inhibit degranulation, production of LTC4, and production of super oxide anions by eosinophils.[13,14] PDE inhibitors, a class of drugs inducing increases in intracellular cyclic AMP level, an effect also found with β2-agonists, have been demonstrated to inhibit *in vitro* expression of ICAM-1 and VCAM-1 by vascular endothelial cells.[15] The authors have reported that theophylline, which can inhibit PDE activity and increase intracellular levels of cyclic AMP, decreases both the adhesiveness of blood eosinophils and the expression of VCAM-1 and ICAM-1 on vascular endothelial cells in response to a combination of IL-4 + TNFα.[5] However, we confirmed that tulobuterol does not modify the surface expression of adhesion molecules on eosinophils or endothelial cells. We therefore speculate that tulobuterol mainly modulates the functional status or conformational change of adhesion molecules. Another systemic β2-agonists may exert similar inhibitory effects on the adhesive interaction between eosinophils and endothelial cells.

The inhibition of eosinophil adhesion to vascular endothelial cells by tulobuterol observed in the present study may involve interaction between α4-integrins of eosinophils and VCAM-1 of vascular endothelial cells or interaction between β2-integrins of eosinophils and ICAM-1 of vascular endothelial cell. This hypothesis is based on the findings that HUVEC stimulated with IL-4 + TNFα exhibits an increase in adhesion to eosinophils mainly via interaction between α4-integrins of eosinophils and VCAM-1 of vascular endothelial cells,[7,8] and that β2-integrins of eosinophils play an important role in mediating the increase in adhesion of eosinophils by eosinophil growth factors as well as FMLP.[16] Further studies are required to clarify the role of these adhesion molecules in modifing the effect of tulobuterol on the ad-

hesive interaction between eosinophils and endothelial cells, and to examine whether β2-agonists other than tulobuterol also exert the effects observed in the present study.

The anti-inflammatory effect of tulobuterol observed in the present study is likely to be of clinical significance in the treatment of asthma when this drug is administered systemically via transdermal patches. Tulobuterol is expected to complement use of corticosteroids which do not inhibit the adhesion of eosinophils to vascular endothelial cells *in vitro* as reported by the authors[3] and Kaiser *et al*. [4] In fact, Hozawa *et al*.[17] reported that treatment with a combination of inhaled corticosteroid and tulobuterol patches significantly decreased the number of eosinophils in sputum of adult patients with asthma, compared with inhaled corticosteroid monotherapy.

Long-acting inhalational β2-agonists, including salmeterol, are not expected to exert the anti-inflammatory effect observed with tulobuterol since they are unlikely to modulate the functional status of circulating eosinophils or the adhesiveness of endothelial cells. Combined use of inhalational steroids and salmeterol has been reported to yield a "masking effect" on the airway inflammation associated with asthma, as determined by measurement of numbers of eosinophils in sputum or nitric oxide (NO) level in expired air.[18,19] Inhalational β2-agonists have potent dilatative effects on airway smooth muscle, and are also likely to affect epithelial cells in the airway as well as T cells and eosinophils accumulating in airway tissues. In fact, β2-agonists inhibit the function of T cells at high concentrations via selective effects on Th1 cells. Panina-Bordigon *et al*. found that salbutamol, a β2-agonist, inhibits the production of IL-12 from monocytes in a dose-related fashion.[20] Huang *et al*. reported that salbutamol decreases the Th1/Th2 ratio of cord blood T cells.[21] Malfait *et al*. showed that salbutamol inhibited the production of IFN-γ in an arthritis animal model.[22] Taken together, these findings suggest that inhalational β2-agonists may exacerbate airway inflammation in patients with asthma by inducing deterioration of the balance between Th1 and Th2 in sites of inflammation when local concentrations of β2-agonist increase excessively in the airways. Reports of exacerbation of eosinophil accumulation in airway tissues by use of short-acting β2-agonists[23] mono-therapy and of exacerbation of airway hyper responsiveness to allergens by treatment with short-acting β2-agonists[24] suggest that inhalational β2-agonists have the potential to exacerbate allergic inflammation under certain conditions. Since tulobuterol administered via transdermal patches does not accumulate in high concentrations in the airways, unfavorable effects would be expected to be minimized. In general, β2-agonists have significant bronchodilatative effects even when administered systemically. In addition to their bronchodilatative effects, tulobuterol patches are expected to inhibit the adhesion of eosinophils to vascular endothelial cells. Treatment with tulobuterol patches may thus be useful in controlling allergic airway inflammation including eosinophil accumulation, the fundamental pathological feature of asthma. In conclusion, our study demonstrated an inhibitory effect of tulobuterol, on the adhesion of peripheral blood eosinophils to vascular endothelial cells using a transdermal slow-releasing β2-agonist patch widely used in Japan. Tulobuterol inhibited the adhesion of eosinophils to endothelial cells both when endothelial cells were stimulated with cytokines to induce expression of adhesion molecules and when surface molecules of eosinophils were stimulated. The tulobuterol patch is thus expected to act not only as a bronchodilator but also, possibly, as a modulator of airway inflammation, the fundamental pathological feature of asthma, in which accumulation of eosinophils may be particularly important.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of Ms Akemi Yokote and Ms Nozomi Nozaki.

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