Hypoxia enhances the induction of human amniotic mesenchymal side population cells into vascular endothelial lineage

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Abstract. Human amniotic mesenchymal side population (hAM-SP) cells have pluripotency and weak immunogenicity, and have promising roles in the field GAPDH of regenerative medicine. The aim of the present study was to determine whether hypoxic conditions induce the differentiation of hAM-SP cells into the vascular endothelial lineage. Mesenchymal cells were isolated from enzyme-treated amniotic membranes and stained with Hoechst 33342. The hAM-SP cells were negatively sorted by FACS and cultured in induction medium containing vascular endothelial growth factor (VEGF) under normoxic (20% O_2) or hypoxic (1% O_2) conditions for 1 or 2 weeks. The expression of endothelial markers such as kinase domain region (KDR), fms-like tyrosine kinase (Flt)-1, von Willebrand factor (vWF), vascular endothelial (VE)-cadherin and human vascular cell adhesion molecule (VCAM) at the gene and protein level was evaluated by real-time PCR and fluorescent immunostaining, respectively. The gene expression of KDR, Flt-1, VE-cadherin and vWF peaked after 2 weeks of culture. The protein expression of KDR and VE-cadherin was also enhanced after 2 weeks of culture under hypoxic conditions. To confirm the involvement of hypoxia-inducible factor (HIF) in the induction under hypoxic conditions, the expression of genes which are known to be upregulated by HIF was analyzed by DNA microarray. The expression of these genes increased under hypoxic conditions. hAM-SP cells cultured under hypoxic conditions differentiated into the vascular endothelial lineage, probably due to upregulation of the gene expression associated with angiogenesis through activation of the HIF system.

Introduction

Human amniotic mesenchymal cells (hAMCs), which are derived from amniotic membranes, are an attractive stem cell source in the field of regenerative medicine (1). hAMCs have weak immunogenicity due to their negligible expression of human leukocyte antigen (HLA) class II molecules, low expression levels of HLA class I molecules (2), and high expression levels of immunosuppressive factors, including interleukin-1 receptor antagonist (IL-1ra), IL-10 and collagen XVIII (3,4). There are relatively fewer ethical constraints in using hAMCs, since hAMCs are obtained from the amnion, which is discarded after childbirth. Since the amniotic membrane is derived from the inner cell mass of the blastocyst and is of fetal origin, it is expected that amniotic cells contain pluripotent stem cells. Amniotic epithelial and mesenchymal cells express POU domain class 5 transcription factor 1 (Oct-3/4, ES cell makers), nestin and musashi (neural stem cell markers) (5), suggesting that the cells derived from the amniotic membranes indeed contain undifferentiated cells.

Several methods have been used to induce the differentiation of hAMCs into endothelial cells. When hAMCs were cultured in a medium appropriate for endothelial cell culture (EGM-2™ medium) containing hydrocortisone, human epidermal growth factor (hEGF), fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), human fibroblast growth factor-basic (hFGF-B), the cells changed in morphology from fibroblast-like shape to endothelial cell-like shape, and they also developed the ability to take up acetylated low-density lipoprotein (LDL) and form endothelial-like networks in the Matrigel™ assay (6). VEGF has also been shown to induce hAMCs to differentiate into endothelial cells. When the cells were cultured on Matrigel™, spontaneous differentiation of hAMCs into endothelial cells was also detected. VEGF was shown to enhance the expression of fms-like tyrosine kinase (Flt)-1 and kinase domain region (KDR) in hAMCs, and to also induce the expression of endothelial cell-specific markers such as intercellular adhesion molecule (ICAM)-1, CD34 and von Willebrand factor (vWF) (7). Human amniotic fluid-derived cells have also been used as a source of mesenchymal stem cells. The cells acquired endothelial cell characteristics when cultured in EGM-2 medium or under a shear force created by setting the culture dish on an orbital shaker, and to produce angiogenic factors such as VEGF, placental growth factor (PGF) and hepatocyte growth factor (HGF) when cultured under hypoxic conditions (5% O_2) (8).

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Angiogenesis is induced by hypoxia in several situations, such as in the development of the retinal circulation in premature infants (9), wound repair (10,11), and tumor angiogenesis (12,13). The hypoxia-inducible factor (HIF) system plays an important role in the regulation of angiogenesis under hypoxic conditions. In the presence of hypoxia, HIF upregulates VEGF transcription (14,15), as well as the expression of two VEGF receptors, Flt-1 (16) and KDR (17), which increase the biological activity of secreted VEGF.

Cells that are negatively stained by Hoechst 33342 (DNA-binding fluorescent dye), i.e., cells with the highest efflux capacity for the dye, are known to be a very small and homogeneous population of highly primitive cells, known as side population (SP) cells (18-21). SP cells found in a number of species, including mice, monkeys and humans, and isolated from several organs, including the bone marrow, skeletal muscle and liver (18-21), have demonstrated the potential for differentiation into cell types beyond their organ of origin (22). SP cells have also been isolated from hAMCs (23). hAM-SP cells express Oct-3/4 and have the potential to differentiate into multiple lineages, including several organ- or tissue-specific cells including neurons, osteoblasts, chondrocytes, and adipocytes, as found for the other type of mesenchymal stem cells (23).

The aim of the present study was to clarify whether hAM-SP cells, which can be regarded as an undifferentiated stem cell fraction of AMCs, were effectively induced to differentiate into cells of endothelial lineage by hypoxia. Therefore, we cultured hAM-SP cells in an endothelial induction medium containing VEGF in a hypoxic (1% O_2) or a normoxic (20% O_2) environment.

Materials and methods

Preparation of human amniotic mesenchymal side population cells. The Institutional Ethics Committee approved all protocols (Kitasato University, School of Allied Health Sciences, no. 2009-015). The protocol for the preparation of amniotic mesenchymal SP cells has previously been described (23). Briefly, after informed consent was obtained from a pregnant woman scheduled for caesarean section, the amniotic membrane was separated from the post-partum placenta. The human amniotic membrane consists of two cell layers, the epithelial layer and mesenchymal layer, with the basement membrane between the two. To prepare AMCs, the amniotic membrane was first treated with trypsin to remove the amniotic epithelial cells, and then the remnant layer was treated with an enzyme mixture (0.1% papain, 1 mg/ml collagenase, 0.01% DNase, and 0.1% dispase) to dissolve the mesenchymal layer and disperse cells. The AMCs were stained with Hoechst 33342 and the SP cells were sorted using a cell sorter (EPICS Altra; Beckman Coulter, Fullerton, CA, USA).

The sorted SP cells in the hAMCs (hAM-SP cells) were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DMEM/F12) containing 5% FBS (both were from Sigma-Aldrich, St. Louis, MO, USA), 10 ng/ml human leukemia inhibitory factor (hLIF; Chemicon-Merck Millipore, Billerica, MA, USA), 10 ng/ml hFGF-B (PeproTech, Inc., Rocky Hill, NJ, USA) and 10 ng/ml platelet-derived growth factor-BB (PDGF-BB; PeproTech, Inc.), on a type I collagen-coated dish (Iwaki, Chiba, Japan) in a 5% CO_2 environment at 37˚C. The cells were cultured until they reached 90% confluence and were recovered with 0.1% trypsin (Sigma-Aldrich)-ethylenediaminetetraacetic acid (EDTA; Gibco-Life Technologies, Carlsbad, CA, USA), and sub-cultured at a density of 10^4 cells/cm^2.

Endothelial differentiation culture. The hAM-SP cells at the fourth or fifth passages cultured in cell growth medium were rinsed with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} and then cultured in endothelial induction medium consisting of DMEM/F12 supplemented with 2% FBS and 50 ng/ml VEGF (PeproTech, Inc.) for 1 or 2 weeks in a hypoxic (1% O_2) or normoxic (20% O_2) environment, on a type I collagen-coated dish. As control, the hAM-SP cells were cultured in normal medium consisting of DMEM/F12 supplemented with 2% FBS in the absence of VEGF for 1 or 2 weeks. The endothelial induction medium and normal medium were replenished every two days. The endothelial markers after differentiation of the hAM-SP cells were evaluated by real-time PCR and fluorescence immunostaining.

Real-time PCR. To examine the expression of endothelial cell-specific mRNA, we performed a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay. RNAprotect® Cell Reagent (Qiagen, Hilden, Germany) was used to stabilize the RNA of the cultivated cells and total RNA was extracted from the cells using RNeasy Plus Mini kit (Qiagen). The mRNA was transcribed into cDNA using the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). PCR was carried out by mixing 1 μl of cDNA template, each primer (Table I) and SYBR-Green (Bio-Rad) in a volume of 20 μl. The samples were amplified in a thermocycler. Each sample was analyzed in triplicate by the Chromo4 system (Bio-Rad). Amplification data were obtained using the software of Opticon Monitor (Bio-Rad). The expression levels were quantified relative to the expression level of GAPDH.

Immunochemistry. hAM-SP cells subjected to induction were fixed and incubated for 1 h with diluted primary antibodies specific for the endothelial cells. The primary antibodies used as endothelial markers were: anti-human vascular cell adhesion molecule (VCAM)-1 mouse IgG (1:100; Immunotech, Beckman Coulter, Inc.), anti-human KDR mouse IgG (1:100; Sigma-Aldrich), anti-human vascular endothelial (VE)-cadherin mouse IgG (1:100; R&D Systems, Inc., Minneapolis, MN, USA), and anti-human vWF mouse IgG (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The secondary antibodies were Alexa Fluor 488-conjugated anti-mouse goat IgG (1:500; Molecular Probes-Life Technologies). The immunostained cells were analyzed by confocal laser scanning microscopy (CLSM) (IX70; Olympus Corporation, Tokyo, Japan).

Expression of the Oct-3/4 protein was evaluated by fluorescent immunostaining following induction with VEGF under hypoxic conditions for 1 or 2 weeks. The primary antibody used was anti-human Oct-3/4 rabbit IgG (1:200; Santa Cruz Biotechnology, Inc.), and the secondary antibody was Alexa 488-conjugated anti-rabbit goat IgG (1:500; Molecular

Table I. Primer sets used for RT-PCR analysis of the endothelial differentiation.

	Forward primers	Reverse primers
GAPDH	5'-GGCC TCCA AGGA GTAA GACC-3'	5'-AGGG GTCT ACAT GGCA ACTG-3'
KDR	5'-AGCC AGCT CTGG ATTT GTGG A-3'	5'-CATG CCCT TAGC CACT TGGA A-3'
Flt-1	5'-GCGC TTCA CCTG GACT GACA-3'	5'-GAAA CTGG GCCT GCTG ACAT C-3'
VCAM-1	5'-ATTG ACTT GCAG CACC ACAG-3'	5'-ATCT CCAG CCTG TCAA ATGG-3'
vWF	5'-AGAT GTTT GCCT ACGG CTTG-3'	5'-CAGC CTGT GACC CTCT TCTC-3'

RT-PCR, reverse transcription-polymerase chain reaction; KDR, kinase domain region; Flt-1, fms-like tyrosine kinase-1; VCAM-1, vascular cell adhesion molecule-1; vWF, von Willebrand factor.

Probes). Immunostained cells were analyzed by confocal laser microscopy using a CLSM. Each RGB image was separated into red, green and blue by color deconvolution with the ImageJ 1.40 software (National Institutes of Health, Bethesda, MD, USA), and the numbers of pixels in green color (endothelial markers) and red color (Oct-3/4) were calculated.

Microarray processing. All experiments were performed using commercially available microarrays for humans (Human Genome U133 Plus 2.0 Array, Affymetrix, Santa Clara, CA, USA). The hAM-SP cells cultivated with VEGF under hypoxic (1% O_2) conditions for 2 or 0 weeks were removed from the culture dishes using trypsin and washed with PBS. Total RNA was isolated from the cells using an RNeasy Mini kit® (Qiagen). The isolation and purification of the total RNA were carried out according to the manufacturer's protocol (Qiagen). The quality and amount of starting RNA were confirmed by agarose gel electrophoresis in addition to the ratio of absorbance ($1.9< A_{260\ nm}/A_{280\ nm} <2.0$). Total RNA was used to prepare a biotinylated target cRNA according to the manufacturer's recommendation (Affymetrix). Briefly, 1 μg of mRNA was used to generate the first-strand cDNA using a T7-linked oligo(dT) primer. After second-strand synthesis, *in vitro* transcription was performed using a synthetic biotinylated nucleotide analog (biotinylated uridine-triphosphate), which yielded an ~50-100-fold amplification of RNA. The cRNA was fragmented prior to overnight hybridization. The arrays were then washed, stained with streptavidin-phycoerythrin, and scanned (GeneChip Scanner 3000®; Affymetrix).

After scanning, the array images were visually inspected to confirm the scanner alignment and the absence of significant bubbles or scratches on the chip surface. The 3'/5' ratios for GAPDH were 1.34 and 1.63, which were within the acceptable limits; BioB spike controls were also present on all the chips, with BioC, BioD and Cre being present in increasing intensities. BioB, BioC, BioD and Cre are genes from *Escherichia coli* or bacteriophage P1 and were added before hybridization to check the hybridization quality. Background intensities were 93.8 and 102.6 and noise factors were 5.08 and 5.12, which were sufficiently low.

Statistical analysis. All data are presented as the means + SD. Bonferroni's post hoc test was used for multiple-group comparisons. P-values <0.05 were considered to indicate statistically significant differences.

Results

For evaluation of the endothelial differentiation potency, the gene expression of endothelial markers such as KDR (24), Flt-1 (25), VCAM (26) and vWF (27) was evaluated by real-time PCR. While the expression of KDR, VCAM and vWF did not change after 1 week of culture with VEGF under hypoxic conditions, the expression of Flt-1 increased (Fig. 1).

After 2 weeks of induction with VEGF under hypoxic conditions (Fig. 2), the hAM-SP cells showed enhanced expression of KDR, Flt-1, VCAM and vWF. The cells cultivated only under hypoxic conditions in the absence of VEGF or the cells induced with VEGF under normoxic conditions showed no significant changes in the expression of genes as compared to those in the control (cultivated without VEGF under normoxic conditions). On the other hand, the cells induced with VEGF under hypoxic conditions showed significantly increased expression levels of KDR, VCAM, Flt-1 and vWF as compared to the cells induced with VEGF under normoxic conditions as well as to the cells cultured only under hypoxic conditions in the absence of VEGF or the cells induced with VEGF under normoxic conditions (Fig. 2). Thus, culture under hypoxic conditions enhanced the cellular differentiation by VEGF into the endothelial lineage.

Immunocytochemical assay for endothelial markers such as VCAM, KDR, vWF and VE-cadherin (28) was performed following induction of hAM-SP cells with VEGF under normoxic/hypoxic conditions for 2 weeks. Positive staining for KDR and VE-cadherin proteins was observed following induction with VEGF under both normoxic and hypoxic conditions, while staining for the VCAM and vWF proteins was negative (Fig. 3A). Human pulmonary artery endothelial cells (HPAECs) were used as positive control, and showed positive staining for all markers (data not shown). The number of pixels in green color was calculated with the ImageJ software (Fig. 3B). KDR was significantly induced by VEGF under both normoxic and hypoxic conditions as compared with that in the control conditions ($P<0.01$). VE-cadherin expression was increased by VEGF under normoxic conditions as compared with that in the control conditions ($P<0.05$), and was induced even more significantly under hypoxic condition as compared with that in normoxic conditions ($P<0.01$). To evaluate if any undifferentiated cells remained after the induction, immunocytochemical staining for Oct-3/4 was carried out after induction with VEGF under hypoxic condi-

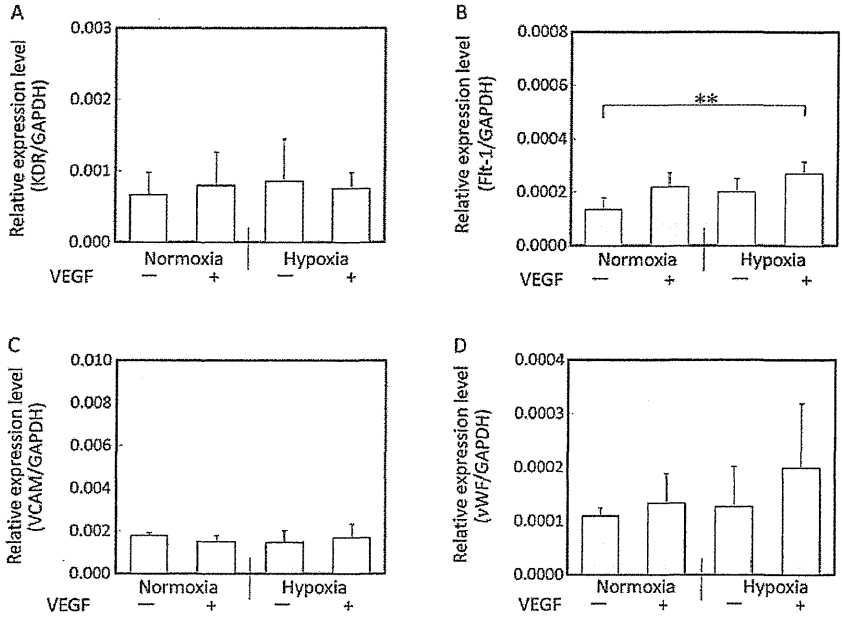

Figure 1. Gene expression of endothelial cell markers evaluated by real-time PCR after 1 week of induction with VEGF. (A) KDR, (B) Flt-1, (C) VCAM, (D) vWF. Cells were induced by VEGF under normoxic or hypoxic conditions. The expression levels relative to the GAPDH expression level were compared. While no changes in the expression of KDR, VCAM or vWF were observed after induction with VEGF under hypoxic conditions, the expression of Flt-1 increased. Data shown are the means + SD (n=5); (**P<0.01). VEGF, vascular endothelial growth factor; KDR, kinase domain region; Flt-1, fms-like tyrosine kinase-1; VCAM, vascular cell adhesion molecule; vWF, von Willebrand factor.

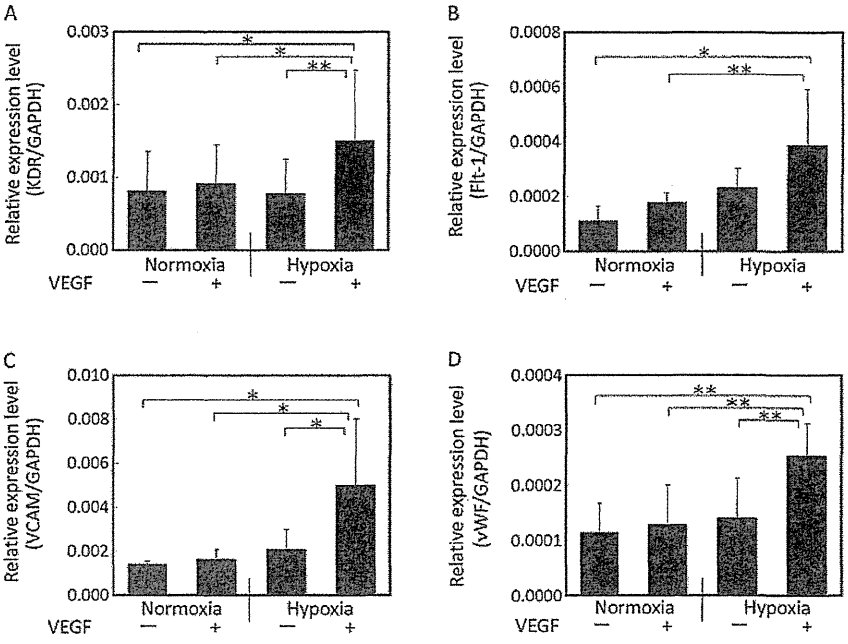

Figure 2. Gene expression of endothelial cell markers evaluated by real-time PCR after 2 weeks of induction with VEGF. (A) KDR, (B) Flt-1, (C) VCAM, (D) vWF. Cells were induced by VEGF under normoxic or hypoxic conditions. The expression levels relative to the GAPDH expression level were compared. Following cultivation under hypoxic conditions in the presence of VEGF, significant increase in the expression levels of KDR, Flt-1, VCAM and vWF was observed. Data shown are the means + SD (n=5); (*P<0.05 and **P<0.01). VEGF, vascular endothelial growth factor; KDR, kinase domain region; Flt-1, fms-like tyrosine kinase-1; VCAM, vascular cell adhesion molecule; vWF, von Willebrand factor.

tions for 1 or 2 weeks (Fig. 4). Strong expression of Oct-3/4, which is a marker gene of undifferentiated cells (29,30), was observed in the hAM-SP cells prior to the induction. While some expression of Oct-3/4 protein was still observed after 1 week of induction with VEGF under hypoxic conditions, the red staining for this protein disappeared almost entirely after 2 weeks of induction. The expression of Oct-3/4 protein decreased significantly with the induction time.

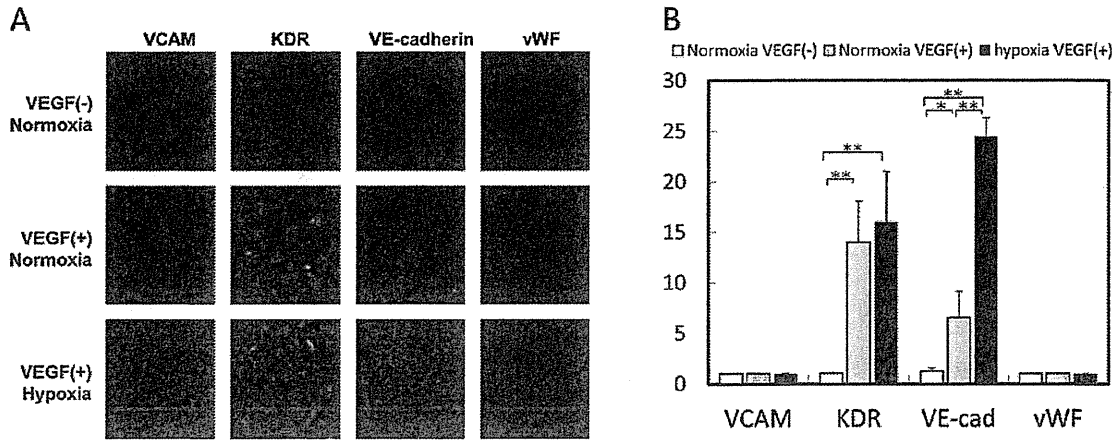

Figure 3. (A) Fluorescent immunostaining for endothelial cell markers following induction with VEGF for 2 weeks under hypoxic or normoxic conditions. The primary antibodies used were: anti-human VCAM mouse IgG, anti-human Flk-1 mouse IgG and anti-human VE-cadherin mouse IgG, and the secondary antibodies were Alexa Fluor 488-conjugated anti-mouse goat IgG. (B) The number of pixels in green color extracted by color deconvolution was calculated with the ImageJ software. Data shown are the means + SD (n=4); (*P<0.05 and **P<0.01). KDR expression was significantly increased by cultivation with VEGF under both normoxic as well as hypoxic conditions. Staining for VE-cadherin was stronger following cultivation under hypoxic than normoxic conditions. Negative staining was found for VCAM and vWF. VEGF, vascular endothelial growth factor; VCAM, vascular cell adhesion molecule; KDR, kinase domain region; vWF, von Willebrand factor.

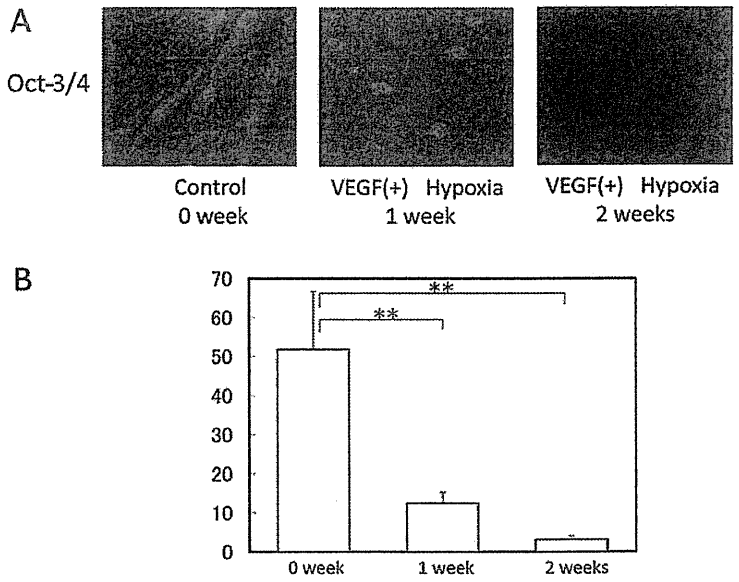

Figure 4. (A) Fluorescent immunostaining for Oct-3/4 following induction with VEGF for 1 or 2 weeks under hypoxic conditions. The primary antibody used was anti-human Oct-3/4 rabbit IgG, and the secondary antibody was Alexa 488-conjugated anti-rabbit goat IgG. The immunostained cells were analyzed by confocal laser scanning microscopy. The control group showed positive staining for Oct-3/4; Oct-3/4 expression was still observed after 1 week of induction under hypoxic conditions, while it was no longer detected after 2 weeks of induction. (B) The number of pixels in red color was calculated with the ImageJ software. Data shown are the means + SD (n=4); (**P<0.01). VEGF, vascular endothelial growth factor.

We also analyzed gene expression by microarray analysis and focused on the gene expression of stem cell markers (31) and neural stem cell markers. The gene expression of stem cell markers, such as Nanog homeobox (NANOG) (32,33), Oct-3/4 (29,30) and growth differentiation factor 3 (GDF3) (34), was decreased by less than half in the cells cultivated with VEGF under hypoxic conditions for 2 weeks as compared to those cultivated for 0 weeks (prior to induction). The gene expression of Kruppel-like factor 4 (KLF4) (35), v-myc myelocytomatosis viral oncogene homolog (MYC) (36), sex determining region Y (SRY)-box 2 (SOX2) (37), RNA exonuclease 1 homolog (REX1) (38), fibroblast growth factor 4 (FGF4) (39), telomerase reverse transcriptase (TERT) (40) slightly decreased (or remained the same) following induction with VEGF under hypoxic conditions for 2 weeks. The neural stem cell markers such as nestin (41) and musashi (42) were decreased after induction with VEGF under hypoxic conditions for 2 weeks (Table II).

To confirm the involvement of HIF in the induction under hypoxic conditions, we selected downstream genes of HIF-1. The results revealed that the gene expression of VEGF-A (15), Flt-1 (16), erythropoietin (EPO) (43), enolase

Table II. Gene expression of stem cell markers and neural stem cell markers by microarray analysis.

Gene	Detection	Hypoxia VEGF(+) 2/0 week	Author/(ref.)
Stem cell markers			
Kruppel-like factor 4 (gut) (KLF4)	P/P	0.98	Li *et al* (35)
v-myc myelocytomatosis viral oncogene homolog (MYC)	P/P	0.88	Cartwright *et al* (36)
Nanog homeobox (NANOG)	A/A	0.08	Chambers *et al* (32) Mitsui *et al* (33)
POU domain class 5 transcription factor 1 (Oct-3/4)	A/A	0.47	Nichols *et al* (29) Niwa *et al* (30)
Sex determining region Y (SRY)-box 2 (SOX2)	A/A	1.00	Avilion *et al* (37)
RNA exonuclease 1 homolog (REX1)	A/A	0.83	Ben-Shushan *et al* (38)
Growth differentiation factor 3 (GDF3)	A/A	0.24	Levine and Brivanlou (34)
Fibroblast growth factor 4 (FGF4)	A/A	0.81	Yuan *et al* (39)
Telomerase reverse transcriptase (TERT)	P/P	0.67	Yang *et al* (40)
Neural stem cell markers			
Nestin (NES)	P/A	0.09	Park *et al* (41)
Musashi homolog 1 (MSI1)	A/A	0.71	Kaneko *et al* (42)

Detection indicates whether the transcript was present (P) or absent (A) calls. Present and absent calls mean that the expression levels are above or below the threshold of detection.

Table III. Hypoxia-inducible factor-1 target genes upregulated by hypoxic culture.

Gene	Detection	Hypoxia VEGF(+) 2/0 week	Author/(ref.)
Vascular endothelial growth factor A (VEGF-A)	P/P	2.0	Forsythe *et al* (15)
Fms-like tyrosine kinase-1 (flt-1)	P/P	2.7	Gerber *et al* (16)
Erythropoietin (EPO)	A/A	6.6	Wang and Semenza (43)
Enolase 1 (ENO-1)	P/P	2.0	Semenza *et al* (44)
Adrenomedullin (ADM)	P/P	6.3	Nguyen and Claycomb (45)
EGL nine homolog 3 (EGLN-3)	P/P	17.9	Pescador *et al* (46)

Detection indicates whether the transcript was present (P) or absent (A) calls. Present and absent calls mean that the expression levels are above or below the threshold of detection.

(ENO)-1 (44), adrenomedullin (ADM) (45) and Egl nine homolog (EGLN)-3 (46), which are known to be upregulated by HIF-1, were increased by more than 2-fold in the cells cultivated with VEGF under hypoxic conditions for 2 weeks as compared to those cultivated with VEGF under normoxic conditions (Table III).

Discussion

To clarify whether hAM-SP cells can be effectively induced to differentiate into endothelial lineage cells by hypoxia, hAM-SP cells were cultured in endothelial cell induction medium containing VEGF under hypoxic (1% O_2) or normoxic (20% O_2) conditions. Our data revealed that under hypoxic conditions: i) the gene expression of endothelial lineage markers such as KDR, Flt-1, VCAM and vWF was induced; ii) the expression of endothelial marker proteins including KDR and VE-cadherin was induced; and iii) expression of the HIF target genes was upregulated in the hAM-SP cells. Cultivation in the presence of VEGF under hypoxic conditions for 2 weeks enhanced the expression of endothelial lineage markers. While the expression of Oct-3/4 was still observed after 1 week of induction with VEGF under hypoxic conditions, it disappeared almost completely after 2 weeks. These results suggest that induction of hAM-SP cells for 1 week was inadequate to induce differentiation of the cells into endothelial cells, while induction for 2 weeks led to pronounced differentiation of the cells into the endothelial lineage. However, protein expression of VCAM and vWF could not be detected under these conditions. Therefore, the characteristics of hAM-SP cells following induction by VEGF under hypoxic condition for 2 weeks were not entirely identical to those of endothelial cells, and it is suggested that extended culture time would have created more mature endo-

thelial cells, since enhanced mRNA expression of vWF and VCAM was observed.

hAMCs have been reported to be induced by EGM-2, which contain several growth factors, including VEGF; however, the differentiated cells did not express mature endothelial cell markers, such as vWF and VE-cadherin (6). Also, VEGF receptors 1 (Flt-1) and 2 (KDR) were basally expressed in hAMCs, and enhanced expression of endothelial-specific markers such as Flt-1 KDR and ICAM-1 was observed following exposure to VEGF (7). These results indicate that while hAMCs may have the potential to differentiate into endothelial cells, they are a heterogeneous population of cells. hAM-SP cells have a larger population of undifferentiated cells, while the hAMCs contain these cells only at the rate of 0.1-0.2%. In the present study conducted using hAM-SP cells, low expression levels of KDR and Flt-1 in the hAM-SP cells and a high expression level of Oct-3/4 were observed prior to induction. The gene expression of KDR, Flt-1 and vWF, as well as the protein expressions of VE-cadherin increased following induction with VEGF under hypoxic conditions. hAM-SP cells include several stem cells in a more pluripotent state than non-SP cells, and are, therefore, preferable as the source of cells for use in the field of regenerative medicine.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) have also been used as candidate cells in the field of regenerative medicine. Reduced oxygen tension in the physiological range (4-7%) (47) has been shown to enhance the proliferation of these cells. Attention has been focused on the effects of hypoxia on the differentiation of pluripotent cells into various mesenchymal lineages. Although the exact outcome depended on the oxygen tension, time in culture, and use/non-use of hypoxic preculture, the beneficial effects appeared more often on osteogenic, chondrogenic and adipogenic differentiation (48). Although the target cells of these studies were not endothelial cells, they did show that hypoxia clearly plays an important role in the differentiation of mesenchymal stem cells. Hypoxia was also found to be an important factor inducing the differentiation of hAM-SP cells into the endothelial lineage.

It is well-known that VEGF transcription is upregulated under hypoxic conditions by the effects of HIF-1. HIF-1 also upregulates two VEGF receptors, Flt-1 (16) and KDR (17), which increase the biological activities of secreted VEGF (49). To confirm the involvement of HIF in the effective induction of hAM-SP cells under hypoxic conditions, we analyzed the changes in the expression of downstream genes of HIF-1. Our results revealed that the gene expression of VEGF-A, Flt-1, EPO, ENO-1, ADM and EGLN-3, which are known to be upregulated by HIF, (15,16,43-46) increased following induction with VEGF under hypoxic conditions for 2 weeks. These results indicate that under hypoxic conditions, the HIF system is activated, which enhances the expressions of VEGF and VEGF receptors leading to the differentiation into the endothelial lineage.

The hypoxic environment (3-5% of oxygen) is physiologically normal for embryonic stem cells and its pluripotency is regulated by the family of HIFs (50). From the microarray data and Oct-3/4 staining, stem cell markers are gradually decreased by induction of VEGF under hypoxic conditions. These results indicate that hypoxic conditions would also contribute to maintaining an undifferentiated state of hAM-SP cells and maximizing the differentiation into vascular endothelial cells by VGEF by suppressing the differentiation into the other types of cells.

In conclusion, the hAM-SP cells cultivated in the presence of VEGF under hypoxic conditions differentiated into the vascular endothelial lineage, possibly due to upregulation of the gene expression associated with angiogenesis through activation of the HIF system.

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Omalizumab Attenuates Airway Inflammation and Interleukin-5 Production by Mononuclear Cells in Patients with Severe Allergic Asthma

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Key Words
Omalizumab · Eosinophilic inflammation · Interleukin-5 · Peripheral blood mononuclear cells

Abstract
Background: Omalizumab, an anti-immunoglobulin E monoclonal antibody, has shown an inhibitory effect on airway inflammation, which may be associated with clinical improvement of severe asthma. This study evaluated changes in airway inflammation and cytokine release by the peripheral blood mononuclear cells (PBMCs) of Japanese patients with severe asthma after administration of omalizumab. ***Methods:*** Sixteen Japanese patients with severe asthma who were allergic to house-dust mites were enrolled in this study. Eight received omalizumab every 2 or 4 weeks for 16 weeks, and 8 control subjects were treated with conventional drug treatment. Changes in clinical scores for sputum eosinophils and levels of fraction of exhaled nitric oxide (FeNO) were measured at the time of enrollment and at week 16. Cytokines from PBMCs stimulated by house-dust mite *(Dermatophagoides farinae)* or ionomycin/phorbol myristate acetate (PMA) were measured at baseline and at week 16. ***Results:*** In the omalizumab-treated group, decreases in sputum eosinophils and FeNO were observed following treatment. Furthermore, the ex vivo production of interleukin (IL)-5 by PBMCs in response to both mite allergen and ionomycin/PMA decreased significantly. In contrast, interferon (IFN)-γ production was unchanged. There were no changes in any of the parameters observed in the control group. ***Conclusion:*** Omalizumab exerts inhibitory effects on airway inflammation in Japanese patients with severe allergic asthma. This treatment attenuates production of IL-5 by PBMCs stimulated with both a specific allergen and a nonspecific activator. Reduction of the Th2 inflammatory cascade likely contributes to clinical benefits; however, further studies are required to clarify these results due to the small sample size in this study.

Introduction

Asthma is a chronic allergic airway inflammatory disease causing airway hypersensitivity and airflow limitation. Various cells are involved in this allergic airway inflammation and regulate a Th2 network by generating and releasing chemical mediators such as cytokines and chemokines [1]. Immunoglobulin E (IgE) plays an important role as a mediator in the Th2 inflammatory cas-

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cade and progression of the asthmatic airway. IgE cross-linking with receptors (FcεRI and FcεRII) on a variety of cell surfaces, including mast cells, basophils and dendritic cells (DCs), induces release of Th2 cytokines and inflammatory mediators which promote allergic airway inflammation, ultimately leading to the development of asthma symptoms.

Omalizumab (Xolair) is a recombinant humanized IgG1 monoclonal anti-IgE antibody that binds to the IgE molecule at the same epitope on the Fc region that binds to FcεRI, and subsequently decreases the amount of free IgE. Less IgE available to bind causes downregulation of the expression of FcεRI on inflammatory cells, such as mast cells, basophils and DCs. Interrupting the binding of IgE to FcεRIs inhibits mast cells and basophil activation and the subsequent degranulation and release of inflammatory mediators, providing a mechanism to control asthmatic airway inflammation [2–7]. In fact, the anti-IgE antibody has beneficial clinical effects for patients with severe asthma, including inhibiting the frequency of acute exacerbation, improving their clinical symptoms and quality of life [8, 9], improving peak expiratory flow rates (PEFR) in the morning [10] and reducing steroid consumption [8, 11]. Based on these results, the use of anti-IgE antibody for severe allergic asthma is recommended by guidelines including the Japanese Guidelines for Diagnosis and Management of Asthma, the Global Initiative for Asthma and the National Heart, Lung and Blood Institute Expert Panel Report 3 [12–14].

Several mechanisms of anti-inflammation underlying the clinical efficacy of omalizumab have been demonstrated. Treatment of asthma patients with omalizumab for 12 weeks decreased eosinophils in sputum and bronchial tissue when allergen challenges were performed. This inhibitory effect of omalizumab on eosinophilic airway inflammation is related to an inhibition of both early and late asthmatic reactions to allergen provocation and an improvement in PEFR [15]. Omalizumab also reduces lymphocytes in airway mucosa and peripheral blood, which produce Th2 cytokines and chemokines and regulate the Th2 inflammatory cascade in asthma. Treatment with omalizumab for 16 weeks decreased $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes, and B lymphocytes in bronchial lumen in mild-to-moderate persistent asthma [16]. Omalizumab also reduced the number of lymphocytes expressing interleukin (IL)-2, IL-13 or granulocyte-macrophage colony-stimulating factor (GM-CSF) in peripheral blood [17]. Th2-type T cells deliver Th2 cytokines and chemokines, which activate eosinophils, mast cells, basophils and bronchial epithelial cells. Diminishing the number of Th2-type T cells using omalizumab likely results in a reduction of airway tissue remodeling and functional changes. However, the mechanisms producing the anti-inflammatory effects of omalizumab remain unclear because the studies listed were designed to observe the pathological condition rather than to evaluate cell function.

In this study, we examined the influence of omalizumab on the inflammatory cell profile of asthma airways in a clinical setting with a focus on eosinophils. Eosinophil airway infiltration is a typical feature of asthma that broadly correlates with asthma severity [18] and is associated with a greater risk of exacerbation [19]. We also determined the mechanism of airway cell accumulation by investigating modulation of the cytokine-producing profile of peripheral blood mononuclear cells (PBMCs) stimulated by either a specific allergen or a nonspecific agonist before and after omalizumab administration for 16 weeks.

Subjects and Methods

Subjects and Study Protocol

Subjects between 20 and 80 years of age with severe persistent allergic asthma according to the Japanese Guidelines for Diagnosis and Management of Asthma [12] were eligible. This was a 16-week, open-label, prospective study at the Allergy and Asthma Center of the Saitama Medical University Hospital. The protocol was approved by the institutional review board of the hospital and all subjects gave their written informed consent before participating. Asthma was diagnosed based on a history of recurrent wheezing, dyspnea, chest tightness and either reversible airflow limitations (FEV_1 <70% of predicted or a previous best value that increased by >15% after the inhalation of 200 μg salbutamol) or methacholine-induced airway hyper-responsiveness. Inclusion criteria were: (1) a positive skin test or in vitro reactivity to a perennial aeroallergen, (2) a serum total IgE level of 30–700 IU/ml and a body weight of 30–150 kg, (3) receiving more than 1,600 μg/day of inhaled budesonide or equivalent and/or oral steroid, (4) receiving combinations of long-acting inhaled beta-2 agonist (LABA), leukotriene receptor antagonist (LTRA) and/or theophylline (TP), (5) no change of current treatment regimen for at least 12 weeks before the study, and (6) failing to achieve 'well controlled' according to the Japanese guidelines (daily requirement of inhaled β2-agonists and presence of wheeze, cough, chest tightness or shortness of breath at rest that interfered with normal daily activity) [12]. Exclusion criteria were: (1) a smoking history, (2) history of immunosuppressants, (3) pregnant or breast-feeding women, and (4) serious systemic diseases (e.g. cancer or renal failure).

Eligible patients were assigned to the omalizumab or control group. Treatment allocation was based on whether the subject agreed to use omalizumab as their regular maintenance treatment (omalizumab group) or not (control group). Omalizumab was administered subcutaneously, based on the serum total IgE level and

body weight of the subjects at baseline. The subjects in the control group remained on their current asthma therapy. Use of rescue medication dependent on level of severity was permitted as required throughout the study and the intervention period was 16 weeks. Throughout the study, all subjects recorded their symptom scores and PEFR in a diary. In addition, all subjects underwent an asthma control test (ACT), spirometry, exhaled nitric oxide (FeNO), sputum induction and a cytokine profile analysis of PBMCs at baseline and at week 16.

Monitoring PEFR and Asthmatic Symptoms – Asthma Questionnaire

All subjects monitored PEFR twice daily (early morning and at bedtime before drug inhalation) with a mini-Wright peak flow meter for the 12 weeks prior to the start of this study (run-in period) and then for up to 16 weeks during the treatment period. The weekly mean of the 3 highest exhalations (mean PEFR) was recorded in their diaries as well as asthma symptoms (cough, dyspnea and wheezing). To evaluate disease control, ACT was assessed at baseline and at week 16 [20, 21].

FeNO Measurements

FeNO was measured using a portable NO analyzer (NIOX MINO Airway Inflammation Monitor, Aerocrine, Solna, Sweden) that provides FeNO measurements at 50 ml/s exhalation flow rate, expressed in parts per billion (ppb) [22]. The details of the procedure have been described elsewhere [23]. At least two successive recordings at 2-minute intervals were made and the mean of the peak values of two reproducible readings was used in the analysis of the results. All FeNO measurements were performed under supervision of one investigator. Subjects were not to consume food or beverages prior to the taking of measurements.

Sputum Induction

Sputum was induced as described previously [24, 25]. In brief, salbutamol was delivered using a metered-dose inhaler prior to sputum induction. Fifteen minutes later, sterile hypertonic saline (4.5%) was inhaled using an ultrasonic nebulizer. Sputum was collected at 5-minute intervals for up to 30 min; all initial samples were discarded. Induced sputum samples were treated with Hanks' balanced salt solution (HBSS; 1 ml) containing 1% dithiothreitol (Sigma, St. Louis, Mo., USA) until the mixture was homogeneous. Samples were centrifuged at 400 *g* for 10 min. Cytospin slides of resuspended pellets were stained with May-Giemsa for differential cell counts. At least 500 inflammatory cells were counted for each sample. Cytospin slides were judged as adequate when <50% of squamous epithelial cells were present.

Mononuclear Cells and Measurement of Cytokines

PBMCs were obtained from 40 ml of heparinized blood, isolated by the Ficoll-Hypaque density gradient technique, and adjusted to a final concentration of 2×10^6 cells/ml in RPMI culture medium (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine). To assess the effects of omalizumab, PBMCs were cultured for 72 h at 37°C in 5% CO_2 in the presence or absence of a combination of ionomycin (300 nM, Sigma) and phorbol myristate acetate (PMA, 5 nM, Sigma) or 1 μg/ml *Dermatophagoides farinae (Df)* antigen [26, 27]. The supernatants were then collected and stored at –80°C until use.

Table 1. Subject characteristics

	Omalizumab	Control
Number	8	8
Gender (M/F)	3/5	4/4
Age, years*	51.6±7.2	53.4±5.9
Duration of asthma, years*	23.9±7.6	15.3±2.5
Allergic rhinitis (+/–)	8/0	7/1
IgE, IU/ml*	178.6±70.6	355.3±87.4
FEV_1 (% of predicted)	77.9±6.9	80.1±4.9
Allergen (perennial/seasonal)	8/6	8/7
Equivalent budesonide dose, μg/day*	2,514±323	1,457±95
Patients using oral PSL*	5	2

PSL = Oral prednisone therapy.
* $p < 0.05$ omalizumab versus control.

The supernatants were analyzed by a Bio-Plex Human 27 cytokine assay kit and the Bio-Plex suspension array system (Bio-Rad, Hercules, Calif., USA). All assays were conducted in duplicate.

Statistical Analysis

Statistical analyses were performed with Prism4 (Graphpad Software Inc., San Diego, Calif., USA). All data are presented as mean ± SEM, except for FeNO and sputum eosinophil ratio, which are presented as median [interquartile range (IQR)]. A paired t test was applied to test for changes within groups and a Wilcoxon signed-rank test was used for changes from baseline in FeNO and sputum eosinophil ratio. A Wilcoxon rank-sum test was used for differences in change from baseline between the 2 groups. A p value <0.05 was considered statistically significant.

Results

Subjects

A total of 16 subjects were enrolled in this study and a summary of their demographic and baseline characteristics is shown in table 1. All subjects had severe persistent allergic asthma according to the Japanese Guideline for Prevention and Management of Asthma [12]. The duration of disease was longer, there were more subjects using oral prednisone and the inhaled corticosteroid (ICS) dose and total IgE were higher in the omalizumab group than in the control group. Other medications were similar between the 2 groups. There was no difference in the respiratory function and complications of allergic rhinitis or sensitization to allergens. All subjects were sensitized to *Df*.

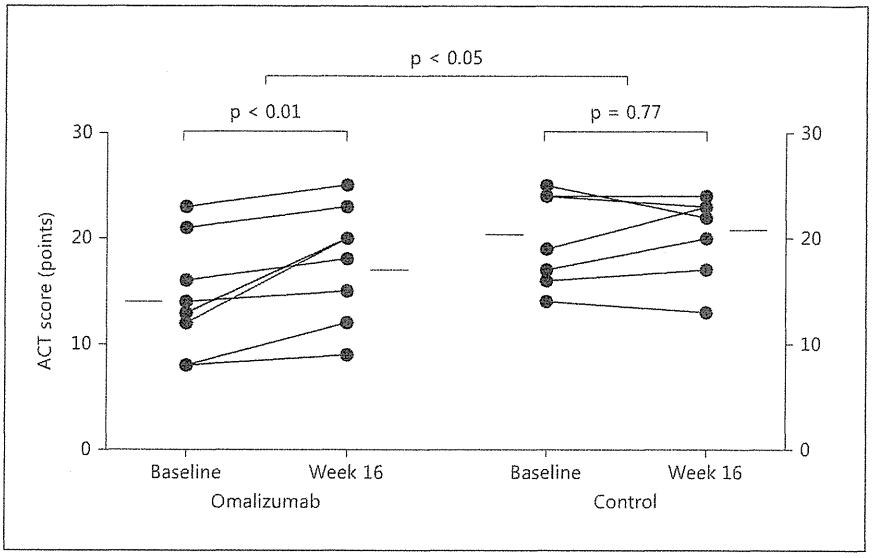

Fig. 1. Changes in ACT score from baseline to the end of week 16. The omalizumab group (n = 8) demonstrated a significant increase in ACT score compared to the control group (n = 8). Horizontal bars represent the means.

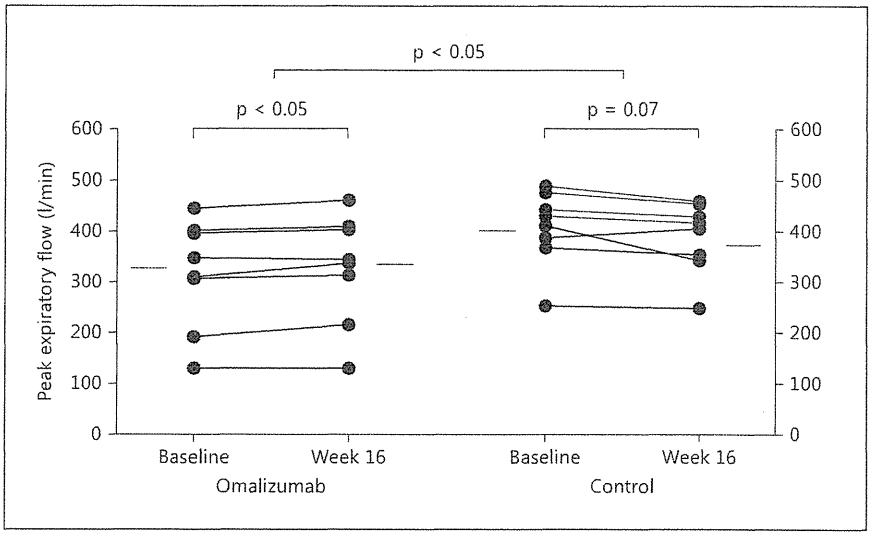

Fig. 2. Changes in morning PEFR from baseline to the end of week 16. The omalizumab group (n = 8) demonstrated a significant increase in morning PEFR compared to the control group (n = 8). Horizontal bars represent means.

Clinical Efficacy

In the omalizumab group, ACT score increased from baseline to week 16 (14.4 ± 2.0 to 17.8 ± 1.9, $p < 0.01$; fig. 1) as did morning PEFR (316.1 ± 38.2 to 327.1 ± 38.4, $p < 0.05$; fig. 2). There was also an improvement in the daily symptom score (data not shown). There were no significant changes observed for the control group in ACT score (20.4 ± 1.5 to 20.6 ± 1.3, $p = 0.77$; fig. 1), morning PEFR (407.3 ± 26.4 to 388.6 ± 25.0, $p = 0.07$; fig. 1) or daily symptom score (data not shown).

Effects on Airway Inflammation

FeNO decreased in the omalizumab group [21.0 (IQR 10.5–41.8) to 16.5 (IQR 7.3–29.3), $p < 0.05$; fig. 3], while no significant change was observed in the control group [11.0 (IQR 5.0–24.5) to 11.5 (IQR 5.0–24.5), $p = 0.62$; fig. 3]. Sputum was obtained from 7 subjects in each group. The ratio of eosinophils in sputum in the omalizumab group decreased from baseline to the end of week 16, but it was not a significant change [0.6 (IQR 0.0–2.8) to 0.0 (IQR 0.0–1.0), $p = 0.23$; fig. 4]. In contrast, the control group modestly increased from baseline to the end of

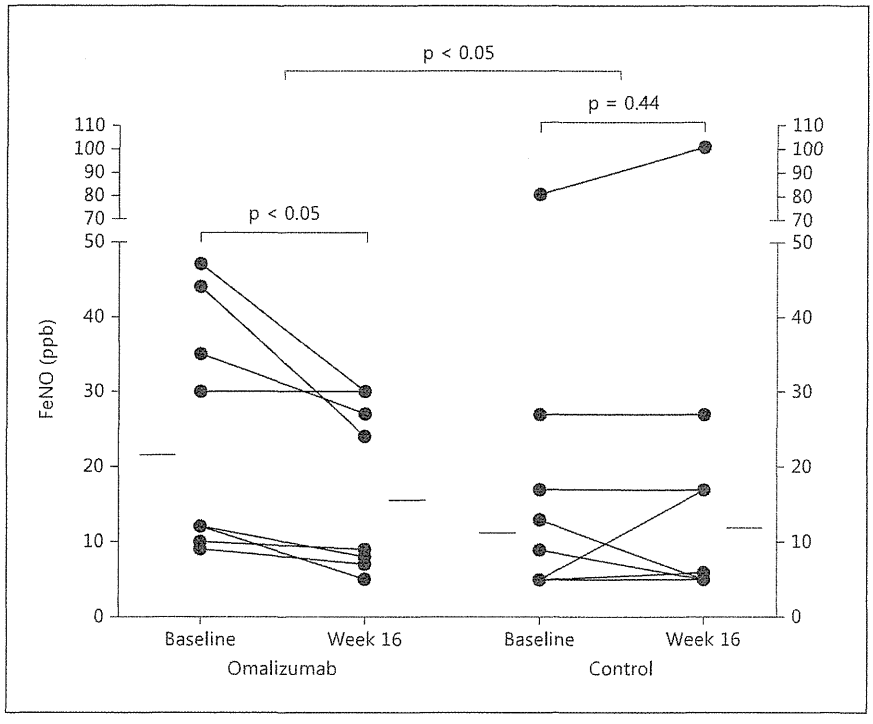

Fig. 3. Changes in FeNO from baseline to the end of week 16. The omalizumab group (n = 8) demonstrated a significant decrease in FeNO compared to the control group (n = 8). Horizontal bars represent medians.

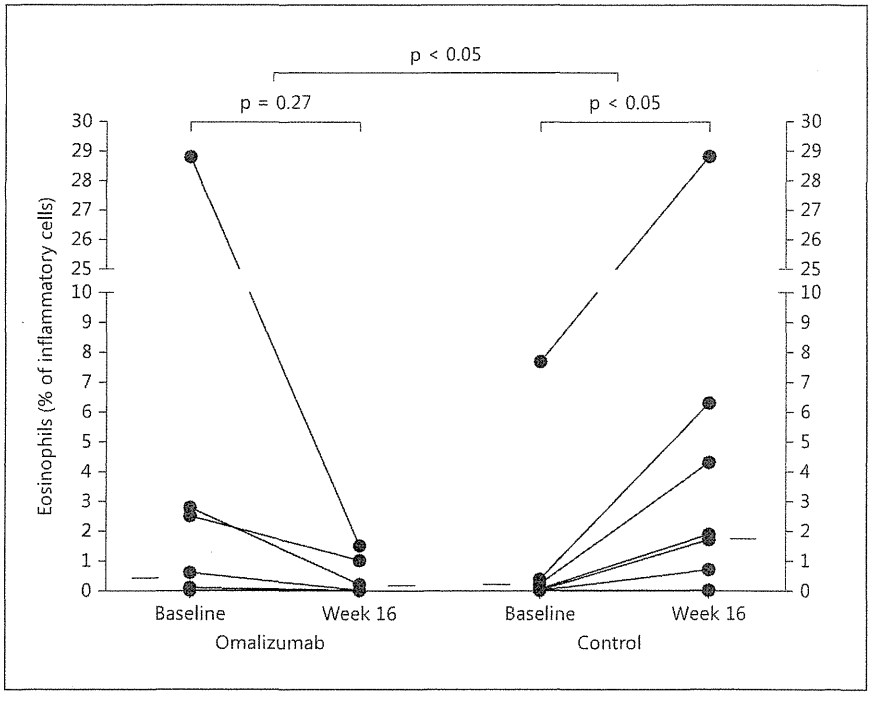

Fig. 4. Changes in sputum eosinophils (%). The omalizumab group (n = 7) showed a decrease from baseline to the end of week 16, but it was not significant. In contrast, the control group (n = 7) showed an increase from baseline to the end of week 16. There was a significant difference in the changes in eosinophil ratios in sputum between the groups. Eosinophils were identified by May-Giemsa staining and from morphological criteria. Horizontal bars represent medians.

week 16 [0.1 (IQR 0.0–0.4) to 1.9 (IQR 0.7–6.3), $p < 0.05$; fig. 4]. There was a significant difference in the amount of change in the sputum eosinophil ratio between the 2 groups ($p < 0.05$). There was no significant change in macrophage, neutrophil, lymphocyte or total cell counts in sputum from baseline to the end of week 16 within either group, and there was no difference between the groups (data not shown).

Effects on ex vivo Production of Cytokines by PBMCs

When PBMCs were stimulated with a specific antigen *(Df)*, IL-5 production was significantly decreased by the end of week 16 in the omalizumab group (60.8 ± 19.6 to 23.5 ± 9.2, $p < 0.05$) compared to the control group (23.1 ± 11.0 to 20.2 ± 9.1, $p < 0.05$), as shown in figure 5a. When PBMCs were stimulated with a combination of ionomycin and PMA, IL-5 levels also significantly decreased (omalizumab: 2,240 ± 484.6 to 1,359 ± 361.9, $p < 0.05$ and control: 753.2 ± 143.9 to 952.9 ± 113.2, $p = 0.26$; fig. 5b). In contrast, there was no change in IFN-γ when PBMCs were stimulated with *Df* (omalizumab: 571.4 ± 90.0 to 705.9 ± 121.1, $p = 0.09$ and control: 644.8 ± 85.2 to 737.9 ± 90.2, $p = 0.25$; fig. 6a). There was also no change in IFN-γ when PBMCs were stimulated with a combination of ionomycin and PMA (omalizumab: $1.9 \times 10^5 \pm 0.7 \times 10^5$ to $1.4 \times 10^5 \pm 0.4 \times 10^5$, $p = 0.55$ and control: $2.6 \times 10^5 \pm 0.7 \times 10^5$ to $2.9 \times 10^5 \pm 1.1 \times 10^5$, $p = 0.78$; fig. 6b).

Discussion

In this study, we confirmed that after 16 weeks of treatment with omalizumab, clinical indices such as symptoms, ACT score and PEFR were improved in a small group of Japanese subjects with severe, persistent asthma. These subjects also demonstrated a reduction in FeNO, which is an indirect parameter of eosinophilic airway inflammation, as well as decreased sputum eosinophils, which is a direct parameter of allergic airway inflammation. In addition, we found a reduction in the ex vivo production of IL-5 (a representative Th2 cytokine), but not IFN-γ (a representative Th1 cytokine) from PBMCs in severe asthmatics who were treated with omalizumab. These results suggest that omalizumab improves the clinical condition as well as the inflammatory status of the airways of subjects with severe asthma, and both of these results are likely associated with a systemic reduction of Th2 cytokine(s). This study is the first to show that omalizumab affects the cytokine-producing profile of PBMCs stimulated by either a specific allergen or a nonspecific agonist in Japanese asthmatics.

We observed that the eosinophil count in sputum was attenuated in subjects treated with omalizumab but not in those given conventional therapy after 16 weeks of treatment. This result is similar to the findings in several other reports. For example, omalizumab treatment for 12 or 16 weeks reduced eosinophils in sputum and infiltration into the bronchial wall [15, 16]. EG2+ cells in the bronchial wall were also significantly reduced after 12 weeks of treatment with omalizumab or a placebo, there was a clear reduction in eosinophil counts in the omalizumab group, which was significantly different from the placebo group [28]. Omalizumab as an add-on treatment to ICS for 28 weeks reduced the elevation of blood eosinophil counts in patients with severe allergic asthma [11]. The suppression of eosinophilia leads to clinical benefits for asthmatics. Eosinophils are the main effector cells for asthma and play a central role in patient outcome. Exacerbation rate is reduced when asthma is controlled based on sputum eosinophil count [29, 30]. The frequency of asthma exacerbations and the eosinophil ratio in blood and sputum decrease significantly with mepolizumab administration, which is a monoclonal antibody for IL-5 [31]. In this study, FeNO decreased significantly. Eosinophils in sputum also decreased, but not significantly, due to outliers. Above all, it is important to note that omalizumab treatment as an add-on to conventional therapy inhibited residual eosinophil accumulation in airways despite the combination with high doses of ICS, LABA, LTRA and TP.

Potential mechanisms for the ability of omalizumab to decrease airway eosinophilia and provide the clinical benefits seen in our study are discussed below. We analyzed which cytokine release from PBMCs was modulated by omalizumab add-on treatment. Our study indicated that omalizumab for 16 weeks reduced ex vivo IL-5 release from PBMCs stimulated by both nonspecific stimuli and *Df*. This result supports previous observed reductions in circulating IL-5, in addition to decreasing circulating eosinophil counts [32]. As IL-5 has been shown to energize eosinophils and prolong their survival, decreasing IL-5 via omalizumab appears to be involved in reducing the effect of eosinophils.

Omalizumab can also reduce other Th2 cytokines such as IL-4 and IL-13. A study of patients with mild-to-moderate persistent asthma evaluated the inflammatory cells and bronchial biopsies following 16 weeks of treatment with omalizumab or placebo. Omalizumab led to a significantly greater reduction in eosinophil counts in bronchial submucosa than the placebo, with greater reduc-

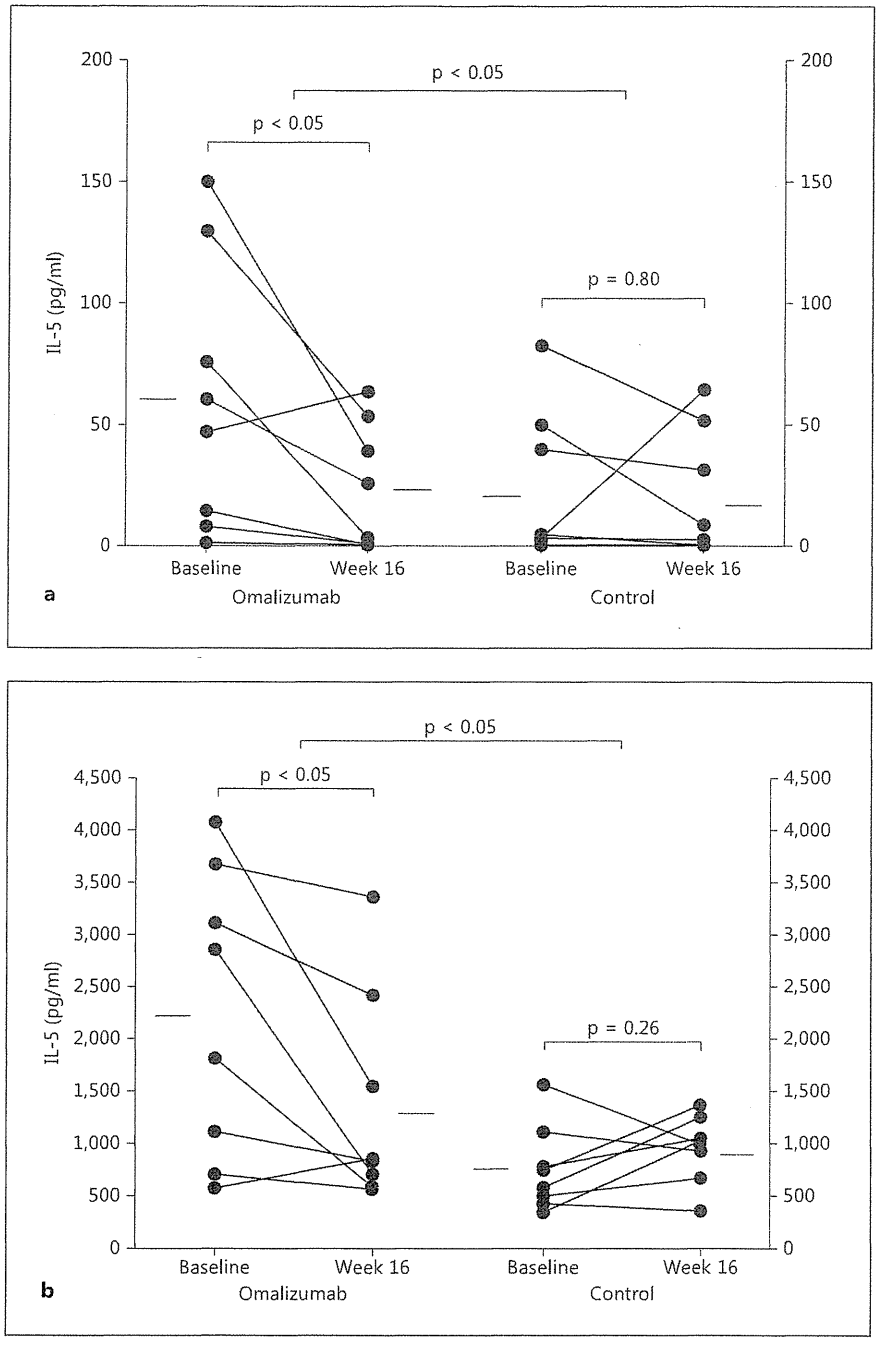

Fig. 5. IL-5 release from PBMCs of severe allergic asthmatics stimulated with *Df* antigen (**a**) or ionomycin and PMA (**b**). The omalizumab group (n = 8) demonstrated a significant decrease in IL-5 compared to the control group (n = 8). Horizontal bars represent means.

tions in cells immunostaining for IL-4 [16]. Following 12 weeks of treatment with omalizumab or placebo in moderate-to-severe asthma, there were significant reductions in the number of IL-13$^+$ T lymphocytes in the omalizumab group compared to in the placebo group [17]. Omalizumab treatment in patients with moderate-to-severe allergic asthma reduced circulating levels of IL-13, in addition to reducing circulating eosinophil counts [32]. Th2 cytokines induce asthmatic airway inflammation by activating mast cells and basophils, which promote eosinophil recruitment [33]. Omalizumab ameliorating the Th2 cascade seems to be a reasonable mechanism underlying

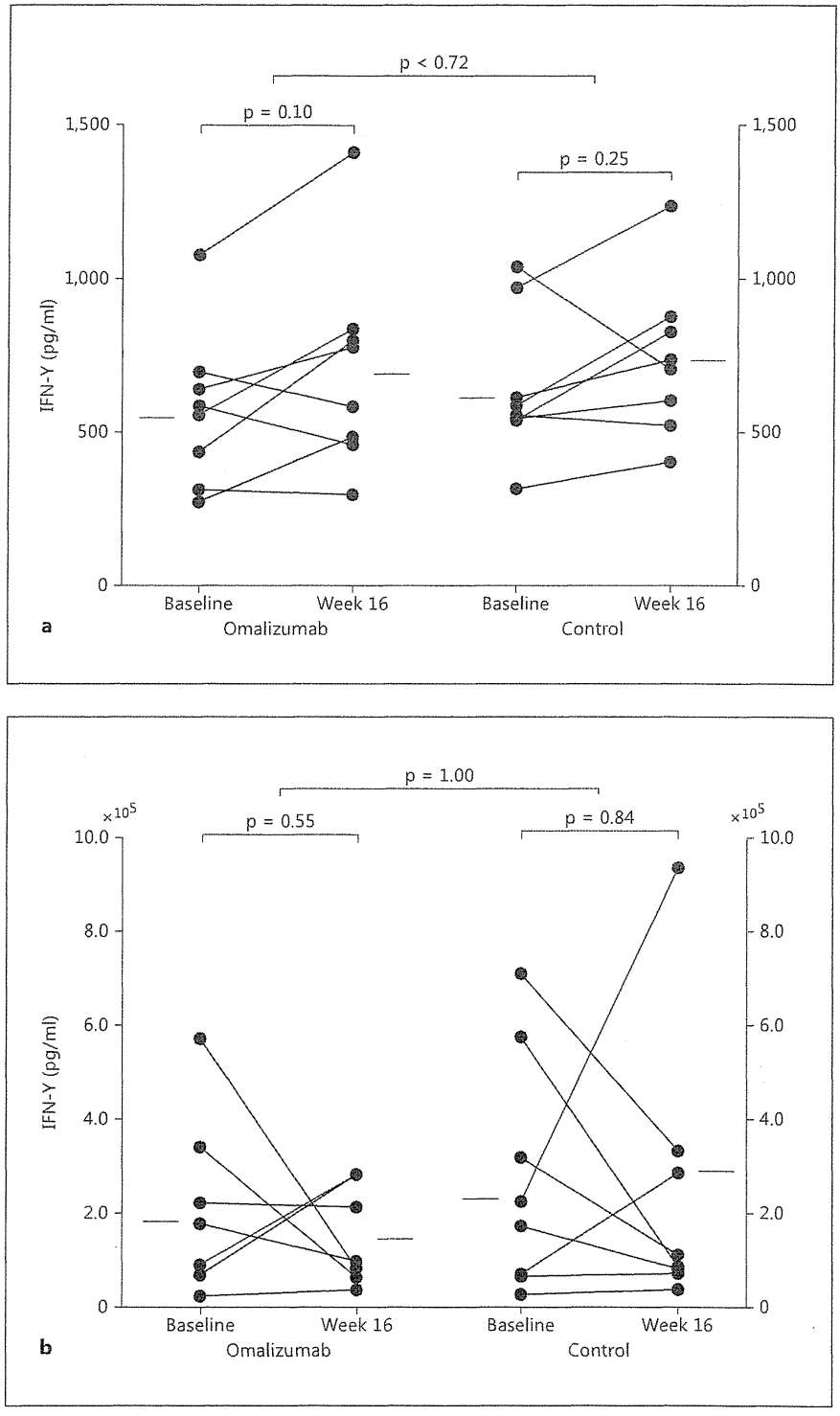

Fig. 6. IFN-γ release from PBMCs of severe allergic asthmatics stimulated with *Df* antigen (**a**) or ionomycin and PMA (**b**). Neither the omalizumab group (n = 8) nor the control group (n = 8) showed any changes in IFN-γ. Horizontal bars represent means.

the clinical benefit in severe persistent asthma. However, ex vivo release of IFN-γ (Th1 cytokine), with either stimuli, was not modulated in subjects treated with omalizumab. This result is consistent with a previous study in which omalizumab did not alter IFN-γ release from $CD4^+$ T cells when DCs and $CD4^+$ T cells were cocultured [34]. This preservation of the Th1 response would attenuate a Th2 shift, suggesting that omalizumab indirectly creates a balance between Th1 and Th2.

The reduction of Th2 cytokines is mainly the result of the decreasing numbers of Th2 cells. Compared with the placebo, omalizumab administered to patients with mild-to-moderate persistent asthma for 16 weeks significantly decreased the number of eosinophils as well as the number of $CD4^+$ T, $CD8^+$ T, B lymphocytes and IL-4-positive cells in the airway mucosa [16]. Similarly, omalizumab decreased $CD4^+$ T lymphocytes in the bronchial lumen 24 h after an allergen challenge in patients with mild allergic asthma [28]. These results suggest that omalizumab decreases Th2 cytokines, possibly via suppression of proliferation and/or accumulation of Th2 cells in the bronchial wall.

In this study, IL-5 release from PBMCs was reduced. A possible explanation may be that omalizumab attenuates DC-dependent T-cell proliferation and the production of Th2 cytokines in response to allergen. Schroeder et al. [34] reported that omalizumab decreased the production of lymphocytes by downregulating DCs. They also reported that the expression of FcεRI on DCs decreased after 3.5 months of omalizumab administration, that the antigen-presenting ability of DCs was weakened and that the release of IL-5 and IL-13 from $CD4^+$ T and DC cocultures stimulated with allergen was reduced [35]. Reduction of allergen-driven Th2 cell proliferation with attenuation of the activity of antigen-presenting cells such as DCs by neutralizing IgE may also occur in omalizumab-treated subjects leading to decreased IL-5. However, in this study, there was no change in the release of other Th2 cytokines (IL-4 and IL-13) from PBMCs, regardless of the stimuli. Other studies have shown that omalizumab decreases Th2 cytokines and $CD4^+$ T cells in blood and bronchial tissue, but to varying degrees. T cell production of cytokines invoked by antigen stimulation occurs without proliferation [36, 37], suggesting that some Th2 cytokines are not affected unless Th2 cell proliferation is reduced. Unique transcriptional mechanisms distinct from those regulating the IL-2 or IL-4 gene seem to control the IL-5 gene [38]. Consequently, these facts suggest that omalizumab might modulate a unique lymphocyte cascade of Th2 cytokine release, in particular, IL-5.

We found that the release of ex vivo IL-5 from PBMCs by nonspecific stimulation (ionomycin and PMA) was reduced in asthmatics treated with omalizumab, but not in those treated with conventional therapy. For IL-5 synthesis, both protein kinase C activation and $Ca2^+$ influx by T cells are required. In addition, activation of transcription factors, including NF-AT, AP-1 and NF-κB, is also required [39, 40]. Omalizumab might modulate this cascade, leading to IL-5 synthesis. Reduction of allergen-driven Th2 cell proliferation due to omalizumab might result in a decrease of IL-5 production by ionomycin and PMA stimulation as well as *Df* antigen. Even if omalizumab does not affect all Th2 cytokines, maintenance of the ex vivo release of IFN-γ from PBMCs in patients treated with omalizumab would correct the Th1/Th2 imbalance.

This study has a few limitations. It contained a potent selection bias since the assignment was dependent on whether or not the patient agreed with add-on omalizumab therapy. This bias may influence the subject characteristics of the treatment groups. The statistical power of the study might be insufficient to unequivocally prove the findings because the number of subjects was small. As more cases accumulate, we will need further investigation on the effect of omalizumab on lymphocytes.

In conclusion, this study indicates that 16 weeks of add-on treatment with omalizumab has a positive effect on clinical and inflammatory parameters in patients with severe, persistent allergic asthma who are not adequately controlled by standard therapy. This clinical benefit appears to be attributed to the inhibition of a Th2-type immunological process. Our results suggest that omalizumab has the potential to improve asthma outcome especially by decreasing the production of IL-5, which inhibits eosinophil respiratory tract inflammation. Omalizumab suppression of the cascade of Th2 cytokines and eosinophils supports the use of omalizumab as an add-on therapy.

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Disclosure Statement

The authors declare no financial or other conflicts of interest.

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A cohort study of mortality predictors in patients with acute exacerbation of chronic fibrosing interstitial pneumonia

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ABSTRACT

Objectives: To assess clinical, laboratory and radiographic findings associated with outcomes and to clarify more practical ways to predict hospital mortality in patients with acute exacerbation (AE) of chronic fibrosing interstitial pneumonia (CFIP).

Design: Single-centre retrospective cohort study.

Setting: University Hospital in Japan.

Participants: We identified 51 consecutive patients with AE of idiopathic CFIP through multidisciplinary discussion. Patients who had connective tissue disease, drug-induced lung disease, pneumoconiosis, hypersensitivity pneumonitis, sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis and eosinophilic pneumonia were excluded.

Interventions: There were no interventions.

Main outcome measures: The main outcome was determination of in-hospital mortality predictors. Other outcomes included clinical, laboratory and radiographic differences between non-survivors and survivors in patients with AE of CFIP.

Results: The mean age of the patients with AE of CFIP was 71 years. Compared with survivors, non-survivors had a significantly shorter duration of symptoms before admission, lower prevalence of peripheral distribution of ground-glass opacity and centrilobular emphysema (CLE) on thin-section CT, lower peripheral lymphocyte count, higher brain natriuretic peptide titre, lower Pao_2:Fio_2 (P:F) ratio, higher prevalence of systemic inflammatory response syndrome (SIRS) and higher SIRS score on admission (p=0.0069, 0.0032, 0.015, 0.040, 0.0098, 0.012, 9.9×10^{-7} and 5.4×10^{-6}, respectively). Multivariate analysis revealed SIRS (HR=6.2810, p=0.015), CLE (HR=0.0606, $p=3.6\times10^{-5}$) and serum procalcitonin level (HR=2.7110, p=0.022) to be independent predictors of in-hospital mortality. A Kaplan-Meier estimate on the basis of stratification according to the presence or absence of SIRS and CLE demonstrated a distinct survival curve for each subset of patients.

Conclusions: Distinct survival curves documented by stratification according to the presence or absence of SIRS and CLE may provide basic information for a rational management strategy for patients with AE of CFIP on admission.

ARTICLE SUMMARY

Article focus

- Several independent predictors of mortality in patients with acute exacerbation (AE) of idiopathic pulmonary fibrosis have been identified. However, more practical ways to predict hospital mortality, which may be of use in routine medical care, are required for AE of chronic fibrosing interstitial pneumonia (CFIP).
- This study was undertaken to identify practical mortality predictors in patients with AE of CFIP.

Key messages

- Systemic inflammatory response syndrome and centrilobular emphysema, which have not previously been evaluated as factors possibly affecting outcome, were the most significant predictors of in-hospital mortality in patients with AE of CFIP. Stratification according to the presence or absence of these two factors documented distinct prognoses for the subsets of patients and thus may be helpful for enabling more appropriate management strategies in the future.

Strengths and limitations of this study

- This study's strength was the identification of the novel, non-invasive and easily applicable predictors of in-hospital mortality in patients with AE of CFIP. The major limitation of the study was the single-centre retrospective design.

INTRODUCTION

In patients with a pre-existing chronic process of pulmonary fibrosis, such as idiopathic pulmonary fibrosis (IPF), an acute exacerbation (AE) is the development of an acute lung injury superimposed on the underlying disease. Although AE was first described in patients with IPF,[1] it has also been reported in underlying diseases other than IPF, such as interstitial pneumonia associated with connective tissue disease,[2 3] fibrotic non-specific interstitial pneumonia (NSIP),[2 3] hypersensitivity pneumonitis[4] and