Midazolam Inhibits IgE Production through Suppression of Class Switch Recombination

Hirotomo Kusama,1 Ryoki Kobayashi,2 Tomoko Kurita-Ochiai,2 Tomomi Hashizume-Takizawa,2 Manabu Ishikawa,1 Yu Fujita,1 and Koh Shibutani1

Departments of 1Anesthesiology, 2Oral Immunology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Abstract

Midazolam (MDZ) is reported to have immunomodulatory properties that affect immune cells. However, little is known about the effects of MDZ treatment on Immunoglobulin (Ig) E responses. Therefore, we examined whether MDZ is able to suppress total IgE production, followed by IgE class switch recombination (CSR), in a mouse model. To assess the effects of MDZ on IgE-CSR, splenic B cells were cultured with LPS, IL-4 and anti-CD40 antibody in the presence or absence of MDZ for 72 h. Total-IgE, interferon-gamma (IFN-γ) responses and surface IgE-positive (sIgE+) B cells were analyzed by ELISA and flow cytometry. To confirm IgE-CSR, total RNA was isolated from splenic B cells and levels of CSR-associated molecules, such as germ-line transcript ε (εGLT), germ-circle transcript ε (εCT), activation-induced cytidine deaminase (AID) and inhibitor of differentiation 2 (Id2) were compared. MDZ significantly decreased total IgE production and numbers of sIgE+ B cells. Significantly reduced levels of both εGLT- and εCT-specific mRNA were detected in MDZ-treated B cells. In contrast, Id2-specific mRNA transcript, which is a negative regulator for Ig-CSR, was increased on MDZ-treated B cells. Furthermore, MDZ-treated B cell significantly increased IFN-γ production and IFN-γRα expression. These results suggest that MDZ inhibits εGLT and εCT expression, and IgE synthesis via induction of IFN-γ production. MDZ may be useful for preventing IgE-mediated allergic diseases.

Introduction

Immunoglobulin (Ig) E plays an important role in the onset and development of Type-I allergic diseases. IgE binds to the high-affinity IgE receptor FcεRI on mast cells and basophils, and cross-linking of FcεRI by IgE and specific antigens induce the release of proinflammatory mediators, such as histamine, leukotrienes and various cytokines (1). Serum concentration of IgE is correlated with FcεRI expression levels on the surface of basophils and mast cells (1-3), and with disease severity, particularly that of cutaneous lesions (4, 5). Thus, controlling IgE production is a promising therapeutic option for allergic disease. In fact, a recombinant humanized anti-IgE monoclonal antibody, omalizumab, was recently shown to be highly effective in the treatment of severe asthma (6).

IgE is produced by plasma B cells, which are mainly regulated by cytokines. For example, interleukin (IL)-4 has been shown to induce IgE isotype class switching and production of IgE by B cells (7). While interferon-gamma (IFN-γ) has been reported to inhibit IgE, but favor IgG2a class switching in B cells (8). Although IgE has a relatively short half-life in plasma, mechanisms that tightly control IgE class switch recombination (CSR) are thought to contribute to low levels of plasma IgE (9-11). The regulation of CSR in B cells is coordinated with germ line transcription (GLT) of Cε genes and induction of activation-induced cytidine deaminase (AID) expression (12). Stimulation of CD40 on B cells by CD40 ligand (CD40L) on activated T cells is critical for AID induction. On the other hand, cytokines play an important role in the induction of GLT of distinct Cε genes. For example, ε-GLT is induced by IL-4 and IL-13 (13). In contrast, IFN-γ and IL-21 inhibit the ε-GLT (14, 15).

Sedatives play an important role in the management of
critically ill patients. In addition to their effects on the central nervous system, sedative drugs can have multiple effects on the immune system, including protective immunity against infection and tumors, as well as involvement in inflammatory processes in other diseases, such as cerebral ischemia or acute lung injury (16, 17). In addition to its action in the central nervous system as an agonist that acts differently on central versus peripheral benzodiazepine receptors, midazolam also exerts inhibitory effects on endothelial cells (18), monocytes (19) and macrophages (20). However, little is known about the influence of midazolam in IgE antibody production by B lymphocytes. Therefore, in this study, we examined the effects of MDZ on total IgE levels, as well as IgE CSR, in splenic B cells stimulated with LPS, IL-4 and anti-CD40 antibody. Furthermore, we investigated the immunoregulatory effects of MDZ on IFN-γ production and its receptor expression in splenic B cells.

Materials and Methods

Mice

Female BALB/c Cr Slc mice were purchased from Sankyo Laboratories (Tokyo, Japan) and were given regular mouse chow and water ad libitum. Mice were aged 8 to 12 weeks when used for experiments. All mice were maintained in temperature-controlled clean racks with a 12-h light/dark cycle. Mice were used in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Nihon University School of Dentistry at Matsudo (Approval number AP13 MD007).

B cell preparation

Mouse splenocytes were isolated by forcing the spleens through a metal mesh, and were suspended in RPMI 1640 medium supplemented with 2% fetal bovine serum (FBS) (21). Splenocyte suspensions were centrifuged at 1200 rpm for 8 min, and red blood cells were dissolved in lysing buffer. Lymphocytes were washed twice in RPMI 1640 and resuspended in medium. Cell number and viability were assessed by trypan blue exclusion assay after adjustment to 10⁶ cells/ml. B-cells were then isolated by negative selection with anti-mouse CD3, CD11b and CD11c-labeled magnetic microbeads. B-cells obtained by these treatments were >90% pure, as determined by flow cytometry with anti-B220 staining.

In vitro culture

In order to assess the effects of MDZ on CSR of B lymphocytes, mouse splenic B cells (10⁶ cells/ml) were cultured with LPS (10 μg/ml) as growth factor, IL-4 (10 ng/ml) and anti-CD40 (2.5 μg/ml) antibody as substitution for T cell help, in the presence or absence of MDZ for 72 h. The concentration of MDZ was determined using the method of Miyazaki et al. (22).

Total-IgE and IFN-γ ELISA

In order to assess total-IgE and IFN-γ levels in B cell culture, culture supernatants were collected and assayed using an ELISA kit specific for IgE (BD, San Diego, CA) (23) and IFN-γ (Thermo, Rockford, IL) (24).

Flow cytometry

In order to analyze the frequency of surface-IgE⁺ (sIgE⁺) B cells, 2 × 10⁵ mononuclear cells were incubated with phycoerythrin (PE)-labeled anti-IgE antibody (BioLegend, San Diego, CA) or biotin-labeled IFN-γRα (BioLegend, San Diego, CA) and Alex Fluor 647 B220 (BioLegend, San Diego, CA) at 4°C for 30 min. Samples were then subjected to FACS analysis (BD Biosciences, San Jose, CA) (25).

Quantitative real-time PCR analysis

Total RNA was purified from splenic B cells using Trizol reagent (Invitrogen, Tokyo, Japan) in accordance with the manufacturer’s protocol. Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) was used to generate cDNA from purified total RNA obtained from each mouse. Quantitative real-time RT-PCR analyses were performed using a Thermal Cycler Dice real-time PCR system (Takara, Shiga, Japan) in accordance with the manufacturer’s protocol. The initial denaturation step was carried out at 95°C for 5 s, followed by 40 cycles of 95°C for 15 s, 60°C for 25 s, and 60°C for 35 s. Each gene was tested in triplicate. Target RNA levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Levels of εGLT, germ-circle transcript ε (εCT), activation-induced cytidine deaminase (AID), inhibitor of differentiation 2 (Id2) and GAPDH cDNA were determined by quantitative PCR using the following primer pairs:

εGLT: 5′-GCACAGGGGGGCAGAAGAT-3′,
5′-CCAGGGTCAGGAAGCGACTG-3′
exCT: 5′-TTGGACTACTGGGGTCAAGG-3′,
5′-CAGTGCCCTTACAGGGCTTC-3′
AID: 5'-GGCTGAGGTTAGGGTTCCATCTCAG-3' 5'-GAGGGAGTCAAGAAAGTCACGCTGGA-3'
Id2: 5'-ATCGTCTTGCCCAGGTGTCGTTCT-3' 5'-AGCATCCCCCAGAACAAGAAGGTG-3'
GAPDH: 5'-TGTGTCCGTCGTGGATCTGA-3' 5'-TTGCTGTTGAAGTCGCAGGAG-3'

Statistical analysis

Student's t-test (two-tailed) was used for all statistical analyses and P values of <0.05 were considered to be statistically significant.

Results

MDZ inhibits IgE responses

We examined the immunoregulatory effects of MDZ on IgE antibody production in cultured splenic B cells. When we cultured purified mouse B cells stimulated with LPS, IL-4 and anti-CD40 antibody in the presence of various concentrations of MDZ, significantly reduced levels of IgE antibody were detected in B cell cultures treated with 10 μg/ml MDZ (Fig.1). Therefore, we used 10 μg/ml MDZ in subsequent experiments. We confirmed IgE production by flow cytometric analysis. Cultured B cells were stained with Alex Fluor 647-conjugated anti-mouse B220 and PE-conjugated anti-mouse IgE, and were analyzed by flow cytometry on a side scatter (SSC) /B220 plot (A) or IgE/B220 plot (B). The B cell population (Gate R1) was further gated for B220 binding and SSC properties (A) by selecting those that were stained with anti-IgE Abs. The results are representative of three different experiments. Panel C summarizes the results of three different experiments with triplicate cultures. Results are expressed as means±SD. *P<0.05 when compared with MDZ-free control.
including spleen, immunohistochemical staining showed that B220 is expressed by B cells. Therefore, B cells were distinguished on the basis of different SSC and B220+ properties (R1, Fig. 2A), and the cells were further stained with PE-conjugated anti-IgE antibodies (Fig. 2B). Interestingly, significant reductions in surface IgE expressing B cells was seen in B cell culture on treatment with MZD (Fig. 2B, 2C). Taken together, these results indicate that IgE production was diminished when B cell cultures were treated with MZD.

**Suppression of IgE CSR by MDZ**

In order to examine the mechanisms by which MDZ suppresses IgE production, we examined the effects of MDZ on CSR-mediated regulatory genes in splenic B cell culture.

**MDZ increases IFN-γ production and IFN-γRα expression in splenic B cells**

As the number of surface IgE+ B cells and the frequency of IgE CSR were lower in MDZ-treated culture than in non-treated culture, we speculated that soluble factors may be involved in MDZ-mediated suppression of CSR. To examine the mechanisms responsible for inhibition of IgE production, we assessed the production of IFN-γ, which is known to regulate IgE CSR in activated B cells. MDZ significantly increased IFN-γ production in B cell culture (Fig. 4A). Furthermore, when we examined IFN-γRα expression on MDZ-treated B cells, expression of IFN-γRα on B cells was significantly increased by MDZ (Fig. 4B).
**Discussion**

In this study, we demonstrated that MDZ inhibited the production of total IgE, as well as the percentage of surface IgE+ B cells in the spleen in concert with suppression of IgE CSR. Furthermore, IFN-γ secretion and receptor expression were increased in splenic B cells cultures treated with MDZ.

BDZ, particularly MDZ, has been widely used at high doses for anesthetic induction and maintenance, as well as sedation during locoregional anesthetic procedures (26, 27). IgE plays a critical role in the pathogenesis of allergy and asthma. Therefore, the combined action of suppression of IgE production and anesthesia promotion makes MDZ an ideal agent for use in allergy prevention in addition to its sedative effects.

MDZ significantly suppressed CSR to IgE through the inhibition of eGLT and εCT expression. On the other hand, expression of Id2 was increased by MDZ. It has been shown that Id2 suppresses IgE CSR by inhibiting the εGLT-inducing activities of E2A and Pax5 (28, 29). Therefore, MDZ may inhibit γGLT and εCT expression through increased expression of Id2. Although the mechanism through which MDZ enhances Id2 expression is unclear, the production of TGF-β may have increased through MDZ, as TGF-β inhibits IgE CSR through induction of Id2 (11).

MDZ effectively suppressed the levels of IgE production, as well as surface IgE+ B cells in splenic B cells cultured with LPS, IL-4 and anti-CD40 antibodies. Isotype switching by B cells is division-associated and regulated by cytokines. IL-4 and IL-5 are essential for IgE production and IgE-mediated allergic responses because of their function in recruiting B cells, and eosinophils involved in allergic inflammatory reactions (30, 31). Meanwhile, IFN-γ has strong inhibitory effects on IgE production (32) via the suppression of ε GLT followed by downregulation of CSR to ε (14). In this study, MDZ significantly increased the production of IFN-γ, as well as the receptor expression in splenic B cell cultures. One of the cytokines produced in large quantities by activated B cells is IFN-γ. Although IFN-γ-producing B cells have been identified in mice infected with pathogens such as *Toxoplasma gondii* and *Borrelia burgdorferi* (33, 34), the molecular mechanisms that regulate IFN-γ production by B cells are not known. It has previously been shown that B cells produce IFN-γ in response to antigen (35), and to cytokines such as IL-12 and IL-18 (36, 37). In addition, B cells cultured in the presence of polarized Th1 cells and antigen develop into IFN-γ-producing B effector 1 cells that are capable of promoting differentiation of naïve T cells into Th1 effectors (33). Therefore, our data suggest that combined signals through B-cell receptor (BCR) and CD40 ligation, IL-4 and other signals induced by MDZ are necessary to induce differentiation of naïve B cells into IFN-γ-producing effectors.

In summary, although the mechanisms by which MDZ enhances IFN-γ secretion in splenic B cells remain uncertain at the cellular and molecular levels, MDZ attenuated IgE secretion and production of surface IgE+ B cells via suppression of IgE CSR and IFN-γ secretion. Our results suggest that intravenous injection of MDZ prevents IgE-related allergic diseases and exerts a sedative effect.

**Acknowledgments**

We would like to thank Dr. Michiharu Shimosaka (Department of Dental Anesthesiology, Nihon University School of Dentistry at Matsudo) for helpful suggestions. We would also like to thank to Professor Koh Shibutani (Department of Dental Anesthesiology, Nihon University School of Dentistry at Matsudo) and Professor Masafumi Yamamoto (Department of Oral Immunology, Nihon University School of Dentistry at Matsudo) for critical reading of this paper and helpful comments. This work was supported by a Grant-in-Aid for Challenging Exploratory Research (25670676) from the Japan Society for the Promotion of Science.

**References**


