Midazolam Suppresses Maturation of Murine Dendritic Cells and Priming of Lipopolysaccharide-induced T Helper 1-type Immune Response

Noriyuki Ohta, M.D., Ph.D.,* Yoshifumi Ohashi, M.D.,† Chihiro Takayama, M.D.,† Takashi Mashimo, M.D., Ph.D.,‡ Yuji Fujino, M.D., Ph.D.§

ABSTRACT

Background: Dendritic cells (DCs), as antigen-presenting cells, play a key role in the induction and regulation of adaptive immune response. Midazolam is reported to have immunomodulatory properties that affect immune cells. However, the effect of midazolam on DCs has not been characterized. We examined the immunomodulatory properties of midazolam on DC-mediated immune response.

Methods: After allowing murine bone marrow–derived DCs induced by granulocyte macrophage colony stimulating factor to mature, we analyzed their expression of costimulatory molecules (CD80 and CD86), major histocompatibility complex II molecules, and the secretion of interleukin-12 p40. In vitro, we evaluated the effect of midazolam on maturing DCs in mixed cell cultures containing DCs and T cells. In vivo, we investigated the contact-hypersensitivity response.

Results: Midazolam suppressed the expression of CD80, CD86, and major histocompatibility complex II molecules from murine DCs. Treated with midazolam, DCs also secreted less interleukin-12 p40. In mixed cell cultures with CD3-positive T cells, midazolam-treated DCs showed less propensity to stimulate the proliferation of CD3-positive T cells and the secretion of interferon-γ from CD4-positive T cells. Midazolam-treated DCs impaired the induction of contact-hypersensitivity response. Treatment with ligands for peripheral benzodiazepine receptor inhibited the up-regulation of CD80 during DC maturation.

Conclusion: Midazolam inhibits the functional maturation of dendritic cells, interfering with the lipopolysaccharide-induced T helper 1-type immune response in the mouse through the activation of peripheral benzodiazepine receptors.

The first step in the induction of adaptive immune response is when antigen-presenting cells present antigen to naïve T cells. Dendritic cells (DCs) are the most potent antigen-presenting cells and play a key role in the regulation of immune response. Thus, the possibility of harnessing the power of DCs to fight infectious disease and cancer and to regulate inflammatory disease has received prominent attention in research immunology.1–3

DCs residing in peripheral tissue are phenotypically and functionally immature. On stimulation with cytokines, mi-
crobes, or pathogen-associated molecular patterns, such as lipopolysaccharide, DCs mature. This maturation involves decreased antigen uptake capacity, high levels of major histocompatibility complex (MHC) class II and costimulatory molecules (CD40, CD80, and CD86), and the production of interleukin 12 (IL-12). Once loaded with antigens and activated by maturation stimuli, DCs act on lymphocytes through cytokines and costimulatory molecules, leading to the differentiation of lymphocytes into T helper 1 (Th1) cells and other effectors.

Sedatives play an essential role in the management of critically ill patients. In addition to their effects on the central nervous system, sedative reagents can have multiple effects on the immune system, including protective immunity against microbes and tumors, as well as involvement in inflammatory processes in other forms of disease, such as cerebral ischemia or acute lung injury. Besides 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, monocytes, macrophages. Little attention has been paid to the effects of midazolam on human blood monocyte differentiation to DCs.

In this study, we tested the hypothesis that midazolam affects DCs by changing the polarity of the immune response they induce. Using murine bone marrow–derived DCs (BM-DCs), we found that midazolam inhibits the maturation of murine BM-DCs and impedes the ability of DCs to prime the Th1-biased immune response. Furthermore, our evidence suggests that the suppressive effect of midazolam is mediated by the peripheral benzodiazepine receptor (PBR) rather than the central benzodiazepine receptor (CBR).

Materials and Methods

Animals
Female C57BL/6 and Balb/c mice (aged 4–6 weeks) were purchased for cell samples (Japan CLEA, Tokyo, Japan). Animals were housed with food and water available ad libitum in the specific pathogen-free central animal facility of Osaka University Medical School (Suita, Japan). All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Osaka University Medical School (Suita, Japan) and carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reagents and Antibodies
Recombinant mouse granulocyte macrophage colony stimulating factor was purchased from R&D Systems (Minneapolis, MN). Fluorescein isothiocyanate-conjugated dextran (FITC-dextran [40,000 molecular mass]) and lipopolysaccharide from Escherichia coli 055:B5 were obtained (Sigma-Aldrich, St Louis, MO). FITC- or phycoerythrin-conjugated monoclonal antibodies (mAbs) were used to detect the expression of CD11c (HL3), MHC class II (I-Ab), (AF6-120.1), CD80 (16-10A1), and CD86 (GL1) were purchased from BD Biosciences (San Diego, CA). For intracellular cytokine detection, we used mAbs for tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin 4 (11B11, MP6-XT22, and XMG1.2; BD Biosciences). Ligands Ro5-4864 and PK11195, specific to PBR, and clonazepam, specific to CBR, were purchased from Sigma-Aldrich. Ligands were dissolved separately in dimethyl sulfoxide, reserved, and diluted with culture medium immediately before use.

Isolation and Culture of DC
Using a previously described method with minor modifications, DCs were generated from murine bone marrow cells. In brief, murine bone marrow was flushed from the tibiae and femurs of C57BL/6 mice and then depleted of red cells with ammonium chloride. Bone marrow cells were suspended in complete media (RPMI-1640 supplemented with 10% fetal bovine serum albumin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells for each mouse were plated on 10-cm plates at 5 × 10^5/ml and cultured in the presence of 20 ng/ml recombinant mouse granulocyte macrophage colony stimulating factor at 37°C in 5% CO₂. On days 3 and 5 of the culture, floating cells were gently removed and fresh medium supplemented with recombinant mouse granulocyte macrophage colony stimulating factor was added. On day 7, nonadherent cells and loosely adherent proliferating DCs were harvested and, by centrifugation at 600g for 20 min at room temperature, purified by 14.5% Accudenz density gradients (Accurate Chemical & Scientific Corporation, Westbury, NJ). Purified samples of DCs were cultured for another 2 days with and without midazolam and then stimulated with 100 ng/ml lipopolysaccharide for 24 h.

The viability of cultured cells was assessed by trypan blue exclusion testing. Cell viability of more than 90% was observed in all experimental cultures used in this study.

Flow-cytometric Analysis of Surface Molecules
The expression of surface molecules on DCs was analyzed under flow cytometry. At each step of staining, to prevent nonspecific binding of antibodies, 1–2 × 10^5 cells were incubated for 15 min on ice in staining buffer containing anti-CD16/CD32 mAbs. Cells were stained with specific antibodies. After mAb staining, 7-amino actinomycin D viability staining solution (eBioscience, Inc., San Diego, CA) was applied to stain dead cells. Samples were analyzed using BD FACSCalibur and BD CellQuest Pro (BD Biosciences). We used FITC- or phycoerythrin-labeled mAbs to stain for MHC class II, CD80, CD86, and CD11c. Using flow cytometry, dead cells were gated out by staining with 7-amino actinomycin D and only live cells were phenotypically assessed.

Interleukin-12 p40 Enzyme-linked Immunosorbent Assay
DCs were cultured in the presence versus absence of midazolam, followed by stimulation with lipopolysaccharide (100...
ng/ml) for 12 h. Following manufacturer instructions, we analyzed culture supernatants using IL-12 p40 enzyme-linked immunosorbent assay kits (R&D Systems).

**Intracellular Cytokine Assay**

For intracellular cytokine assay, T cells were restimulated with ionomycin and phorbol myristate acetate in the presence of BD GolgiStop (BD Biosciences). Following manufacturer instructions, intracellular cytokines were detected using standard Cytofix/Cytoperperm Plus Kits (BD Biosciences). We used phycoerythrin-labeled mAbs to stain TNF-α, IFN-γ, and interleukin 4, and FITC-labeled mAbs for staining CD4.

**Quantification of Antigen Uptake**

As described by Sallusto et al., endocytosis was quantified. In brief, 2 × 10⁵ cells were equilibrated at 37°C or 4°C for 10 min and then pulsed with 1 mg/ml FITC-dextran. Cold staining buffer was added to stop the reaction. Cells were washed three times and stained with phycoerythrin-conjugated anti-CD11c antibodies before analysis with BD FACS Calibur. Nonspecific binding of FITC-dextran to DCs was assessed by evaluating FITC-dextran uptake at 4°C. Mean fluorescence intensity at 4°C was used as the measure of antigen uptake.

**Reverse Transcription–Polymerase Chain Reaction Analysis of PBR-specific Messenger RNA (mRNA)**

After 7-day culture, BM-DCs were purified by magnetic sorting using magnetic-activated column sorting column and beads coated with anti-CD11c antibody (Miltenyi Biotech, Bergisch Gladbach, Germany). Total RNA from BM-DCs was prepared with RNeasy mini kit (QUIAGEN, Hilden, Germany) and complementary DNA was synthesized (Superscript III; Invitrogen Life Technologies, Tokyo, Japan). To amplify PBR complementary DNA, the following primers were used: 5′-AGTGTCCTTCACGGAA-CACAC-3′ and 5′-AAGCTGGTGACTCTGACAGACT-3′. After heating at 95°C for 10 min, complementary DNA was amplified for 35 cycles, each cycle consisting of 95°C for 15 s then 60°C for 60 s. Amplified products were separated by electrophoresis on 1.8% agarose gel and stained with 1 μg/ml ethidium bromide.

**Allogeneic Mixed Cell Culture Reaction**

DCs induced from C57BL/6 mice (haplotype I-Ab) were incubated in the presence and absence of midazolam. Subsequently, DCs were stimulated by 100 ng/ml lipopolysaccharide for 12 h and treated with 50 μg/ml mitomycin C. To enrich CD3-positive T cells from splenocytes, splenocytes isolated from 6-week-old female Balb/c mice (haplotype I-Ad) were purified in magnetic-activated column sorting. Enriched CD3-positive T cells were cocultured with mitomycin C–treated DCs. In 96-well round-bottom plates, mixed samples were cultured for 4 days in RPMI-1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. Cell proliferation was estimated based on uptake of [3H]thymidine. For this purpose, cells were pulsed with [3H]thymidine for the final 18 h of mixed cell culture. Then, radioactivity was measured using a liquid scintillation counter (PerkinElmer, Waltham, MA). Intracellular cytokines were detected, following manufacturer instructions, using standard Cytofix/Cytoperperm Plus Kits, as described.

**Contact Hypersensitivity Model and Model with Adoptive Transfer of DCs**

In positive control groups for the original contact hypersensitivity (CHS) model, 2,4-dinitro-1-fluorobenzene (DNFB) was used as a contact-sensitizing agent, and DNFB, 0.5%, was dissolved in 4:1 acetone/olive oil was applied to the shaved abdomen on day 0. On day 5, mice were challenged by epicutaneous application of DNFB on the right ear. After 24 h, using a spring-loaded micrometer (Mitutoyo Corporation, Kawasaki, Japan), we measured right (challenged) and left (unchallenged) ear thickness. Increased ear thickness was evaluated by simple subtraction: thickness of challenged ear − thickness of unchallenged ear.

A CHS model involving adoptive transfer of BM-DCs pulsed with peptide-antigen was designed as described in a previous report and used. DCs were prepared as described in isolation and culture of DC. After culture in the presence or absence of midazolam, 5 × 10⁵ DCs (100 μl saline) were pulsed with 100 μg/ml 2,4-dinitrobenzene sulfonic acid (DNBS: a water-soluble analog of DNFB) and injected subcutaneously on day 0. After 5 days, mice were challenged on both sides of the right ear by the application of 10 μl DNFB, 0.2%, in 4:1 acetone/olive oil solution. At the time of initial immunization, negative control animals were injected with 100 μl saline and exposed to DNFB 5 days later. After 24 h, using a spring-loaded micrometer, ear thickness was evaluated as described above.

**Statistical Analysis**

In a preliminary study, power analysis showed a group size of eight (N = 8) as sufficient to detect a 50% reduction of surface-molecule expression by midazolam with a power of 0.8 at an α level of 0.05. Normality of distribution was tested using the Kolmogorov-Smirnov test. Data are expressed as mean ± SD or median (25–75% interquartile range), as appropriate. Comparison of means was performed using the Student t test or ANOVA and the post hoc Tukey test. Comparison of medians was performed using the Mann–Whitney U test. All analyses were performed using two-tailed tests. A P value of less than 0.05 was considered statistically significant. Statistical analysis was conducted using JMP version 8.0 (SAS Institute, Cary, NC) and STATISTICA 06 software (StatSoft, Inc., Tulsa, OK).
Results

Midazolam Inhibits Maturation of Murine DCs

At first, we examined whether midazolam inhibits the maturation of murine DCs. Our preliminary investigations of DC culture showed that midazolam has no effect on DC viability in concentrations up to 200 μM (data not shown). To examine how midazolam influences the maturation of DCs, we cultured immature DCs in the absence versus presence of 15 μM midazolam. We measured the expression of costimulatory molecules and MHC class II molecules after 24-h lipopolysaccharide stimulation. As DCs matured, we observed higher expression of MHC class II, CD80, and CD86 molecules (fig. 1A). Furthermore, midazolam suppressed the expression of CD80 molecules in a dose-dependent manner (fig. 1B).

Midazolam Inhibits Secretion of Interleukin-12 p40

Mature DCs are important for the synthesis and secretion of cytokines that affect T-cell differentiation, being responsible for the quality of immune response. DCs produce proinflammatory cytokines. In particular, IL-12 production is a marker of DC maturation and can be used as a method of selecting the Th1-dominant adjuvant. Here, we tested the expression of IL-12 p40 from lipopolysaccharide-stimulated DCs. As figure 2A shows, midazolam inhibited IL-12 p40 secretion.

Midazolam Induces Immature DCs with High Endocytotic Capacity

Evaluation of surface molecule and IL-12 p40 expression indicated that exposure to midazolam significantly suppresses phenotypical and functional maturation of DCs generated in vitro. These results did not, however, exclude the possibility that midazolam causes a general inhibition of DC function. In addition, we investigated whether midazolam exposure alters the ability of DCs to capture antigen through the uptake of FITC-dextran. As figure 2B shows, DCs exposed to midazolam for 7 days and then left in the presence of lipopolysaccharide for 24 h displayed increased endocytotic capacity for FITC-dextran. All our findings—including those for surface molecule and IL-12 p40 expression as well as those for endocytotic activity—strongly suggest that midazolam prevents DC maturation.

Midazolam Inhibits T-cell Proliferation and Th1-type Immune Response

To clarify how midazolam-mediated alteration of DC function might affect DC-induced immune response, we investigated
how this medication affected mixed cell culture reaction in DC lymphocytes. In mixed cell culture reaction, DCs from C57BL/6 mice (haplotype I-Ab) cultured with allogeneic T cells from Balb/c mice (haplotype I-Ad) induced Balb/c T cells to proliferate and differentiate into Th1-secreting IFN-γ and TNF-α. In mixed cell culture reaction, DCs matured with lipopolysaccharide in the presence or absence of midazolam were tested for their capacity to stimulate allogeneic T cells.

Coculturing DCs without midazolam effectively enhanced proliferative responses assessed by increase of [3H]thymidine uptake by T cells. By contrast, with DCs that had been treated with midazolam, there was less [3H]thymidine uptake by allogeneic T cells (fig. 3A).

Another determinant of DC potency for priming T cells is how well they are able to adhere to and form clusters on T cells (fig. 3B). Compared with control, we found that midazolam-treated DCs formed fewer clusters on T cells.

In mixed cell culture reaction, DCs matured by lipopolysaccharide also induced the cocultured CD4-positive T cells to differentiate into IFN-γ-secreting Th1 T cells. We evaluated whether midazolam had any effect on this function. Intracellular cytokine analysis revealed a lower density of IFN-γ- and TNF-α-producing CD4-positive cells stimulated by midazolam-treated BM-DCs (figs. 4A and B).

**Midazolam-treated DCs Failed to Elicit CHS that Would Normally Have Been Induced by Th-1 Type Immune Response**

Induced mainly by Th1-type T cells, including CD4-positive and CD8-positive T cells, CHS is a prototypical in vivo model of cell-mediated immune response. In the original model, CHS was induced by epicutaneous immunization with DNBS into flanks and subsequent epicutaneous challenge with haptens, such as DNFB, to ears, followed by swelling of the challenged ears. CHS can be also induced by a single subcutaneous injection (day 0) of 5 × 10^6 DCs that...
have been pulsed with DNBS, followed, on day 5, by epicutaneous challenge with DNFB. We used this CHS model with adoptive transfer of DCs to assess the in vivo effect of midazolam on DC-induced Th1-type immune response in the whole animal. Immunization with midazolam-treated DCs elicited less CHS response than immunization with vehicle-treated DCs (fig. 5).

**Effect on Expression of CD80 during DC Maturation of Ligands Specific to Peripheral and Central Benzodiazepine Receptors**

Next, we investigated how benzodiazepine receptors are involved in the suppressive effect of midazolam by testing with ligands Ro5-4864 and PK11195, specific to PBR, and clonazepam, specific to CBR. The effect of these ligands on DC maturation was examined by evaluating surface-expression of CD80. Although no CD80 suppression was observed in the presence of clonazepam (within the range of concentration of PBR ligands), the dose-dependent suppression of these molecules was detected in the presence of Ro5-4864 and PK11195 (fig. 6A).

**Identification of mRNA Specific for PBR**

We next assessed the existence of PBR-specific mRNA in BM-DCs. From total RNA isolated from BM-DCs, complementary DNA was synthesized. After analysis for PBR-specific mRNA by reverse transcription–polymerase chain reaction, mRNA specific for PBR was identified (fig. 6B).

**Discussion**

In the current study, we demonstrated that midazolam inhibits the phenotypic maturation of DCs and suppresses the DC-induced induction of the Th1-type immune response in...
munosuppressive conditions that are often complicated in inflammation can be sometimes beneficial, but during the immune response, suppressive activity to reduce or avoid excessive midazolam's suppressive activity remain controversial. In invading pathogens. In critical care, the beneficial effects of midazolam might compromise the immune response against infection. Previous investigators have suggested that development toward Th1 is regulated by DC-derived cytokines, such as IL-12. In addition, our study showed that lipopolysaccharide-stimulated DCs that normally occurs in the presence of lipopolysaccharide. As maturation progresses, DCs become increasingly able to stimulate T cells to proliferate and differentiate into effector T cells. By contrast, lipopolysaccharide-stimulated DCs treated with midazolam showed a reduced ability to up-regulate the stimulating capacity of allogeneic T cells. Previous investigators have suggested that development toward Th1 cells is regulated by DC-derived cytokines, such as IL-12. In addition, our study showed that lipopolysaccharide-stimulated DCs differentiate naive T cells into IFN-γ-producing Th1 T cells. Our current finding that midazolam inhibits DC production of IL-12, shows that midazolam affects the action of DCs on the differentiation of T cells to Th1 T cells.

We conjectured that, in the whole animal, midazolam probably impairs the ability of DCs to initiate immune response mediated mainly by Th1 T cells. To test this hypothesis, we used CHS, a well-established model for testing the delayed hypersensitivity that is induced by Th1-type immune response. After confirming that subcutaneous immunization with DNBS-DC made mice susceptible to CHS, we designed a CHS model for the adoptive transfer of DCs. We found that, when mice were similarly exposed to DNBS-DCs cultured with midazolam, CHS was reduced. This result provides evidence that midazolam suppresses the DC-mediated induction of Th1-type immune response in the whole animal.

In mice, repeated administration of diazepam has been reported to impair cell-mediated immunity regulated by the Th1-type immune response. We speculate, in view of the central role of DCs in the induction of immune response, that the suppressive effects of benzodiazepine on cell-mediated immunity is probably mediated by the effect of benzodiazepine on DCs.

With practical development in prospect for controlled Th1-response induction, such as ex vivo manipulation of DCs for DC therapy to treat malignant tumors or infectious disease, our findings, which show the effect of midazolam on DC-induced Th1-response, suggest a candidate means to counter-regulate the immune response induced by DC therapy. Considering the effects on DCs and the Th1-type immune response observed in the current study, midazolam might compromise the immune response against invading pathogens. In critical care, the beneficial effects of midazolam’s suppressive activity remain controversial. In sepsis, suppressive activity to reduce or avoid excessive inflammation can be sometimes beneficial, but during the immunosuppressive conditions that are often complicated in the course of sepsis, the suppressive effects of midazolam may compromise the eradication of infection. However, as a clinically administered drug, midazolam may be a good candidate in safely controlling DC-mediated immune response. Pharmacologic experiments using ligands specific to PBRs and CB1Rs showed that, although clonazepam did not suppress surface-molecule expression on DCs, PBR ligands did—in a manner similar to midazolam—suppress the up-regulation of CD80 during DC maturation. The existence of PBR-specific mRNA and our results using PBR- and CB1R-specific ligands, strongly suggest the involvement of PBRs in the suppressive effect of midazolam on DCs. It has been reported that PBR ligands are involved in autoimmune arthritis in MRL/lpr mice. In another instance, treatment with PBR ligands was shown to suppress arthritic lesions in MRL/lpr mice. Because DCs play a central role in the induction and regulation of immune response, such evidence may suggest that action on DC-PBR ligands mediates the suppression of the immune responses observed in autoimmune arthritis.

This study has several limitations. First, our results are based on experiments using mice; no human tissue was tested. The BM-DC culture system used in most of the experiments reported here has become a well-established system as a widely used means for investigating DC biology. Culturing DCs induced from murine bone marrow with recombinant mouse granulocyte macrophage colony stimulating factor has generated many results that are comparable with standard human DC culture derived from blood monocytes. It is possible that the results reported here would differ in human cells. Our results need to be confirmed using human cultured DCs derived from blood monocytes.

In addition, the findings of our in vitro studies are of questionable relevance to in vivo conditions found in whole animals, where conventional DCs, plasmacytoid DCs, and other DCs are present. Although the BM-DCs generated in our study resemble commonly encountered monocyte-derived DCs in conventional DC subsets, BM-DCs have no counterpart in conventional DC subsets of lymphoid-organ–resident DCs or plasmacytoid DCs. Further investigation of in vivo subsets of DCs is required to clarify the effect of midazolam on in vivo DCs.

The effects of midazolam on DCs in this study were evaluated after exposure to 15 μM midazolam. In addition, we tested for dose-dependent responses by exposing DCs to 0–50 μM midazolam and measuring the expression of surface molecules. Changes in surface-molecule expression at 5 μM midazolam (actual clinical concentration) show the same tendencies observed with 15 μM midazolam. We chose 15 μM midazolam as a proof of principle that was supported by the response of CD80 expression at the lower concentration (5 μM), which is more clinically relevant. In a pharmacokinetic-modeling study of midazolam-based sedation, EC50 concentration for deep sedation (e.g.,
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Ramsay Sedation Scale score higher than 6) is reported to be 6.75 μM (2,200 ng/ml). When midazolam is administered to patients who have immunosuppression induced by therapeutic intervention or by diseases such as sepsis, the immunosuppressive activity of midazolam on DCs may increase immunosuppression. Thus, even if DCs are exposed to concentrations of midazolam that are lower than those investigated in the current study—such as those that occur in actual patients during critical care—there may be a similar inhibitory effect on DC-mediated immune responses. Furthermore, in clinical situations where very deep sedation is necessary (e.g., therapeutic hypothermia), midazolam concentrations exceeding 30 μM (10,000 ng/ml) have been reported. Thus, it is possible in critical care settings that clinically administered midazolam may suppress DC-mediated immune response.

In summary, midazolam can modify the immune response induced by DCs. Administration of midazolam may provide a nontoxic means of modulating the immunostimulatory capacity of DCs. During sedation and analgesia in critical care, the use of midazolam may suppress DC-mediated immune responses.

References