Improved outcome of allogeneic bone marrow transplantation due to breastfeeding-induced tolerance to maternal antigens

Kazutoshi Aoyama,1,2 Motoko Koyama,2 Ken-ichi Matsuoka,1 Daigo Hashimoto,3 Tatsuo Ichinohe,4 Mine Harada,2 Koichi Akashi,2 Mitsune Tanimoto,1 and Takanori Teshima3

1Biopathological Science, Okayama University Graduate School of Medicine and Dentistry, Okayama; 2Department of Medicine and Biosystemic Science and 3Center for Cellular and Molecular Medicine, Kyushu University Graduate School of Medical Science, Fukuoka; and 4Department of Hematology/Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Exposure of offspring to noninherited maternal antigens (NIMAs) during pregnancy may have an impact on transplantations performed later in life. Using a mouse model, we recently showed that bone marrow transplantation (BMT) from NIMA-exposed offspring to the mother led to a reduction of graft-versus-host disease (GVHD). Since offspring can also be exposed to NIMAs by breastfeeding after birth, we tested whether breast milk could mediate the tolerogenic NIMA effect. We found that oral exposure to NIMAs by breastfeeding alone was sufficient to reduce GVHD, and that in utero exposure to NIMAs is required for maximal reduction of GVHD. The tolerogenic NIMA effects disappeared when donor mice were injected with CD25 monoclonal antibodies during the lactation period, suggesting a CD4+CD25+ regulatory T cell–dependent mechanism. Our results suggest a previously unknown impact of breastfeeding on the outcome of transplantation. (Blood. 2009;113:1829-1833)

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potential curative treatment for malignant hematologic diseases; however, HSCT from haploidentical-related donors is complicated by a high incidence of severe graft-versus-host disease (GVHD).

The fetus and mother must tolerate each other’s alloantigens during pregnancy. Fetal and maternal antigens transmitted through the bidirectional transplacental passage during pregnancy might induce tolerance to noninherited maternal antigens (NIMAs) in the offspring and inherited paternal antigens (IPAs) in the mother.1-4 Exposure of the fetus to allogeneic cells may induce a long-lasting tolerance specific to the alloantigens of the donor cells.5,6 We recently demonstrated using a mouse model that a “child-to-mother” bone marrow transplantation (BMT) from a NIMA-exposed donor reduces the mortality and morbidity of GVHD, but a “mother-to-child” BMT from a mother donor exposed to IPAs from the fetus does not.7 We therefore tested the hypothesis that breastfeeding plays an important role in the build-up of the tolerogenic NIMA effect in a mouse model of a “child-to-mother” BMT because breast milk is rich in maternal major histocompatibility complex (MHC) antigens in both soluble and cellular forms.8-11 Offspring were typed for the H-2 locus by flow cytometry using monoclonal antibodies (mAbs) specific for H-2Kb and H-2Kd (BD Pharmingen, San Diego, CA) as previously described.7 The resultant H-2b offspring were nursed by either a B6D2F1 mother (NIMA [in utero + oral]) or a B6 foster mother (NIMA [oral]). Controls were H-2b mice not exposed to H-2b. To produce NIMA-exposed mice via breastfeeding, B6 neonates were nursed by a B6D2F1 foster mother (NIMA [oral]). Mice were weaned at 3 weeks after birth. These mice were used as BMT donors at 6 to 10 weeks of age. BMT was performed as previously described.7 All animal experiments were performed under the auspices of the Institutional Animal Research Advisory Committee at Okayama University.

BMT

Mice underwent transplantation as previously described.13 In brief, after lethal total body irradiation (TBI; x-ray) was delivered in 2 doses at 3-hour intervals to minimize gastrointestinal toxicity, mice were intravenously injected with 5 × 106 T cell–depleted bone marrow (TCD-BM) cells plus 0.5 to 2 × 105 T cells, CD4+ T cells, or CD25-depleted CD4+ T cells from the donors on day 0. T-cell depletion, CD25 depletion, and splenic CD4+ T-cell isolation were performed using Auto-MACS (Miltenyi Biotec, Tokyo, Japan) as previously described.7,14 Hybridomas secreting anti-CD25 mAbs (clone PC61) were obtained from the ATCC (Manassas, VA). For in vivo depletion of CD25+ cells,14,15 mice were injected subcutaneously with 75 µg/g body weight of anti-CD25 or irrelevant mAbs on days 1 and 8 after birth. They were then housed in sterilized microisolator cages, and given autoclaved hyperchlorinated drinking water for the first 3 weeks after BMT and filtered water thereafter. Survival after BMT was monitored daily and the degree of clinical GVHD was assessed weekly by a scoring system that evaluated changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10) as previously described.16

Methods

Mice

Female C57BL/6 (B6, H-2b) and B6D2F1 (H-2b/d) mice were purchased from Charles River Japan (Yokohama, Japan). B6D1F1 (H-2b/q) mice were produced by mating a DBA/1 (H-2q) female (Japan SLC, Shizuoka, Japan) and a B6 male. NIMA-exposed H-2b mice were produced by mating a B6 (H-2b) male and a B6D2F1 (H-2b/d) female as previously described.7,9,12 Offspring were typed for the H-2 locus by flow cytometry using monoclonal antibodies (mAbs) specific for H-2Kb and H-2Kd (BD Pharmingen, San Diego, CA) as previously described.7 The resultant H-2b offspring were nursed by either a B6D2F1 mother (NIMA [in utero + oral]) or a B6 foster mother (NIMA [in utero]). Controls were H-2b mice not exposed to H-2b. To produce NIMA-exposed mice via breastfeeding, B6 neonates were nursed by a B6D2F1 foster mother (NIMA [oral]). Mice were weaned at 3 weeks after birth. These mice were used as BMT donors at 6 to 10 weeks of age. BMT was performed as previously described.7 All animal experiments were performed under the auspices of the Institutional Animal Research Advisory Committee at Okayama University.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

© 2009 by The American Society of Hematology
Flow cytometry

Flow cytometric analysis was performed as previously described. The mAbs used were biotinylated anti–mouse CD25 (clone 7D4); FITC- or PE-conjugated anti–mouse CD4, CD8, H-2Kβ, H-2Kδ (BD Pharmingen); and Foxp3 (eBioscience, San Diego, CA). For intracellular interferon γ (IFN-γ) staining, splenocytes were incubated for 4 hours with Leukocyte Activation Cocktail and BD GolgiPlu (BD Pharmingen) at 37°C. Then the cells were permeabilized with BD Cytofix/Cytoperm solution (BD Pharmingen) and stained with FITC-conjugated anti–IFN-γ mAbs. Dead cells were identified as 7-amino-actinomycin D (BD Pharmingen)–positive cells. At least 5000 live samples were acquired for analysis.

Cell culture

Cell culture was performed as previously described. Splenic CD4+ T cells were cultured at a concentration of 10⁶ cells/well with 4 × 10⁵ irradiated (30 Gy) splenocytes. After culturing for 72 hours, cells were pulsed with H-thymidine (1 μCi [0.037 MBq] per well) for further 18 hours. Proliferation was determined using Topcount NXT (Packard Instruments, Meriden, CT).

Statistical analysis

Mann-Whitney U tests were used to analyze cell counts and clinical scores. We used the Kaplan-Meier product limit method to obtain the survival probability and the log-rank test was applied to compare the survival curves. We defined a P value less than .05 as statistically significant.

Results

Breastfeeding is required for the induction of maximum NIMA effects to reduce GVHD

Lethally irradiated B6D2F1 mice received a transplant of 5 × 10⁶ TCD-BM from the controls together with 2 × 10⁶ T cells from either NIMA (in utero + oral), NIMA (in utero), NIMA (oral), or control mice. NIMA (in utero + oral) T cells produced less severe GVHD than control T cells, as previously reported. Interestingly, in recipients of NIMA (in utero) and NIMA (oral) T cells, the tolerogenic NIMA effect was partial when considering the mortality and morbidity of GVHD. These results demonstrate that in utero exposure to NIMAs alone could mediate the tolerogenic NIMA effect and that further oral exposure to NIMAs is required for induction of the maximum NIMA effect.

Breastfeeding alone is sufficient to mediate the tolerogenic NIMA effect

We then examined whether NIMA exposure by breastfeeding could induce T-cell hyporesponsiveness to NIMAs. Compared with

Breastfeeding-mediated tolerogenic NIMA effect is dependent on CD4+CD25+ cells

We recently demonstrated that CD4+CD25+ regulatory T cells (T₉) play an important role in the tolerogenic NIMA effect. Therefore, we considered whether these T₉ play a role in the induction of tolerogenic milk effects. When B6D2F1 mice received a transplant of CD25-depleted CD4+ T cells from NIMA (oral) mice, the tolerogenic NIMA effect disappeared, suggesting that T₉ in the donor inoculum were involved in the tolerogenic milk effect.

We hypothesized that NIMA-reactive T₉ are produced during the lactation period. Newborn B6 mice fed by a B6D2F1 foster mother were
injected subcutaneously with 75 μg/g body weight of anti-CD25 mAbs on days 1 and 8 after birth to deplete CD25+ T<sub>R</sub> in vivo. Injection of anti-CD25 mAbs significantly reduced the numbers of Foxp3<sup>+</sup> CD4<sup>+</sup> T<sub>R</sub> in spleens at 3 and 8 weeks after birth (n = 3/group, mean ± SD; F) and survival of B6D2F1 mice that received a transplant of CD4<sup>+</sup> T cells from anti-CD25-treated or control-treated NIMA-b/d donors or B6 donors (G) are shown (n = 11/group). (A,F) The results are representative of 3 replicate experiments. (B-E,G) Data from 2 similar experiments are combined. *P < .05; **P < .01 versus controls.

Figure 2. Breastfeeding-mediated tolerogenic NIMA effect is dependent on CD4<sup>+</sup>CD25<sup>+</sup> cells. A newborn B6 mouse was fed by either a B6 mother (control) or a B6D2F1 foster mother (NIMA-b/d). (A) CD4<sup>+</sup> T cells (10<sup>6</sup>) isolated from spleens of NIMA-b/d mice or controls were adoptively transferred into irradiated B6D2F1 mice. Numbers of IFN-γ<sup>+</sup> donor CD4<sup>+</sup> T cells in spleens 5 days after the transfer are shown as mean plus or minus SD (n = 5/group). (B,C) Lethally irradiated B6D2F1 mice received a transplant of 5 × 10<sup>6</sup> TCD-BM from control B6 mice together with 2 × 10<sup>6</sup> T cells from NIMA-b/d, controls, or syngeneic B6D2F1 donors. Survival after BMT (B; n = 11/group) and numbers of double-positive thymocytes at 40 days after BMT (C) are shown. (D) B6D2F1 mice received a transplant as described for panels B and C with T cells from a B6 mouse fed by either a B6D2F1 (NIMA-b/d) or B6D1F1 (NIMA-b/q) foster mother. Survival after BMT is shown (n = 14/group). (E) BMT was performed as described for panels B and C with 0.5 × 10<sup>6</sup> CD25-depleted CD4<sup>+</sup> T cells from NIMA-b/d or control donors. Survival after BMT is shown (n = 10/group). (F,G) Newborn B6 mice were nursed by a B6D2F1 mother and subcutaneously injected with 75 μg/g body weight of anti-CD25 or irrelevant mAbs 1 and 8 days after birth. The numbers of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells in spleens at 3 and 8 weeks after birth (n = 3/group, mean ± SD; F) and survival of B6D2F1 mice that received a transplant of CD4<sup>+</sup> T cells from anti-CD25-treated or control-treated NIMA-b/d donors or B6 donors (G) are shown (n = 11/group). (A,F) The results are representative of 3 replicate experiments. (B-E,G) Data from 2 similar experiments are combined. *P < .05; **P < .01 versus controls.

Finally, we evaluated whether the NIMA effect persists when NIMA-exposed donors are older. B6D2F1 mice received a transplant of T cells from 28-week-old NIMA-exposed mice and age-matched control mice. Again, GVHD was reduced in mice that received a transplant from older NIMA mice (Figure 3).

BREASTFEEDING AND GVHD 1831

BLOOD, 19 FEBRUARY 2009 • VOLUME 113, NUMBER 8

For personal use only.on April 8, 2017. By guest
www.bloodjournal.org
tolerance prevents pathological reactions against environmental and food antigens, and its failure results in an exacerbated inflammation typical of allergies and asthma. Oral tolerance can be mediated by several mechanisms such as deletion, immune deviation, and suppression by Tregs. We recently demonstrated that Tregs play an important role in the tolerogenic NIMA effect. Similarly, in the mouse model of the NIMA cardiac allografts, graft acceptance is associated with a higher frequency of IL-10– and TGF-β–producing CD4+CD25+ cells. In this study, we further showed that the tolerogenic NIMA effect mediated by breastfeeding was also dependent upon Tregs. The tolerogenic NIMA effect was abrogated by depletion of CD25+ T cells of donor inocula, as well as the in vivo depletion of CD25+ cells in neonates on days 1 and 8 after birth, during the breastfeeding period. Injection of anti-CD25 mAbs significantly reduced generation of Foxp3+ Tregs during this period as shown in Figure 2F. However, this cell population had recovered to normal levels by 8 weeks of age when the mice were used as BMT donors, as shown in previous studies; thus, abrogation of tolerogenic NIMA effects is not due to a decrease in the numbers of Foxp3+ Tregs. A recent study addressing the kinetics of Treg generation during ontogeny demonstrated that the largest single-day gain in numbers of Foxp3+ thymocytes occurred between days 3 and 4, followed by a steady increase in number over the first 3 weeks. Thereafter, Tregs were continuously generated in the thymus. Thus, both de novo generation of Tregs in the thymus and peripheral expansion of Tregs appear to be responsible for Treg repopulation within 8 weeks. Interestingly, the mechanisms of oral tolerance differ with different antigen doses. A single administration of high-dose antigens causes clonal deletion, whereas repeated administration of low doses of antigens leads to development of Tregs. Thus, repeated oral exposure to NIMAs by breastfeeding might help to produce NIMA-specific Tregs. However, a single intravenous injection of allogeneic cells can induce T-cell responses against alloantigens in murine neonates. This route of antigen exposure may be critical in determining immunity or tolerance.

Our results suggest the antigen specificity of the NIMA effect in vivo. Oral administration of the self-antigen induces antigen-specific Tregs. Breast milk–mediated transfer of an antigen to the neonate from an antigen-exposed mother produces Tregs that suppress asthma. Such an antigen specificity may be mediated by "adaptive" Tregs that are generated from classical T-cell subsets or "natural" Tregs under certain conditions of antigenic stimulation, but not "natural" Tregs that develop in the thymus during the early stages of fetal and neonatal T-cell development. In fact, we could not demonstrate antigen-specificity of Tregs isolated from NIMA-exposed mice in vitro. Antigen-specific expansion of Tregs in vivo may require a T-cell–deficient environment and stimulation with the corresponding antigens in the presence of IL-2. Therefore, re-exposure to NIMAs in a lymphopenic environment following pretransplantation conditioning might allow for the generation of “adaptive” Tregs, thus contributing to antigen specificity in vivo. Further studies are required to evaluate antigen specificity of the NIMA effect. In addition, it should be noted that oral tolerance can be achieved in the absence of Foxp3+CD25+ Tregs. Thus, there could be multiple mechanisms underlying the tolerogenic NIMA effect cause by breastfeeding.

In clinical HSCT with grafts from NIMA-exposed donors, GVHD is mild or absent in some, but not in others. A history of breastfeeding as well as the presence of microchimerism and anti-HLA antibodies should be considered. MHC allele mismatch combinations between the donor and recipient could matter. A recent clinical study identified high- and low-risk HLA allele mismatches for severe GVHD. The age of donors may also affect the outcome. In our study, the tolerogenic NIMA effect lasted for at least 28 weeks, suggesting that both child and...
adult donors can confer the NIMA effect. Lastly, calcineurin inhibitors used for GVHD prevention inhibit activation and expansion of TcR, thereby suppressing the NIMA effects. The beneficial NIMA effects were observed only if patients were not administered cyclosporine in kidney transplantation. Immunosuppressants such as sirolimus and mycophenolate mofetil, which do not interfere with TcR, may therefore be of use in NIMA-associated HSCT. In conclusion, we demonstrated that breastfeeding plays an important role in the buildup of the tolerogenic NIMA effect, suggesting that novel strategies can be established to induce host-specific tolerance orally in allogeneic HSCT.

Acknowledgments

This study was supported by research funds from the Ministry of Education, Culture, Sports, Science and Technology (Tokyo, Japan; no. 20659153; T.T.), Health and Labor Science Research grants (Tokyo, Japan; T.I., T.T.), and Mitsubishi Pharma Research Foundation (Tokyo, Japan; T.T.).

Authorship

Contribution: K. Aoyama conducted the research and wrote the paper; M.K., K.-i.M., D.H., and T.I. conducted the research; K. Akashi, M.H., and M.T. designed the study; and T.T. designed and organized the study.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Takaroni Teshima, Center for Cellular and Molecular Medicine, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582 Japan; e-mail: tteshima@canancer.med.kyushu-u.ac.jp.

References

Improved outcome of allogeneic bone marrow transplantation due to breastfeeding-induced tolerance to maternal antigens

Kazutoshi Aoyama, Motoko Koyama, Ken-ichi Matsuoka, Daigo Hashimoto, Tatsuo Ichinohe, Mine Harada, Koichi Akashi, Mitsune Tanimoto and Takanori Teshima