Practical Evaluation of Engraftment and Mixed Chimerism Using PCR Amplification of a Microsatellite in the Class II *Eb* Gene in Murine MHC-mismatched, Nonmyeloablative Bone Marrow Transplantation

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Background: Although engraftment following murine allogeneic bone marrow transplantation (BMT) is most commonly confirmed by H2 typing using flow cytometry, recipient mice can be seriously injured during peripheral blood (PB) sampling. Therefore, we developed an alternative DNA-based assay that does not require the large volume of PB necessary for flow cytometry.

Methods: A minute volume of PB from the tail vein was used to evaluate the engraftment by PCR amplification of a microsatellite in the class II *Eb* gene. Dilution experiments were performed to evaluate the sensitivity of this assay for detecting donor cells in mixed cell populations compared with flow cytometry analysis.

Results: Early engraftment and mixed chimerism were confirmed, based on the length variation of the microsatellite in the class II *Eb* gene. The degree of donor chimerism in the donor-recipient cell mixture could be estimated semiquantitatively in a dilution experiment. The sensitivity of this assay by the naked eye approached 10% of the degree of donor chimerism.

Conclusion: PCR amplification of a microsatellite in the class II *Eb* gene can be a useful alternative to flow cytometry for evaluating early engraftment and mixed chimerism following murine nonmyeloablative BMT. ***(Korean J Hematol 2007;42:91-97.)***

Key Words: Mixed chimerism, *Eb* gene, Nonmyeloablative

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INTRODUCTION

The mouse model of allogeneic bone marrow transplantation (BMT) has played an important role in extending our understanding of the biology of BMT.[1] Recently, the intentional induction of a mixed chimerism following nonmyeloablative conditioning has been developed in a mouse model as a strategy for reducing BMT-related toxicity, overcoming the major histocompatibility complex (MHC) barrier,[2] and providing an immunological platform for cell therapy,[3] tumor vaccines,[4] or organ transplantation.[5] It is important to confirm the state of mixed chimerism for integrating additive therapies into the post-BMT course without developing graft versus host disease (GVHD).[4,6] In the murine model, H2 typing is most commonly performed using flow cytometry to evaluate early engraftment and the degree of donor chimerism.[2] However, this method is based on the presence or absence of haplotype-specific antigens and available antibodies. Moreover, it is often limited by the need for expensive equipment and by an inability to collect sufficient cells without animal sacrifice for serial assessment of BMT in mice.

We have established a unique murine model of a stable mixed chimerism in MHC-mismatched nonmyeloablative BMT[2] and investigated its potential for clinical application.[7-10] While performing that research, we sampled peripheral blood (PB) from the retro-orbital venous plexus to obtain sufficient uncoagulated PB for flow cytometry and evaluated the allogeneic mixed chimeric state. Unfortunately, the recipient mice were sometimes injured seriously or died during PB sampling. These unexpected injuries had an adverse effect on our murine BMT-related experiments. Therefore, we sought to develop an alternative, DNA-based assay that could detect engraftment/mixed chimerism using a minute amount of PB without causing serious sampling injuries. Since a highly polymorphic microsatellite in the class II *Eb* gene can be used to trace the MHC in mice,[11] we used microsatellite polymorphism in the class II *Eb* gene to evaluate engraftment/mixed chimerism following mouse BMT.

MATERIALS AND METHODS

1. Mice

Female BALB/c (8 weeks old, H-2k^d) and C57BL/6 (6 to 7 weeks old, H-2k^b) mice were purchased from Samtago (Gwangju, Korea). The mice were maintained under specific pathogen-free conditions in an animal facility with controlled humidity (55±5%), light (12/12 h, light/dark), and temperature (22±1°C). The air in the animal facility was passed through a high-efficiency particle arrestance (HEPA) filter system designed to exclude bacteria and viruses. The animals were allowed mouse chow and tap water *ad libitum*. The Animal Care and Use Committee of the Catholic University of Korea approved the protocols used in this study.

2. Preparation of mixed chimera

Allogeneic mixed chimeras were prepared using established procedures.[2] In brief, one day before BMT, the recipient mice (8-week-old BALB/c, H-2k^d) were injected intraperitoneally with 200 μL of phosphate-buffered saline (PBS) containing 40 μL of reconstituted anti-asialoGM1 (anti-ASGM1; Wako Chemicals, Osaka, Japan). Recipient mice were exposed to a single x-ray dose of 500cGy from a Mevatron MXE-2 (Siemens, NY) with a focus-to-skin distance of 100cm and a rate of 200cGy/min. Donor (6- to 7-week-old C57BL/6, H-2k^b) bone marrow cells were collected into Cedarlane cytotoxicity medium (RPMI 1,640 medium supplemented with 25mm HEPES buffer and 0.3% bovine serum albumin) by flushing the shafts of the femurs and tibias of C57BL/6 mice. Resuspended bone marrow cells were depleted of T cells by incubation with anti-Thy-1.2 microbeads (Miltenyi Biotec, Auburn, CA) ac-

cording to the manufacturer's instructions. Within 6 h after irradiation, 2×10^7 T cell-depleted bone marrow cells, in a final volume of 0.2mL of PBS, were infused into recipient mice.

3. Flow cytometry analysis

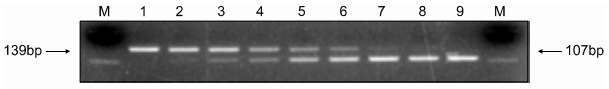
Early engraftment and chimerism were determined using the relative ratio of peripheral blood lymphocytes (PBLs) expressing recipient MHC class I molecules to PBLs expressing donor molecules on day 21. Briefly, peripheral blood was collected from the retro-orbital vein using heparinized capillary tubes. Donor (H-2K^b) and recipient (H-2K^d) cells were distinguished during lymphoid gating by staining with fluorescein isothiocyanate-labeled anti-H-2K^b and phycoerythrin-labeled anti-H-2K^d antibodies (PharMingen, San Diego, CA), respectively. Samples of peripheral blood were stained with FITC-anti-H-2k^b and PE-anti-H-2k^d (PharMingen) or Ig isotype controls (PharMingen), followed by treatment with an RBC lysis kit (Optylase B, Immunotech-Coulter, Marseille, France). Stained cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (both from Becton Dickinson, Mountain View, CA). The percentage of donor-derived cells was calculated by dividing the number of donor cells by the total number of donor and recipient cells that showed positive staining.[2)]

4. PCR amplification of a microsatellite in the class II *Eb* gene

We collected 20 μL of peripheral blood from a tail vein 3 weeks after MHC-mismatched, T-cell-depleted, nonmyeloablative bone marrow transplantation according to our protocol.[2)] Genomic DNA was prepared using an extraction kit (Dr. Gentle; Takara Bio, Otsu, Japan) according to the manufacturer's instructions. A highly polymorphic microsatellite containing tandem repeats (TRs) of two tetranucleotide units, TGGA and GGCA, located at the 3' end of the second intron in the class II *Eb* gene was amplified. Genomic DNA (50ng from a mixed chimeric mouse) was amplified using primers specific for the microsatellite in the class II *Eb* gene, 5'-CGACTGTAGAACCTTAGCCTG-3' and 5'-TGGAGCTGTCCTCCTTGTAG-3', and yielded 107-bp (donor, C57BL/6, H-2^b) and 139-bp (host, BALB/c, H-2^d) fragments of the *Eb* TR allele.[12)] PCR amplification was performed under the following conditions: an initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and a final incubation at 72°C for 7 min in a thermal cycler (GeneAmp 9700, Perkin-Elmer, Foster City, CA, USA). All of the amplified DNA samples were electrophoresed in a 2% agarose gel stained with ethidium bromide and photographed (Fig. 1).

5. Dilution experiment for the sensitivity of PCR amplification of a microsatellite in the class II *Eb* gene in the allogeneic mixed chimeric state

Spleen cells from both recipient (BALB/c, H-2k^d) and donor (C57BL/6, H-2k^b) mice were removed using ACK lysis buffer, washed, and resuspended in complete culture medium (RPMI 1,640 medium supplemented with 10% heat-inactivated fetal calf serum, 1mM sodium pyruvate,



Lane M; 100bp ladder marker
Lane 1; D to R=0% to 100%
Lane 2; D to R=5% to 95%
Lane 3; D to R=10% to 90%
Lane 4; D to R=25% to 75%
Lane 5; D to R=50% to 50%
Lane 6; D to R=75% to 25%
Lane 7; D to R=90% to 10%
Lane 8; D to R=95% to 5%
Lane 9; D to R=100% to 0%

Fig. 1. Assessing the degree of mixed chimerism in an arbitrary cell mixture using PCR amplification of a microsatellite in the class II *Eb* gene of the murine MHC. Donor (D, C57BL/6, H-2k^b mice) and recipient (R, BALB/c, H-2k^d) splenocytes were mixed *in vitro* in various proportions (D to R=0 to 100, 5 to 95, 10 to 90, 25 to 75, 50 to 50, 75 to 25, 90 to 10, 95 to 5, and 100 to 0) in dilution experiments. PCR amplification of the class II *Eb* gene and flow cytometry analysis were performed using the cell mixtures. The DNA fragments amplified from the donor and recipient were 107 and 139bp, respectively. The lanes show the results for artificial mixtures of donor and recipient splenocytes.

5×10^{-5}M 2-ME, 20mM HEPES, 100U/mL penicillin, and 100 μg/mL streptomycin). Donor splenocytes were mixed with recipient splenocytes in various proportions for the dilution experiments. PCR amplification of the class II *Eb* gene and flow cytometry analysis were performed using the cell mixtures. The sensitivity of each method was assessed.

RESULTS

1. Sensitivity of PCR amplification of a microsatellite in the class II *Eb* gene

Donor and recipient DNA could be distinguished simply by PCR amplification of a microsatellite in the class II *Eb* gene, even in the allogeneic mixed chimeric state. A highly polymorphic microsatellite made up of two tetranucleotide blocks, $(TGGA)_x$ and $(GGCA)_y$, is located at the 3' end of the second intron in the *Eb* gene. The number of repeats differs according to the H-2 haplotype.[12] We investigated whether the length polymorphism of this satellite was applicable to the characterization of H-2 haplotypes and could be used to determine engraftment and the degree of donor chimerism in murine MHC-mismatched nonmyeloablative BMT. As donors and recipients possessed alleles that differed by 32 bases, it was possible to analyze this transplant with ethidium bromide staining without the use of radioactivity. Dilution experiments indicated that residual donor or recipient DNA extracted from a cell mixture could be detected at the 5 to 10% level (Fig. 1). By contrast, flow cytometry could detect donor or recipient cells at the 1% level (Fig. 2). Although the DNA-based assay using PCR amplification of a microsatellite in the class II *Eb* gene was slightly less sensitive than flow cytometry analysis at detecting engraftment/mixed chimerism, these observations indicated that it was equally useful for detecting engraftment and a donor-dominant mixed chimeric state (>90% donor chimerism) in murine MHC-mismatched nonmyeloablative BMT.

2. Assessing engraftment/mixed chimerism using a minute amount of peripheral blood from allogeneic mixed chimeric mice

Allogeneic BMT was performed according to our own protocol with the intentional induction of a mixed chimerism. Engraftment/mixed chimerism was evaluated 3 weeks post-transplant

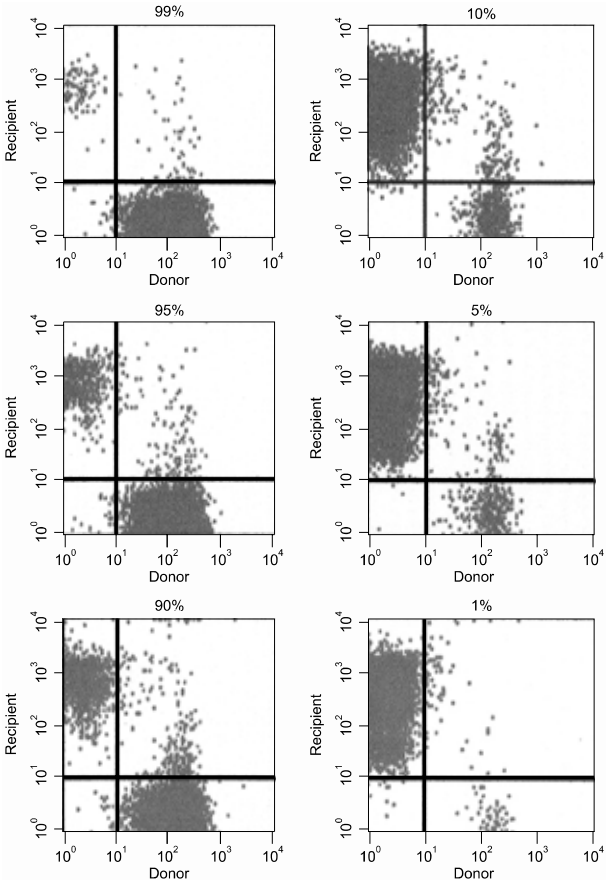


Fig. 2. Assessing the degree of mixed chimerism in arbitrary cell mixtures using flow cytometry. Donor (C57BL/6, H-2k^b mice) and recipient (BALB/c, H-2k^d) splenocytes were mixed *in vitro* in various proportions (D to R=99 to 1, 95 to 5, 90 to 10, 10 to 90, 5 to 95, and 1 to 99) for dilution experiments. Donor (H-2K^b) and recipient (H-2K^d) cells were distinguished during lymphoid gating by staining with fluorescein isothiocyanate-labeled anti-H-2K^b and phycoerythrin-labeled anti-H-2K^d antibodies (PharMingen, San Diego, CA), respectively. Stained cells were analyzed using CellQuest software on a FACSCalibur flow cytometer (both from Becton Dickinson, Mountain View, CA). The percentage of donor-derived cells was calculated by dividing the number of donor cells by the total net number of donor plus recipient cells that showed positive staining.

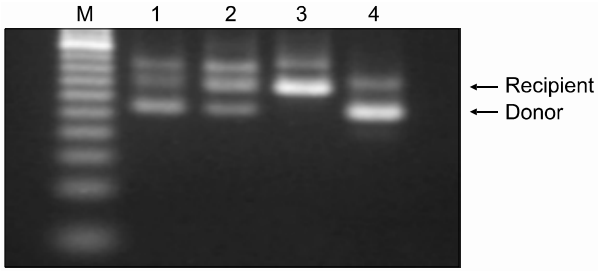


Fig. 3. Donor chimerism in peripheral blood of an allogeneic mixed chimera following MHC-mismatched nonmyeloablative BMT. Engraftment/mixed chimerism at 3 weeks post-BMT was evaluated using PCR amplification of a microsatellite in the class II *Eb* gene. Allogeneic mixed chimeric mice (lanes 1, 2, and 4) showed both the 107-bp (donor, C57BL/6, H-2^b) and 139-bp (host, BALB/c, H-2^d) fragments, whereas non-transplanted recipient mice as a negative control (lane 3) showed only the 107-bp (host, BALB/c, H-2^d) fragment. The relative ratios of donor-derived cells in the PB from those mice were 76, 42, 0, and 89% of all lymphocytes, respectively. The DNA-based assay developed in this study roughly correlated with the results of the flow cytometry assay.

using PCR amplification of a microsatellite in the class II *Eb* gene with a minute PB sample. The PCR products obtained from the PB of allogeneic mixed chimeric mice (lanes 1, 2, 4) showed both 107-bp (donor, C57BL/6, H-2^b) and 139-bp (host, BALB/c, H-2^d) fragments, whereas the product from non-transplanted recipient mice used as a negative control (lane 3) showed only the 139-bp (host, BALB/c, H-2^d) fragment on ethidium bromide-stained agarose gels (Fig. 3). To validate the results of the DNA-based assay, we sacrificed the same mice and evaluated the degree of donor chimerism using a flow cytometry assay. The relative ratios of cells of donor origin in the respective PB samples were 76, 42, 0, and 89% of all lymphocytes. These observations suggest that our DNA-based assay using PCR amplification of a microsatellite in the class II *Eb* gene is a practical alternative to flow cytometry for assessing engraftment/mixed chimerism following murine nonmyeloablative BMT.

DISCUSSION

The presence of engraftment/mixed chimerism or leukemic relapse in human BMT[13,14] is usually assessed using PCR of Y-chromosome-specific sequences, short tandem repeat sequences (STRs), or microsatellites,[15-17] whereas DNA-based assays have been used less frequently than flow cytometry analysis in mouse BMT. Consequently, there are few papers on DNA-based assays in mice. O'Neil et al reported that the changes in donor chimerism following mouse BMT could be monitored using PCR amplification of 12 mouse STR markers.[18] However, the resolution in their analysis of mixed chimerism using selected STR markers was poor, and there was no clear difference in allele size between donor and recipient.

Of various molecular markers in mice, microsatellites in the class II *Eb* gene have an extraordinarily high level of polymorphism. Seven length variants are known in the 12 standard haplotypes of the H2 *Eb* gene (b, k, r, v, f, s, u, z, d, q, j, and p).[11] We used the polymorphism of a microsatellite in the class II *Eb* gene of the murine major histocompatibility complex (MHC) to evaluate mixed chimerism/engraftment following mouse BMT, because it had not been used previously. We found that mixed chimerism was obviously detectable at a level approaching 10% with this method.

Although PCR amplification of a microsatellite in the class II *Eb* gene was less sensitive than flow cytometry, it has several important advantages. First, this method can clearly distinguish donor- and recipient-derived cellular portions in as little as 10 to 20 μL of PB, and it does not require the use of polyacrylamide gels, radioisotopes, or expensive equipment. Second, sampling a small volume of blood from the tail vein does not have an adverse effect on the clinical outcome of murine allogeneic BMT, making it possible to obtain serial samples from transplanted

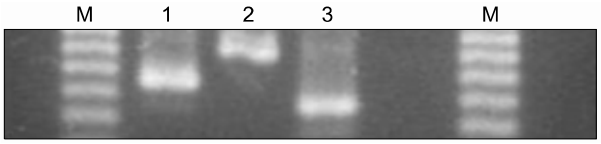


Fig. 4. Assessing NOD/Scid mice using PCR amplification of a microsatellite in the class II *Eb* gene of the murine MHC. The presence of NOD/Scid-derived cells can be detected using PCR amplification of a microsatellite in the class II *Eb* gene of the murine MHC, although NOD/Scid mice do not express the H-2 haplotype antigen. The amplified DNA fragments of the C57BL/6 (H-2^b), BALB/c (H-2^d), and NOD/Scid mice were 107, 139, and 80bp, respectively.

mice. Third, the semiquantitative assessment of engraftment or donor-dominant chimerism using this method makes it possible to conduct subsequent experiments, such as donor lymphocyte infusion (DLI)[3,6] or tumor vaccine.[4] Fourth, this method can be used in murine BMT experiments with NOD/Scid mice, which express few MHC antigens (Fig. 4).

We have provided evidence that PCR amplification of a microsatellite polymorphism of the mouse class II *Eb* gene is a practical method for evaluating mixed chimerism/engraftment following murine BMT in a single PCR amplification. When cell therapy or the manipulation of transplanted mice following engraftment is anticipated, this method will prove convenient in the early post-transplant period.

요 약

배경: 생쥐에서 동종골수이식 후 공여자 조혈모의 생착 확인을 위하여 흐름세포측정법(flow cytometry)을 이용한 H2 판별이 가장 흔히 사용되지만, 말초 혈액을 채취하는 과정에서 이식받은 생쥐에게 치명적인 손상을 줄 수 있다. 저자들은 생착 여부를 판별하는 방법으로 많은 양의 말초 혈액을 필요로 하는 흐름세포 측정법을 대체할 수 있는 DNA 관련 분석법을 개발하고자 하였다.

방법: 생쥐의 꼬리 정맥에서 채취한 극소량의 말초 혈액에서 class II *Eb* 유전자 microsatellite의 중합효소연쇄반응(PCR)을 이용한 증폭을 통하여 공여자 조혈모세포의 생착 여부를 평가하였다. 이와 함께 공여자와 받는이 생쥐의 혼합 세포주에서 공여자 세포의 검출에 대하여 저자들이 개발한 분석법과 흐름세포측정법의 민감도를 비교하기 위하여 희석실험(dilution experiments)을 실시하였다.

결과: Class II *Eb* 유전자의 microsatellite 변이에 대한 PCR 측정법을 이용하여 조기 생착 및 혼합키메리즘이 확인 가능하였다. 공여자와 받는이 생쥐의 혼합세포주에서 공여자의 키메리즘 정도는 희석실험을 통하여 반정량적으로 추정 가능하였다. 이 분석법은 공여자 키메리즘이 10% 이상을 차지하는 경우 키메리즘을 육안적으로도 식별할 수 있는 민감도를 보였다.

결론: 생쥐에서 비골수억제성(non-myeloablative) 골수이식 후 조기 생착 및 혼합 키메리즘의 평가 방법으로 class II *Eb* 유전자의 microsatellite에 대한 PCR 검사법은 흐름세포측정법을 대체하여 유용하게 이용될 수 있다.

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