Recovery of Patient Peripheral Blood Mononuclear Cells after Treatment with Protease Inhibitor in Post-transfusion Graft-versus-host Disease

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Abstract: Post-transfusion graft-versus-host disease (PT-GVHD) is a fatal adverse effect of blood transfusion.1)-3) The mortality rate is said to be 99% or higher.4) In spite of the seriousness of the disease, no definitively effective drug for curing patients of it has been identified. Recently, however, we reported on the potential usefulness of a protease inhibitor, nafamostat mesilate (NM), for treatment of PT-GVHD.5),6) This potential usefulness of NM was suggested by the results in vitro experiments in which possible PT-GVHD effector cytotoxic T cell (CTL) clones and tumor necrosis factor α (TNF α) producing T cell clones established by us were used.7),10) NM inhibited the cytotoxicity of the CTL clones and TNF α production by the TNF α producing clones.6) Based on these results, NM was administered to two patients with PT-GVHD. NM administration resulted in marked recovery of the patient peripheral blood mononuclear cell (PBMC) detected by microsatellite DNA polymorphism analysis distinguishable between donor and patient PBMCs. This is the first report of the marked recovery of PT-GVHD patient PBMC from very low ones which were replaced almost all with donor PBMCs. Moreover, NM administration was associated with body temperature normalization, disappearance of a body rash, liver function improvement and an unusual long survival time after onset of PT-GVHD in both cases. NM is potentially useful drug for treatment of PT-GVHD.

Key words: Post-transfusion graft-versus-host disease; protease inhibitor; microsatellite DNA polymorphism; polymerase chain reaction; cytotoxic T cell; tumor necrosis factor.

Introduction. The development of PT-GVHD is generally thought to be mediated by cytotoxic activity of the transfused donor lymphocytes in blood products and blood components under the conditions of (1) one-way incompatibility of HLAs between donor and recipient, (2) the presence of immunocompetent cells in the graft, and (3) inability of the host to reject the graft.13) Under these conditions, the engrafted donor CTLs are considered to attack recipient tissues.2),3) Along with CTL-mediated injury, humoral factors such as cytotoxic IgG produced by donor-derived B cells and inflammatory cytokines [e.g. TNF α and β, interferon γ (IFN γ)] are thought to play important roles in the development of PT-GVHD.14),15) The clinical symptoms of the disease, fever, skin rash, liver dysfunction, diarrhea, bone marrow aplasia and pancytopenia, become very severe and progress very rapidly. The mortality rate for PT-GVHD is said to be 99% or higher.4) In spite of the seriousness of this disease, no effective drug for radically curing patients of it has been identified. Almost all patients who develop PT-GVHD die within 3–4 weeks after blood transfusion: the exception has been the patient in one atypical PT-GVHD case.16)

Recently, we reported on one strong candidate drug NM, already widely used as an anti-coagulant, for treatment of PT-GVHD, based on in vitro experiment results.5),6) NM is protease inhibitor and speculated to inhibit the cytotoxicity of cytotoxic granules “perforin/granzymes”11),17) which CTLs release while they are exhibiting cytotoxic activity upon appropriate stimulation. Protease inhibitor also inhibits TNF production by T cells and macrophages12) which is said to be closely associated with the development of PT-GVHD.14),18) The potential usefulness of NM was demonstrated in vitro inhibition of the cytotoxicity of CTL clones and TNF β production by TNF β producing T cell clones which we established from PT-GVHD patients' PBMCs and speculated to be effector...
In this report, we describe two PT-GVHD cases in which patient PBMC recovered markedly after NM administration. Moreover, after NM administration, fever decreased to less than 37°C, rash disappeared, liver function improved with decreases in serum aspartate aminotransferase and alanine aminotransferase concentrations, and the patients showed an unusual long survival time after blood transfusion (47 days in case 1, 35 days in case 2 after transfusion). A significant recovery of patient PBMC, body temperature normalization, rash disappearance, liver function improvement and long survival time as in these two cases have not been reported for in any case of PT-GVHD treated by conventional methods (including steroids and cyclosporin A, etc. treatment). These results strongly suggest the potential usefulness of NM for treatment and cure of PT-GVHD.

**Patients and methods.** Case 1. A 77-year-old woman was transfused with 600 ml of unirradiated red cell concentrate from six male donors for serious anemia in December 1996. After transfusion her clinical course was uneventful until day 18, when she developed a fever of over 38°C. On day 19 after transfusion, a rash appeared over her entire body. On the same day, she developed liver dysfunction: her serum aspartate aminotransferase and alanine aminotransferase concentrations were 288 and 222 U per liter, respectively. Her platelet (PLT) count was 64,000 per cubic millimeter, white blood cell (WBC) count was 90,000 per cubic millimeter, red blood cell (RBC) count was 359 × 10^4 per cubic millimeter and hemoglobin (Hb) concentration was 9.7 g/dl. On day 24 after transfusion, samples of her blood and clippings of her fingernail were collected for diagnostic testing for PT-GVHD. On day 26 after transfusion, intravenous administration of NM at 150 mg/day was started which was continued until day 47. Soon after the start of NM administration, a fever decreased to less than 37°C, her rash disappeared and her serum aspartate aminotransferase and alanine aminotransferase concentrations normalized (to 6 and 24 U per liter, respectively, on day 33 after transfusion). On day 35 after transfusion, samples of her peripheral blood were collected for the determination of her non-donor-derived PBMC. She died on day 47 after transfusion.

Case 2. A 69-year-old man underwent cardiac surgery for cardiac infarction in January 1997. During the surgery, he received 600 ml of unirradiated red cell concentrate from three female donors. On postoperative day 14, he developed a fever of over 38°C. On postoperative day 19, his PLT count decrease to 34,000 per cubic millimeter and he developed a rash over his entire body. On postoperative day 22, pancytopenia was detected: his WBC count was 50,000 per cubic millimeter, RBC count was 298 × 10^4 per cubic millimeter, PLT count was 88,000 per cubic millimeter and Hb concentration was 10.1 g/dl. On the same day, he developed liver dysfunction: his serum aspartate aminotransferase and alanine aminotransferase concentrations were 277 and 168 U per liter, respectively. On postoperative day 23, samples of his peripheral blood and clippings of his fingernail were collected for diagnostic testing for PT-GVHD. He was administered with 5 mg/day of OKT-3 (anti-CD3 monoclonal antibody) from postoperative day 24 until postoperative day 33. Intravenous administration of NM (250 mg/day) was started on postoperative day 25 and continued until postoperative day 35. Soon after the NM administration was started, his rash disappeared, his body temperature normalized to less than 37°C and his serum aspartate aminotransferase and alanine aminotransferase concentrations normalized (to 18 and 39 U per liter, respectively, on postoperative day 28). Furthermore, his WBC count gradually recovered (from 200 per cubic millimeter on postoperative day 25 to 800 per cubic millimeter on postoperative day 35). He died on postoperative day 35 due to acute cardiac failure and not due to failures associated with PT-GVHD. On the same day, soon after his death, samples of his peripheral blood were collected for the determination of his non-donor-derived PBMC.

**Diagnostic test for PT-GVHD.** A diagnosis of PT-GVHD was confirmed in both cases based on the results of a molecular method for the detection of donor DNA in the patients, through analysis of dinucleotide or trinucleotide microsatellite repeat length polymorphisms as described previously. The oligonucleotide primers used in the polymerase chain reaction (PCR) to amplify polymorphic loci were D6S89, INT2, HGH, ACTBP2, APOC3. Genomic DNA was amplified by a previously described method. The amplified DNA was subjected to electrophoresis on a 7% polyacrylamide gel and polymorphic alleles were detected by silver staining. DNA was prepared from the patients' peripheral blood samples and fingernail clippings (collected on day 24 after transfusion in case 1 and on postoperative day 23 in case 2) according to a method previously described. DNA prepared from Ficoll purified samples of PBMCs from the peripheral blood collected after development of PT-GVHD, after almost all of the patient PBMCs had been replaced by donor PBMCs, was used for determination of donor type microsatellite length polymorphisms, and DNA prepared from the patient fingernail clippings was used to determine the original...
Detection of recovery of patient PBMC. Ficoll purified samples of PBMCs from the peripheral blood samples collected before and after NM administration (on day 24 and 35 after transfusion in case 1 and on postoperative day 23 and 35 in case 2, respectively) were separated into CD8+, CD4+ (CD:Clusters of differentiation. CD8 is a marker of cytotoxic T cell and CD4 is a marker of helper T cell.) (case 1 and case 2) and CD4−CD8− fractions (case 2) using anti-CD4 monoclonal antibody (mAb)-conjugated DYNABEADS and/or anti-CD8 mAb-conjugated DYNABEADS (Nihon Dynal K.K. Tokyo Japan) as described elsewhere. In case 1, DNA prepared from samples of whole blood, PBMC, CD4+ and CD8+ fractions was amplified by PCR as to all 5 microsatellite DNA markers. In case 2, DNA prepared from samples of whole blood, CD4+, CD8+ and CD4−CD8− fractions was amplified as in case 1 with all 5 microsatellite DNA markers. Since one donor was identified as responsible donor in case 2, DNA prepared from donor derived whole blood was also amplified by PCR as to all 5 microsatellite DNA markers. The amplified DNA was subjected to electrophoresis on a 7% polyacrylamide gel and polymorphic alleles were detected by silver staining. Differences of band patterns on polyacrylamide gel between patient DNA and donor DNA were visually identified.

In case 2, oligonucleotide primers for amplification of amelogenin gene AMEL were used in a PCR to detect the recovery of patient PBMC bearing Y-chromosome.

Results. The diagnosis of PT-GVHD was confirmed in both cases based on the results of the polymorphic microsatellite DNA analysis. Because in both cases, the DNA types of the PBMCs from the blood samples collected after development of symptoms of PT-GVHD (on day 24 after transfusion in case 1 and on postoperative day 23 in case 2) were found to differ from the fingernail DNA types for all 5 microsatellite DNA markers. The amplified DNA was subjected to electrophoresis on a 7% polyacrylamide gel and polymorphic alleles were detected by silver staining. Differences of band patterns on polyacrylamide gel between patient DNA and donor DNA were visually identified.

In case 2, oligonucleotide primers for amplification of amelogenin gene AMEL were used in a PCR to detect the recovery of patient PBMC bearing Y-chromosome.

Discussion. In this report, we describe the effectiveness of the administration of NM in these two patients by determining the extent of recovery of the patients' PBMCs. By the polymorphic microsatellite DNA analysis, we confirmed patient type band patterns appeared on polyacrylamide gel in both cases for all 5 microsatellite DNA markers, using PBMCs from the blood samples collected from the patients before and after NM administration (on day 24 and 35 after transfusion in case 1 and on postoperative day 23 and 25 in case 2, respectively). In detail, for all 5 microsatellite DNA markers, the following results were obtained: On day 24 (before NM administration) in case 1, DNA prepared from samples of whole blood and the CD8+ fraction was of donor type, DNA prepared from PBMCs was a chimera of patient and donor type with the patient type predominating and DNA prepared from the CD4+ fraction was a chimera of patient and donor type with the donor type predominating. In contrast, on day 35 (after NM administration) in case 1, DNA prepared from the whole blood samples and the CD4+ fractions was a chimera of patient and donor type with the patient type predominating and DNA prepared from the PBMC fraction was of patient type and DNA prepared from the CD8+ fraction was a chimera of donor and patient type with the donor type predominating. On the other hand, on day 23 (before NM administration) in case 2, DNA prepared from the samples of whole blood and the CD4+ and CD8+ fractions was of donor type and DNA prepared from the CD4−CD8− fraction was a chimera of patient and donor with the patient type predominating. In contrast, on day 35 (after NM administration) in case 2, DNA prepared from the samples of whole blood and the CD4+ fractions was a chimera of patient and donor type with the patient type predominating. DNA prepared from the CD8+ fraction was a chimera of patient and donor type with donor type predominating and DNA prepared from the CD4−CD8− fraction was of patient type. Representative examples are shown in Fig. 1 (case 1) using oligonucleotide primers ACTBP2 and Fig. 2 (case 2) using oligonucleotide primer APOC3 in the PCR to amplify polymorphic loci. To sum up, the patient type band patterns were seen in DNAs for all cell fractions prepared from samples of whole blood collected after administration of NM on polyacrylamide gel, to an especially marked extent in the CD4+ fraction in both cases. Moreover, the results of amelogenin specific gene detection, clearly showed the recovery of Y-chromosome specific alleles, were completely identical to the results of microsatellite polymorphism analysis in case 2 in every cell fractions (data not shown).
count (700 per cubic millimeter on day 26 after transfusion in case 1 and 200 per cubic millimeter on postoperative day 25 in case 2, both at the start of NM administration) were notable. Since, in case 2, the anti-CD3 mAb OKT-3 was administered with NM, the recovery of the patient PBMCs in this case might have been due in part to an effect of OKT-3. Only one case of OKT-3 administration resulting in recovery of a patient from PT-GVHD had been described previously in literature. However, this was an atypical case of PT-GVHD in that the patient WBC count was normal and the patient’s general clinical condition was fairly good. In marked contrast, in the present two cases, the patients’ symptoms including the pancytopenia were very severe. As far as we know, in six other PT-GVHD cases in which the patients were administered with OKT-3, the patient PBMC did not recover and the patients did not recover from the disease (personal communications). With the evidence in case 1 in which OKT-3 was not administered, NM alone was probably responsible for the recovery of the patient PBMC, the disappearance of the rash, normalization of the fever, liver function improvement and the long survival time in the present two cases.

In our previous report, we showed in vitro data that, same as NM, “chloroquine” also efficiently inhibited cytotoxic activities of CTL clones and TNF α production by TNF α producing clones. Moreover, combination use of NM and chloroquine resulted in showing synergistic inhibitory effect on the cytotoxic activities of CTL clones in vitro. These actions of chloroquine are probably due to interfering CTL alloreactivity by blocking peptides binding to major histocompatibility complexes (MHC) of target cell, and interfering the exocytosis of cellular products (cytokines such as TNF) by inhibiting vesicle fusion. Chloroquine cannot freely be used to the administration in Japan under governmental direction for the present, though the governmental regulation would be released in near future to use to administration in PT-GVHD. At that time, chloroquine might also be powerful drug to cure of

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Table I. Results of DNA polymorphism analysis in case 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>WB</td>
</tr>
<tr>
<td>NM administration (day 24)</td>
<td>D (lane 3)</td>
</tr>
<tr>
<td>After</td>
<td>NM administration (day 35)</td>
</tr>
</tbody>
</table>

Results were obtained using the microsatellite primer ACTBP2 for PCR amplification. Lane 1 and 11 show the DNA size markers of φ×174 digested with HaeIII. Lane 2 shows the patient DNA band patterns derived from her fingernail chippings. DNA samples and band patterns in each lane are indicated in the above table. WB: whole blood. “P” indicates patient band patterns (examples are indicated by solid triangles). “D” indicates donor band patterns (examples are indicated by open triangles). “P>D” indicates chimera of patient and donor band patterns dominating patient band. Recovery of patient band patterns indicate by underlines in the above table.
PT-GVHD along with NM.

PT-GVHD effector clones were classified into two groups as regard to their mediator of killing, 1) Fas/Fas-ligand mediated and 2) perforin/granzyme mediated ones, as shown in our previous report. NM is a protease inhibitor and the inhibitory mechanisms of perforin/granzyme mediated CTL activities are naturally well understood. Up to now, though mechanisms of Fas/Fas-ligand mediated killing are not completely understood, NM is also probably effective on the inhibition of cytotoxic activities of Fas/ Fas-ligand mediated CTL clones same as perforin/granzyme mediated CTL clones, because NM effectively inhibited cytotoxic activities of both type CTL clones. NM would surely inhibit the cytotoxic activities of CD8+ CTLs, though the recovery of patient type band patterns in CD8+ fractions were delayed compared to those in CD4+ fractions which were soon replaced to patient type after administration of NM. Treatment of NM in an earlier stage before patient cell are completely replaced with donor cells and achievement of complete elimination of donor-derived CD8+ CTLs after NM treatment success would lead to complete cure of PT-GVHD.

### Table II. Results of DNA polymorphism analysis in case 2

<table>
<thead>
<tr>
<th>Samples</th>
<th>Fractions</th>
<th>WB</th>
<th>CD8+</th>
<th>CD4+</th>
<th>CD4−CD8−</th>
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<tbody>
<tr>
<td>Before NM administration</td>
<td>D (lane 3)</td>
<td>D</td>
<td>D</td>
<td>P</td>
<td>D</td>
</tr>
<tr>
<td>(day 23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After NM administration</td>
<td>P&gt;D (lane 4)</td>
<td></td>
<td>D&gt;P</td>
<td>P&gt;D</td>
<td>P</td>
</tr>
<tr>
<td>(day 35)</td>
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</table>

Results were obtained using the microsatellite primer APOC3 for PCR amplification. Lane 1 and 12 show the DNA size markers of ø×174 digested with HaeIII. Lane 2 shows the patient DNA band patterns derived from his fingernail clippings. Lane 11 shows the band patterns of DNA derived from donor WB. DNA samples and band patterns in each lane are indicated in the above table. WB: whole blood. "P" indicates patient band patterns (examples are indicated by solid triangles). "D" indicates donor band patterns (examples are indicated by open triangles). "P>D" indicates chimera of patient and donor band patterns dominating patient band. Recovery of patient band patterns indicate by underlines in the above table.

### References