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GUIDELINE

# Guidelines for safety management of granulocyte transfusion in Japan

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**Abstract** Granulocyte transfusion (GTX) has recently been revived by the ability to stimulate granulocyte donors with granulocyte colony-stimulating factor (G-CSF), resulting in a greatly increased number of cells that can be collected. However, there is a paucity of guidelines for assessing the appropriateness and safety management of GTX. The objective of this study was to establish guidelines for the safety management of GTX appropriate for the clinical situation in Japan. The Japan Society of Transfusion Medicine and Cell Therapy, Granulocyte Transfusion Task Force issued the first version of guidelines for GTX considering the safety management of both granulocyte donors and patients who receive GTX therapy. The current guidelines cover issues concerning: (1) the appropriateness of medical institutions, (2) management of granulocyte donors, (3) quality assurance of granulocyte concentrates, (4) administration of granulocyte concentrates, (5) evaluation of the effectiveness of GTX therapy, and (6) complications of GTX therapy. The simple 'bag separation method' without apheresis may be recommended for granulocyte collection in pediatric patients. The first version of guidelines for GTX therapy has been

The Japan Society of Transfusion Medicine and Cell Therapy, Granulocyte Transfusion Task Force.

Although the recommendation and information are believed to be true and accurate at the time of preparation of the guidelines, neither the authors nor the Japan Society of Transfusion Medicine and Cell Therapy accept any legal responsibility for the content of current guidelines.

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established, which may be appropriate for the clinical situation in Japan. Care should be taken to perform the safety management of both granulocyte donors and patients who receive GTX therapy.

**Keywords** Granulocyte transfusion · Guidelines · Granulocyte colony-stimulating factor · Safety management

## 1 Introduction

Neutrophils play an essential role in the body's first line of defense against bacterial and fungal infections, and severe neutropenia, defined as an absolute neutrophil count (ANC) of less than 500/μl, is a well-recognized factor predisposing patients to these infections [1]. A direct correlation between the depth and duration of neutropenia and the risk of infection was demonstrated [2]. Because febrile neutropenia (FN), defined as a fever ≥38.3°C (101°F) with severe neutropenia, is associated with potentially life-threatening infection, patients with FN require treatment with broad-spectrum antibiotics as soon as possible without waiting for the results of blood cultures or other studies [3]. In spite of modern antimicrobials and supportive therapy, infections associated with severe neutropenia have been a major cause of morbidity and mortality in patients undergoing aggressive cancer chemotherapy and hematopoietic stem cell transplantation (HSCT) [4]. Granulocyte colony-stimulating factor (G-CSF) stimulates the proliferation of granulocytic precursors, reduces the transit time through the granulocytic compartment, and potently stimulates neutrophil release from the bone marrow [5]. G-CSF also activates neutrophils to enhance their phagocytic function, including respiratory burst activity and surface CD11b/CD18 antigen expression in vitro and in vivo [6, 7]. G-CSF is widely employed in the clinical setting to treat or prevent neutropenia attributable to hematological disorders, myelosuppressive chemotherapy, or HSCT. In addition, the use of G-CSF for the mobilization of peripheral blood progenitor cells (PBSC) has been adopted as an international standard of care [8].

When infections occur in severe neutropenic patients who do not respond to G-CSF therapy, providing the patient with normally functioning neutrophils seems to be logical. Traditional granulocyte transfusion (GTX) therapy showed marginal efficacy, mainly attributable to the inadequacy of the cell dose ordinarily provided [9]. In the G-CSF era, G-CSF stimulation with or without corticosteroids of healthy individuals is well tolerated and allows the collection of large numbers of neutrophils [10, 11]. Although the evidence for the clinical efficacy of GTX therapy is less clear, many single case reports and small cohort studies have been published. The objective of this study was to establish guidelines for GTX therapy considering the safety management of both granulocyte donors and patients, being appropriate for the clinical situation in Japan.

## 2 Text of the guidelines

### 2.1 Purpose of the guidelines

This document sets out guidelines specifically addressing the issues regarding GTX therapy, especially the safety management of both granulocyte donors and patients who receive GTX therapy. These guidelines include: (1) the appropriateness of medical institutions, (2) management of granulocyte donors, (3) quality assurance of granulocyte concentrates, (4) administration of granulocyte concentrates, (5) evaluation of the effectiveness of GTX therapy, and (6) complications of GTX therapy.

### 2.2 Indications for GTX therapy

A good indication for GTX therapy is prolonged 'reversible' neutropenia with an ANC of less than 500/μl, which is refractory to G-CSF therapy and is associated with severe uncontrolled infection (e.g., sepsis including suspicious cases, abscess in the liver or spleen, cellulites, and marrow myelitis). The cause of neutropenia is typically HSCT or aggressive cancer chemotherapy-induced bone marrow failure that is expected to recover. Because the underlying disease process is the main determinant of the outcome in neutropenic patients, the indication for GTX therapy in hematologic disorders may be limited to patients who have received HSCT or aggressive cancer chemotherapy. Patients with congenital neutrophil dysfunction, such as chronic granulomatous disease and leukocyte adhesion deficiency, may also be indicated for GTX therapy when severe uncontrolled infection is accompanied.

### 2.3 Appropriateness of medical institutions

#### *2.3.1 Transfusion service*

Although blood components are administered to patients in most large-scale community and university hospitals in Japan, some hospitals neither have transfusion services nor employ laboratory technologists licensed by the Japan Society of Transfusion Medicine and Cell Therapy. Because granulocyte concentrates are not supplied from branches of the Japanese Red Cross Blood Center (JRCBC), unlike other allogeneic blood components, they need to be collected from granulocyte donors in hospitals. Thus, the hospital where GTX therapy is performed should have a transfusion service or appropriate system, approved

by the hospital transfusion committee, as described below. In particular, the hospital should appoint a professional medical doctor(s) responsible for managing the overall safety of GTX therapy. In the case of granulocyte collection by employing the apheresis method, the hospital is encouraged to employ a professional medical technologist(s) practicing apheresis therapy.

*2.3.2 Role of the hospital transfusion committee*

Every hospital where GTX therapy is performed should have a multidisciplinary hospital transfusion committee to oversee the provision of safe and appropriate transfusion support. The hospital transfusion committee may comprise doctors and nurses from clinical departments where blood administrations are frequently required, pharmacists, laboratory technologists, as well as representatives of the hospital. The practice of GTX therapy should be approved by the committee.

*2.3.3 Area for collection of granulocyte concentrates*

Blood collection from granulocyte donors should be carried out in a well-cleaned room, and it is recommended to use a reclining phlebotomy seat. In addition, there should be emergency kits including oxygen inhalation for resuscitation if the conditions of donors deteriorate. As described below, granulocyte concentrates should be irradiated before administration to the patient to prevent transfusion-associated graft-versus-host disease (TA-GVHD). Thus, the hospital should have an exclusive irradiation apparatus or an alternative way to irradiate blood components.

2.4 Management of granulocyte donors

*2.4.1 Selection of granulocyte donors*

A phase I/II trial of GTX therapy employing donors selected from pools of community apheresis donors has been reported [12]. Because the JRCBC does not participate in the collection of granulocyte concentrates for GTX therapy, granulocyte donors may be selected from family members or friends of the patient undergoing GTX therapy. The current guidelines do not positively recommend non-family members for granulocyte donors at present, unlike allogeneic HSCT.

*2.4.2 Age of granulocyte donors*

The criteria for granulocyte donor selection should be broadly inline with those used for other blood donations. The age of granulocyte donors should be from 19 to 54 years old, in accordance with the standard for platelet apheresis donors of the JRCBC.

*2.4.3 Blood group of granulocyte donors*

Granulocyte donors should be ABO- and Rh(D)-compatible with the patient, because a relatively large number of red blood cells (RBCs) are contained in a typical granulocyte concentrate. If the hospital has a transfusion service, where the plasma fraction can be removed from granulocyte concentrates in the case of 'the bag separation method' as described below, granulocyte donors with minor incompatibility may also be selected.

*2.4.4 Collection from the same donor*

Granulocyte concentrates may be collected from the same donor in the case of a limited number of available granulocyte donors. Granulocyte collection from the same donor should be conducted on two consecutive days in the case of apheresis donation, but repeated collections from the same donor are not prohibited in the presence of an intermission.

*2.4.5 Cytomegalovirus (CMV) serology*

If the patient is CMV-seronegative, granulocyte donors should also be CMV-seronegative except for life-threatening situations, because most patients who receive GTX therapy are in a patient population that requires CMV-safe components.

*2.4.6 Alloimmunization*

In the case of alloimmunized patients, granulocyte concentrates may be collected from either HLA-matched donors or donors who are selected by leukoagglutination crossmatching, although the best method to accurately assess donor and leukocyte compatibility has yet to be determined [13]. Considering life-threatening situations, granulocyte concentrates may also be collected from an HLA-mismatched donor for GTX therapy for the patient with anti-HLA antibody.

*2.4.7 Medical examinations and laboratory testing*

A doctor responsible for GTX therapy should fully interview granulocyte donors regarding episodes of suspected infectious disease transmission and conduct physical examinations before granulocyte collection. The timing of medical examinations may be optimal at the time of G-CSF administration 12–18 h before granulocyte collection. Laboratory tests for granulocyte donors should be as consistent as possible with those for any allogeneic blood

components supplied from branches of the JRCBC, including blood group ABO and Rh(D); serum antibody screening; infectious disease screening of hepatitis B virus (HBs-Ag and Hbc-Ab), hepatitis C virus (HCV-Ab), human immunodeficiency virus (HIV-1/-2-Ab), human T cell lymphotropic virus type I (HTLV-I-Ab), and syphilis (TPHA); complete blood count; and biochemical analysis (e.g., alanine aminotransferase). In the case of infectious disease screening, the current guidelines recommend performing the tests as many as possible in the hospital, although the results of tests will not immediately be obtained.

*2.4.8 Informed consent*

Informed consent should always be obtained from the granulocyte donor for: (a) granulocyte collection, (b) collection procedures, (c) the administration of G-CSF with or without corticosteroids, (d) use of RBC-sedimenting agents (when employed), and (e) any possible short- and long-term consequences of granulocyte collection. There should always be an opportunity for the donor to reconsider granulocyte donation in the light of a response or lack or response.

*2.4.9 Post-donation care*

Considering the administration of G-CSF to healthy individuals and its potential long-term adverse effects, as described below, a record of granulocyte donors regarding any post-donation complications should be made. Care of granulocyte donors should include observations in the immediate post-apheresis period to minimize the occurrence of delayed complications (e.g., thrombocytopenia). The current guidelines recommend the establishment of a donor registry to collect the necessary data on short- and long-term side effects of G-CSF administration to normal donors [14, 15]. Comprehensive, prospectively obtained registration data are needed to fully evaluate long-term safety concerns among healthy individuals who receive G-CSF.

2.5 Quality assurance of granulocyte concentrates

*2.5.1 Collection of granulocyte concentrates*

*2.5.1.1 G-CSF* For granulocyte mobilization, donors may receive recombinant human G-CSF (non-glycosylated G-CSF [Filgrastim] or glycosylated G-CSF [Lenograstim]) with or without corticosteroid administration. It has been reported that optimal granulocyte mobilization can be achieved in normal donors with a combined regimen of subcutaneous G-CSF at 450 μg and oral dexamethasone (DEX) at 8 mg in a single-dose format designed for clinical GTX therapy [10]. Although the daily administration of G-CSF (e.g., 5 consecutive days) results in higher yields of granulocytes, the current guidelines recommend a single subcutaneous dose of G-CSF (5–10 μg/kg) 12–18 h before each granulocyte collection. As described above, granulocyte collection from the same donor on consecutive days is recommended over 2 days, but repeated collections from the same donor are not prohibited in the presence of an intermission.

*2.5.1.2 Corticosteroids* To maximize the number of granulocytes obtained, corticosteroids have been administered to mobilize granulocytes from the marrow storage pool and to increase circulating granulocyte counts [10, 11]. Usually, DEX at 8 mg is orally administered once 12 h before granulocyte collection. On frequent collection from the same donor, the medical doctor in charge should monitor the donor regarding corticosteroid-induced adverse events, as discussed below.

*2.5.1.3 RBC-sedimenting agent* The RBC-sedimenting agent, traditionally hydroxyethyl starch (HES), may be continuously added to the donor's blood during an apheresis procedure to achieve an adequate separation of granulocytes from RBCs. It has been shown that high-molecular weight (MW) HES resulted in a significantly higher yield compared with low-MW HES [16]. However, high-MW HES products have, at present, not been approved in Japan. In the case of using a high-MW HES for granulocyte collection, it should be approved by the Ethics Committee of the hospital.

*2.5.2 Methods of granulocyte collection*

*2.5.2.1 Bag separation method* The simple 'bag separation method' without apheresis may be recommended for granulocyte collection in pediatric patients [17]. In brief, whole blood (200 or 400 ml) is drawn into the main bag of a triple-collection bag [200- or 400-ml capacity containing 34 or 68 ml, respectively, of citrate–phosphate–dextrose (CPD) solution] employing the gravity-flow principle. After centrifugation at 640*g* for 15 min at 20°C, the plasma layer is separated into the first sub-bag. The buffy-coat layer and the upper one-third of the RBC layer, both of which are rich in granulocytes, are collected into the second sub-bag by applying pressure on the main bag. The remaining RBC and plasma components are returned to the donor using a sterile-connecting device. This process is repeated two or three times, when necessary. It is noteworthy that the bag separation method does not require the use of an RBC-sedimenting agent, such as high-MW HES, which reduces the burden on the donor [17, 18].

*2.5.2.2 Apheresis method* Granulocyte collection may usually be performed on various blood cell separators using the white blood cell cytapheresis set and an exclusive program of the separator's software. It was reported that the use of higher interface offset settings (35 vs. 15) resulted in a significant increase in the granulocyte collection efficiency [19]. Because interface offset settings are dependent on the apheresis systems used, the relevant setting should be evaluated and used for achieving a maximal granulocyte yield in the hospital. The required apheresis procedure for granulocyte collection would present a potential clinical risk for cardiac or cerebrovascular events in donors with preexisting inflammatory or vascular disease, and, as such, should be avoided in these subjects.

*2.5.3 Preparation of granulocyte concentrates*

*2.5.3.1 Gamma irradiation* Granulocyte concentrates contain significant amount of donor lymphocytes and are frequently transfused to immunocompromised patients with neutropenia [20]. Currently, the gamma irradiation of blood components is the only proven effective method for TA-GVHD prevention [20]. The AABB Standards recommend a minimum 25 Gy dose of gamma irradiation to the central portion of the container, with no less than 15 Gy delivered to any part of the bag [21]. 'HLA one-way match' results in the inability to reject donor lymphocytes even if the recipient is immunocompetent, and it occurs at a rather high frequency, one in several hundred blood transfusions from unrelated donors in Japan [22]. The JRCBC disseminated transfusion information regarding TA-GVHD to most Japanese hospitals in December 1999, in which the administration of irradiated blood components except for fresh-frozen plasma is recommended for preventing TA-GVHD. Most Japanese hospitals are generally supplied with 15-Gy (or more)-irradiated blood components from branches of the JRCBC. If hospitals have an exclusive gamma-irradiation apparatus for blood, non-irradiated components are supplied and irradiated at a dose between 15 and 50 Gy in transfusion services [23]. Thus, granulocyte concentrates should be irradiated before administration to the patient at a dose between 15 and 50 Gy. Recent studies have demonstrated that the irradiation of neutrophils did not affect their in vitro functions, including respiratory burst activity and phagocytosis [24].

*2.5.3.2 Storage* There is general agreement that granulocyte concentrates should be administered as soon as possible after collection [21]. The British Committee for Standards in Haematology (BCSH) recommended that granulocytes should be stored in the same donor's citrate-anti-coagulated plasma at room temperature, kept unagitated, and administered within 12 h of preparation [25]. In the case of a limited number of available granulocyte donors, there may be a need for storage of an aliquot of granulocyte concentrates. G-CSF has been shown to inhibit granulocyte apoptosis [26], and may be useful in lengthening the acceptable storage time for granulocyte concentrates and, thereby, improving the logistics of GTX programs [9]. Drewniak and colleagues [27] investigated granulocytes from leukapheresis products mobilized by G-CSF with DEX, where in vitro granulocyte functions were intact at least 24 h. Mochizuki and colleagues [18] also reported the extended storage of granulocyte concentrates mobilized by G-CSF with or without DEX, where in vitro granulocyte functions were maintained for as long as 72 h after collection by the 'bag separation method'. The current guidelines recommend that granulocyte concentrates should be transfused within 48 h after collection.

2.6 Administration of granulocyte concentrates

*2.6.1 Infusion of granulocyte concentrates*

Granulocyte concentrates should be slowly administered over 1–4 h through a standard transfusion set with a screen filter (170–200 μm) within 6 h after collection. In the case of 200 ml of granulocyte concentrates, it should be administered over 1– 2 h in adults and 2–4 h in pediatric patients. In general, granulocyte concentrates are administered every other day until complete recovery from infection is documented. The BCSH guidelines recommend that all granulocytes should be irradiated for patients of any age and transfused as soon as possible after irradiation [25]. Leukocyte reduction filters must not be used, because it makes no sense to use these filters in GTX. Patients should be monitored by pulse oximetry. The blood pressure should also be measured every 15 min during the infusion of granulocyte concentrates.

*2.6.2 Premedication*

The administration of antipyretics or corticosteroids (e.g., 100 mg of hydrocortisone) is appropriate for patients who experience symptoms such as chills and fever. Routine prophylaxis with these agents is not necessary [9].

2.7 Evaluation of effectiveness of GTX therapy

*2.7.1 Success of GTX therapy*

The success of GTX therapy is defined as complete recovery from infection, being documented by: (a) disappearance of clinical symptoms (e.g., fever), (b) negativity of laboratory findings (e.g., C-reactive protein), (c) disappearance or

marked reduction of radiological findings, or (d) negativity of microbiological cultures.

*2.7.2 Discontinuation of GTX therapy*

In general, GTX therapy is continued daily to maintain an ANC of more than 500/μl until neutrophil recovery, clinical improvement, or stability. However, prolonged GTX therapy may be difficult in cases of a limited number of available granulocyte donors. The current guidelines recommend criteria for the discontinuation of GTX therapy as follows: (a) neutrophil recovery or bone marrow engraftment with an ANC of more than 500/μl in patients who received HSCT, (b) recovery from infection without the need of GTX support, (c) refractoriness to GTX therapy even if continued for 7 consecutive days, or (d) occurrence of an adverse event due to GTX therapy.

2.8 Complications of GTX therapy

*2.8.1 Donor-associated side effects*

*2.8.1.1 G-CSF* Short-term side effects: The most commonly reported side effects of G-CSF administration include bone pain, headache, fatigue, nausea, fever (with or without chills and sweats), insomnia, anorexia, and myalgias [28]. All side effects appear to be generally mild and usually resolve after the discontinuation of G-CSF. However, analgesics may be needed for bone pain, which was the most frequent symptom [29, 30]. Suggested contraindications to G-CSF administration in donors include the presence of active inflammatory conditions and hypercoagulable states, with or without previous venous thrombosis and known or suspected atherosclerotic vascular disease [28].

Long-term side effects: The question regarding the long-term safety of G-CSF administration to normal donors, particularly in terms of the leukemogenic potential, has been raised. Theoretically, a prior history of malignancy or a strong family predisposition to acute myeloid leukemia (AML) or myelodysplasia may place individuals at a higher risk of developing hematologic malignancies [14]. There are limited data generated by long-term follow-up studies on normal donors who received G-CSF administration for granulocyte collection. Quillen and colleagues [31] recently reported 2 cases of lymphoid malignancy (one case each of non-Hodgkin's lymphoma and chronic lymphocytic leukemia) in 83 unrelated granulocyte donors who received repeated administrations of both G-CSF and DEX and were followed for a median of 10 years. Although it has been shown that pharmacologic doses of G-CSF affect cytokine production by lymphocytes in vitro and in vivo [32], there is no evidence to date supporting an association between G-CSF and lymphoid malignancy [31]. Bux and colleagues [16] reported on a 2-year follow-up of 183 granulocyte donors, where no severe G-CSF-related adverse events were noted. The Research on Adverse Drug Events and Reports (RADAR) project reviewed clinical literature on adverse events that occur when G-CSF is administered to healthy individuals for PBSC collection [29]. Three PBSC donors were described who developed AML following stem cell mobilization, but the evidence supporting causality is unclear.

*2.8.1.2 Corticosteroids* It remains controversial whether the administration of corticosteroids along with G-CSF stimulation to granulocyte donors increases the risk of posterior subcapsular cataract [33, 34]. However, the administration of corticosteroids to granulocyte donors, especially in frequent donations, should be used with caution.

*2.8.1.3 RBC-sedimenting agent* RBC-sedimenting agents, such as high-MW HES, act as a plasma expander and can cause transient hypertension with flushing and headache. Severe itching following the infusion of HES may be observed in a small number of granulocyte donors [16].

*2.8.1.4 Apheresis donation* During apheresis, anticoagulation is necessary to prevent coagulation and the clumping of collected components. CPD is returned to the donor, and its toxicity occasionally causes symptoms associated with decreased ionized calcium levels (e.g., peri-oral paresthesia). As an antidote to citrate toxicity, calcium prophylaxis may be required during large-volume leukapheresis.

*2.8.2 Recipient-associated side effects*

*2.8.2.1 Transfusion reactions* Mild to moderate fever and chills are relatively common, whereby the slowing of administration may be required. These reactions are preventable on subsequent transfusions by treatment with antipyretics or corticosteroids [12]. However, routine prophylaxis with these agents is controversial. More severe reactions may occur in approximately 1–5% of cases of GTX therapy, including hypotension, pulmonary infiltrates, and respiratory distress [35]. In patients who receive repeated GTX therapy, alloimmunization and platelet refractoriness may develop [36]. The rate of leukocyte alloimmunization has been reported to be 24% [16].

*2.8.2.2 Concurrent use of Amphotericin B* Although an association between pulmonary infiltration and Amphotericin B administration has not been confirmed [37], it is still common practice to separate the administration times if Amphotericin B and granulocyte concentrates are being given concurrently [35]. The current guidelines recommend that granulocyte concentrates should be administered

at least 4 h after stopping Amphotericin B administration in patients who receive both Amphotericin B and granulocyte concentrates.

## 3 Conclusion

The current guidelines may be appropriate for the clinical situation in Japan, in which granulocyte donors cannot be selected from the community pool of apheresis donors of the JRCBC, and high-MW HES products are not approved. Care should be taken to perform GTX therapy considering the safety management of both granulocyte donors and patients. Future randomized controlled trials are needed to clarify the efficacy of GTX therapy and identify which subgroup of patients benefits the most.

**Conflict of interest statement** The authors declare no conflicts of interest.

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# Modified ELISPOT assay may predict T-cell hyporesponsiveness to non-inherited maternal antigens

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ELISPOT assay, noninherited maternal antigens, immunological tolerance

## SUMMARY

Clinical reports have suggested the existence of immunological tolerance to noninherited maternal antigens (NIMA) in human leukocyte antigen (HLA) mismatched allogeneic stem cell transplantation (allo-SCT). We studied the T-cell reactivity using IFN-γ enzyme-linked immunospot (ELISPOT) assay in three HLA fully matched allo-SCT cases and one healthy volunteer family case. In HLA fully matched allo-SCT cases, ELISPOT assay could detect the hyporesponsiveness of T cells from donors to the B cells from recipients. Moreover, ELISPOT assay showed that the T cells from an individual responded to B cell from his mother significantly weakly than those from an unrelated HLA-haploidentical individual. These observations suggest that our IFN-γ ELISPOT assay-based method may predict the presence of immunological tolerance to NIMA.

## INTRODUCTION

Highly transfused renal transplant candidates develop anti-HLA antibodies significantly less frequently against their noninherited maternal HLA antigens (NIMA) than noninherited paternal HLA antigens (NIPA), suggesting the existence of feto-maternal immunological tolerance (Class *et al.*, 1988). Based on this hypothesis, Burlingham *et al.* (1998) showed the superior graft survival rate in NIMA- to NIPA-mis-

 

matched renal transplant recipients from sibling donors. Furthermore, van Rood *et al.* (2002) demonstrated that allogeneic stem cell transplantation (SCT) from NIMA-mismatched siblings showed a lower incidence of severe acute graft-versus-host disease (aGVHD) compared with that from the other family donors. Following these observations, we and others have demonstrated the feasibility of HLA-haploidentical SCT from NIMA-mismatched relatives without T-cell depletion (Ichinohe, Maruya & Saji, 2002; Shimazaki *et al.*, 2003). Stern *et al.* (2008) recently showed the advantage of maternal donor in T cell depleted HLA-haploidentical SCT. These clinical studies have been performed based on the presence of feto-maternal microchimerism as a result of feto-maternal immunological tolerance, nevertheless some cases developed severe aGVHD despite the existence of microchimeric cells (Ichinohe *et al.*, 2004).

Noninherited maternal HLA antigen allografts have been shown to accept better than NIPA allografts *in vivo*, and *in vitro* T-cell response to NIMA is significantly reduced in interleukin (IL)-2, IL-5 and interferon (IFN)-γ production (Andrassy *et al.*, 2003; Akiyama *et al.*, 2005). In human, Tsafrir *et al.* (2000) demonstrated such NIMA effect using umbilical cord blood mononuclear cells by a mixed lymphocyte reaction (MLC), but Hadley *et al.* (1990) could not detect it when using peripheral blood mononuclear cells from healthy individuals. Recently, van den Boogaardt *et al.*, (2005) also failed to detect any differences between NIMA and NIPA in T-cell reaction of MLC and ELISPOT assay *in vitro*.

In this study, we analyzed the T-cell reactivity by devising an IFN-γ ELISPOT assay using sorted responder T cells and stimulator B cells in three cases of HLA fully matched related SCT, and healthy individuals from one family to compare immune responses of T cells between NIMA and other combinations.

## MATERIALS AND METHODS

### Subjects

Three cases of HLA fully matched related SCT, four healthy volunteers including a family member and a one HLA-haploidentical unrelated donor were tested. All individuals were typed for major histocompatibility complex class I and class II at a high-resolution level by DNA-based WAKFlow HLA typing system (Wakunaga Co., Hiroshima, Japan). All the donors provided written informed consents which were approved by the

| | Donor | Engraftment | aGVHD | cGVHD | Outcome |
|---|---|---|---|---|---|
| Case 1 | Brother | Day 14 | Grade 1 (skin 1) | none | Alive in day 187 |
| Case 2 | Brother | Day 12 | Grade 0 | none | Alive in day 155 |
| Case 3 | Brother | Day 23 | Grade 1 (skin 1) | none | Alive in day 153 |

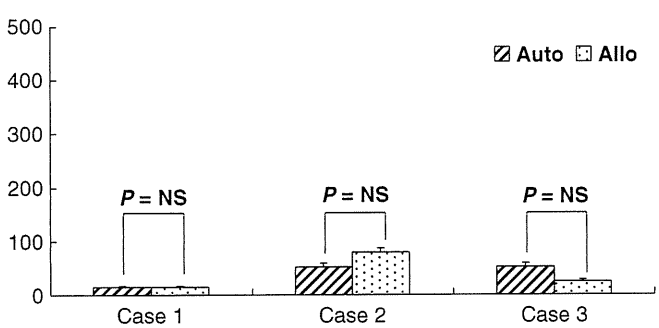


Figure 1. Clinical outcomes in cases of HLA fully matched related SCT and results of a modified IFN-γ ELISPOT assay. The relationship between donor and recipient, the engraftment, the grade of graft-versus-host disease (GVHD), and outcomes in each case are shown in upper table. There is no significant difference in the reaction of T cells from donor against B cells from donor (autologous) and recipient (allogeneic). Acute GVHD was graded by according to standard criteria.

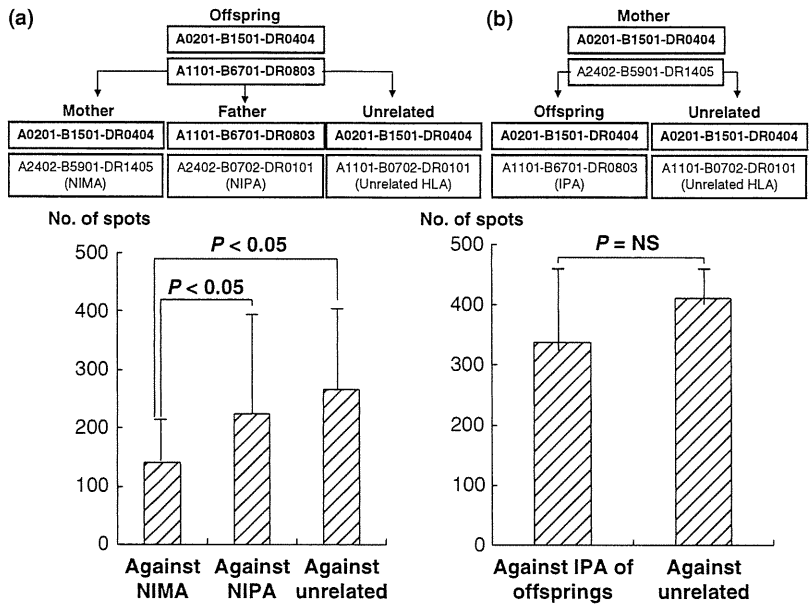


Figure 2. The relationship between responder and stimulator and T-cell responsiveness from the offspring or mother against stimulator B cells using a modified IFN-γ ELISPOT assay. The HLA haplotypes of each individual are shown in upper panel. The offspring, his mother, his father and unrelated donor shared one HLA haplotype (bold characters), and the number of mismatch HLA was same in each combination. T cells from the offspring were activated less remarkably by B cells from his mother (i.e. against NIMA) than those from his father (i.e. against NIPA) and from an unrelated individual (a). T-cells from the mother did not show any specific hyporesponsiveness to stimulator B cells from her offspring (i.e. against IPA) (b). NIMA: noninherited maternal HLA antigen; NIPA: noninherited paternal HLA antigen; IPA: inherited paternal HLA antigen.

institutional review board. The relationship between donor and recipient in HLA fully matched related SCT showed in Figure 1. The HLA-phenotypes of healthy individuals and the relationships in tests are shown in Figure 2. To exclude the effects of mismatched HLA, one haplotype was matched among mother, father, offspring and unrelated control, and the number of mismatches toward NIMA is equal to those in the unrelated control.

### Preparation of blood samples and cell separation

Lymphocytes were separated from peripheral blood using a Ficoll-Hypaque method. $CD3^+$ T cells and $CD19^+$ B cells were separated using MACS cell separation system (Miltenyi Biotec GmbH, Gladbach, Germany). The purity of isolated cell fractions were checked by a FACSCalibur (BD Biosciences, Bedford, MA, USA) and those showing more than 90% purity were used.

### Modified IFN-γ ELISPOT assay

Each purified sample was cultured in RPMI 1640 medium supplemented with 10% pooled human serum and 40 U/ml human recombinant IL-2 (Sigma-Aldrich, St. Louis, MO, USA) overnight at 37 °C in 5% $CO_2$. After preincubation, responder ($4.0 \times 10^6$ cells/ml) and stimulator cells ($6.0 \times 10^5$ cells/ml) were mixed and cultured in 96-well plates (Coaster, Cambridge, MA, USA) for 72 h. ELISPOT plates (Millipore, Billerica, MA, USA) were coated with anti-IFN-γ antibody (10 μg/ml, Sigma) at 4 °C overnight. Stimulated $CD3^+$ T cells were added to the plates and incubated at 37 °C in 5% $CO_2$ for overnight. Then plates were washed, and biotinylated anti-IFN-γ mAb (1 μg/ml; Endogen, Rockford, IL, USA) were added and incubated at room temperature for 2 h. Plates were washed, and SA-AP conjugate horse radish peroxidase (diluted 1/1000; Promega, Madison, WI, USA) was added for 1 h. After washing, AP conjugate substrate

kit (Bio-Rad Laboratories, Hercules, CA, USA) was added. Visualized spot was counted by ELISPOT Reader (Carl Zeiss, Tokyo, Japan).

### Statistical analysis

The Cochran and Cox, and Welch test were used to detect the differences of T cell responses. *P*-values $< 0.05$ with a 95% confidence interval were considered significant.

## RESULTS AND DISCUSSION

In three cases of HLA fully matched related SCT, the reactions of T cells from HLA fully matched related donors to B cells from patients were almost same as those to B cells from donors themselves (Figure 1). Immunological reactions of T cells such as aGVHD in these cases were easily controlled after allogeneic SCT. According to these results, we tested whether modified IFN-γ ELISPOT assay could detect the existence of feto-maternal immunological tolerance in healthy volunteer family members. Our experiments demonstrated that T cells from the offspring were activated less remarkably by B cells from his mother (i.e. against NIMA) than those from his father (i.e. against NIPA) and from an unrelated individual (Figure 2a). The number of mismatched HLA against his mother for the offspring T cells was equal to that against his father and against the unrelated control, implying that T cells from the offspring were hyporesponsive to NIMA compared with NIPA and unrelated antigens.

We have modified the ordinary ELISPOT assay in order to maximally detect the subtle differences in IFN-γ production in response to NIMA or others. First, the significant difference was only observed when purified $CD3^+$ T cells and $CD19^+$ B cells were used as responders and stimulators, respectively. Moreover, we could not detect any differences in T-cell reaction between against NIMA and NIPA with $CD8^+$ T cells only (data not shown), and these observations introduce us that $CD4^+$ T cell might have important ability in the allogeneic recognition of feto-maternal immunological tolerance. Second, responder T cells were stimulated with B cells for 72 h which was longer than usual ELISPOT assay, because this stimulation time demonstrated the most distinguishable results between NIMA and other stimulators (data not shown). Third, use of nonirradiated stimulator B cells seemed to be critical for best readout, because irradiation might damage stimulator cells and blunt their antigen presentation capacity. In comparison our method with previous ones, our method was more 'selective' and 'natural' in immunological reaction of T-cell immunity, and these differences might indicate us to be able to detect the significant difference in T-cell reaction of offspring between to NIMA and to NIPA in our experiments.

In contrast, T cells from the mother did not show any specific hyporesponsiveness to B cells from her offspring when compared with those from unrelated control (Figure 2b). A previous clinical study showed that six of 14 patients receiving SCT from maternal donors developed severe aGVHD, while only two of 20 patients receiving SCT from their offsprings or NIMA-mismatched siblings showed severe aGVHD (Ichinohe *et al.*, 2004). This suggests that the tolerance between mothers to their offspring and vice versa is of different quality or nature, and the tolerance of mother's T cells to the offspring might be more unstable than that of offspring. The persistence of feto-maternal immunological tolerance in healthy individuals varies among cases. Therefore, prediction of the immunological tolerance in HLA-haploidentical SCT is crucial to select an appropriate donor from the family members. The mechanisms involved in the unstable tolerance to her offspring in the mothers are explained by two hypotheses. One is that the mother's T cells become tolerant to the inherited paternal antigens expressed on the fetus during pregnancy, while the fetus' T cells become anergic to NIMA during immunological development before birth (van Rood & Claas, 2000). And this T-cell tolerance of offspring to NIMA is reported to be maintained by mother's breast-feeding after birth (Matsuoka *et al.*, 2006). The other is that T-cells from the mother might have been 'pre-sensitized'minor histocompatibility antigens (mHAs), because mHAs are less immuno-dominant than HLA and it might be difficult for mother's T-cell immunity to get tolerance against offspring's mHAs during pregnancy. Certainly, Verdijk *et al.* (2004) showed the existence of mHA specific cytotoxic T cells in healthy postpartum women, and these observations suggest that the difference of immuno-dominancy between HLA and mHAs. Therefore, we need to take into account the

two histocompatibilities including HLA and mHA to predict the immunological reactivity in HLA-haploidentical SCT between family members. In addition, there are many cytokines influencing to the result of allogeneic SCT, and further studies are required to examine the effect of the genetic polymorphisms of cytokines on the outcome of allogeneic SCT (Dickinson *et al.*, 2004). From this standpoint, our modified ELISPOT assay might be useful to detect the total immunological reaction of donor T cells to recipient including HLA, mHA and genetic polymorphisms of cytokines.

In conclusion, we developed a modified IFN-γ ELISPOT assay to predict the presence of T cell nonresponsiveness to NIMA in comparison with NIPA or unrelated HLAs, which may be useful to select an appropriate donor in HLA-haploidentical allogeneic SCT from family donors. Further studies testing (i) the reproducibility of this assay in other families and (ii) the clinical applicability by comparing the clinical outcome of allogeneic SCT with the *in vitro* results by this ELISPOT assay will be definitely warranted to validate our observations.

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# Loxoprofen Sodium, a Non-Selective NSAID, Reduces Atherosclerosis in Mice by Reducing Inflammation

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*Summary* **Recently, it is suggested that the use of nonsteroidal anti-inflammatory drugs (NSAID) may contribute to the occurrence of cardiovascular events, while the formation of atherosclerotic lesions is related to inflammation. Loxoprofen sodium, a non-selective NSAID, becomes active after metabolism in the body and inhibits the activation of cyclooxygenase. We fed apoE$^{-/-}$ mice a western diet from 8 to 16 weeks of age and administered loxoprofen sodium. We measured atherosclerotic lesions at the aortic root. We examined serum levels of cholesterol and triglycerides with HPLC, platelet aggregation, and urinary prostaglandin metabolites with enzyme immune assay. Atherosclerotic lesion formation was reduced to 63.5% and 41.5% as compared to the control in male and female apoE$^{-/-}$ mice treated with loxoprofen sodium respectively. Urinary metabolites of prostaglandin $E_2$, $F_{1\alpha}$, and thromboxane $B_2$, and platelet aggregation were decreased in mice treated with loxoprofen sodium. Serum levels of cholesterol and triglycerides were not changed. We conclude that loxoprofen sodium reduced the formation of early to intermediate atherosclerotic lesions at the proximal aorta in mice mediated by an anti-inflammatory effect.**

*Key Words*: **non-selective nonsteroidal anti-inflammatory drugs, loxoprofen sodium, atherosclerosis**

## Introduction

As a result of progress in therapy for rheumatoid arthritis (RA), cardiovascular events have become the major cause of death in these patients. RA is associated with an increased and accelerated vascular risk that results in lifespan reduction [*1–3*]. There is an association between disease activity and cardiovascular risk in RA and other inflammatory disorders, such as systemic lupus erythematosus (SLE) [*1*, *4*, *5*]. Several factors may be associated with accelerated arteriosclerosis in patients with RA or SLE. One factor may be the disease itself, while others may include the pharmacological therapies that patients with RA or SLE receive.

Rofecoxib, a highly selective cyclooxygenase(COX)-2 inhibitor, has recently been voluntarily withdrawn from the market due to evidence from the APPROVe trial demonstrating increased cardiovascular events after 18 months

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of use. Combination therapy with the selective COX-2 inhibitors, valdecoxib and parecoxib increased the risk by 3.7-fold [*6*]. Concerns have been raised that a highly selective COX-2 inhibition may promote cardiovascular events by inhibiting prostacyclin and inducing a prothrombotic state [*7*]. In contrast, inhibition of COX-1 mediated production of platelet thromboxane (Tx) by aspirin reduces the risk of myocardial infarction and stroke [*8*].

Selective COX-2 inhibitors are the major anti-pain drugs used in western countries. Loxoprofen sodium is a pro-drug of phenyl mefenamic acid and a non-selective COX inhibitor. It is the dominant anti-pain drug in Japan and most frequently prescribed in East Asian countries [*9*]. In view of the reports related to COX-2 selective inhibitors in the USA, the adverse effects of nonsteroidal anti-inflammatory drugs (NSAIDs) associated with cardiovascular disease were surveyed in Japan in June 2005. However, no evidence was found of any adverse effects of loxoprofen sodium associated with cardiovascular events. The Pharmaceutical and Medical Devices Agency in Japan concluded that it was not necessary to perform safety measures for loxoprofen sodium against cardiovascular events [www.info.pmda.go.jp].

It has been reported that selective inhibition of COX-1 attenuates atherosclerosis in apoE$^{-/-}$ mice [*10*]. However, reports on the effect of selective COX-2 inhibition on the development of atherosclerosis in murine models have been mixed with decreased, increased or unchanged atherosclerotic lesions all noted [*11*, *12*]. The divergence in the results may be the consequence of differences in experimental design, including efficacy and selectivity of the inhibitors, gender of the mice and stage of atherosclerotic lesions.

The surveillance study of adverse effects of loxoprofen sodium in Japan and these studies of non-selective inhibition of COX inhibition on the development of atherosclerosis in animal models demonstrate the ability of non-selective COX inhibition with loxoprofen sodium to reduce early and intermediate atherosclerotic lesion formation in apoE$^{-/-}$ mice, supporting the efficacy of anti-inflammatory approaches in the prevention of atherosclerosis.

## Materials and Methods

*Healthy volunteers*

Three healthy men participated in this study. They received blood examinations and collected urine for 24 h before administration of loxoprofen sodium. After receiving loxoprofen sodium 60 mg/body three times a day for 4 days, they received blood examinations and collected urine again. The ethics committee of Kyoto Prefectural University of Medicine approved the study.

*Animal procedures*

ApoE$^{-/-}$ mice (C57BL/6J-Apoe$^{tm1Unc}$) were originally purchased from Jackson Laboratories (Bar Harbor, ME). ApoE$^{-/-}$ mice were at the 10th backcross into the C57BL/6 background. Mice were maintained on a rodent chow diet containing 4.5% fat (Oriental BioService, Kyoto, Japan) and autoclaved acidified (pH 2.8) water before 8 weeks of age. From 8 (day 57) to 16 (day 113) weeks of age, these mice were maintained on a "Western" high-fat diet (0.2% cholesterol, 21% saturated fat; Oriental BioService, Kyoto, Japan). The atherosclerosis studies were designed to examine the effect of administration of loxoprofen sodium during early atherosclerotic lesion formation in apoE$^{-/-}$ mice. The mice were divided into 3 groups (vehicle group, 7 day loxoprofen sodium group, 56 day loxoprofen sodium group) at 8 weeks of age (day 57), randomly. Animal care and experimental procedures were carried out in accordance with the regulations and with the approval of Kyoto University's Animal Care Committee. The mice were continued on this regimen until 16 weeks of age, when they were killed with an overdose of sodium pentobarbital.

*Administration of loxoprofen sodium or indomethacin to apoE$^{-/-}$ mice*

Randomly selected mice were treated with loxoprofen sodium (4 mg/kg/day) added to the drinking water from 8 (day 57) to 16 (day 113) weeks of age or from 15 (day 106) to 16 (day 113) weeks of age. The dose of loxoprofen sodium used in our study was chosen based on oral dosing in acute studies of carageenan induced footpad edema plethysmometry in rats in which the oral ED50 for this assay in rats is 2 mg/kg, twice a day [*13*]. The dose of the drug was calculated on the basis of the average consumption of water and the body weight, determined weekly. Loxoprofen sodium was kindly provided by Daiichi-Sankyo, Co. (Tokyo, Japan).

*Urinary thromboxane, prostaglandin I, and prostaglandin $E_2$ metabolite excretion*

As platelet $TxA_2$ metabolite, prostaglandin (PG) I metabolite, and $PGE_2$ metabolites, we measured urinary 2,3 dinor $TxB_2$, urinary 2,3 dinor 6 keto $PGF_{1\alpha}$, urinary $PGE_2$ metabolites. From 15 to 16 weeks of age, 24 h urine samples of mice were collected with metabolic cages (Tecniplast, Italy). Mouse and human samples were analyzed by a commercial assay according to the manufacturer's procedure (Cayman Chemical, MI). Urine creatinine (CRE) was measured with dry chem system (SRL, Tokyo, Japan).

*Determination of the platelet aggregation*

ApoE$^{-/-}$ mice were maintained on the "Western" high-fat diet from 8 to 16 weeks of age and were treated with vehicle or 4 mg/kg/day of loxoprofen sodium from 15 to 16 weeks

of age. At 90 min after final dose administration, blood was collected from five mice in each group. Blood was collected in 12.9 mM sodium citrate and gently agitated at 37°C for 30 min, and centrifuged at 250 g for 15 min to obtain plasma [*14*]. Blood samples were also collected from healthy volunteers in 12.9 mM sodium citrate and centrifuged at 150 g for 15 min to obtain platelet rich plasma (PRP) before loxoprofen sodium administration and after receiving loxoprofen sodium 60 mg/body three times a day for 4 days. After separation of PRP, tubes were centrifuged again at 1,200 g for 15 min to obtain platelet poor plasma.

To measure changes in the light transmission rate, PRP samples (200 μl) were incubated for 2 min at 37°C, then with 22.2 μl of adenosine diphosphate (ADP) or collagen solution for 5 min at 37°C. The intensity of light transmitted over 5 min was measured using MCM hematoracer 313 (MC medical. Tokyo, Japan). ADP and collagen solution were purchased from MC medical (MC medical. Tokyo, Japan). ADP added at 1, 2, 4, 8 μM in humans and at 0.5, 1, 2, 4 μM in mice. Similarly, collagen was added at 0.25, 0.5, 1, 2 μg/mL in humans and 0.5, 1, 2, 4 μg/mL in mice. The pressure rate was standardized using a grading curve produced by plotting data with four concentrations of agonist on the x-axis and pressure rate (%) on the y-axis. The concentration of agonist causing a 50% maximum aggregation rate was calculated and applied as the platelet aggregatory threshold index (PATI) [*14*].

*Serum lipids and lipoprotein distribution analysis*

ApoE$^{-/-}$ mice were maintained on a "Western" high-fat diet from 8 to 16 weeks of age and were treated with vehicle or 4 mg/kg/day of loxoprofen sodium from 8 to 16 weeks of age. The sera were obtained from mice fasted overnight (11 animals from each group). Serum albumin, CRE, and free fatty acids (FFA) were analyzed with dry chem system (SRL, Tokyo, Japan). A high performance liquid chromatograph (HPLC) system with two tandem gel permeation columns was used to evaluate the size distribution of plasma lipoprotein particles (Skylight Biotech, Akita, Japan) [*15*, *16*]. Fast performance liquid chromatography was performed on an HPLC system model 600 (Waters) with a Sepharose 6 column (Skylight Biotech, Akita, Japan). Samples were diluted 20 times and analyzed at a flow rate of 350 μl/min by monitoring the concentrations of total cholesterol, and triglycerides with absorbance at 550 nm.

*Analysis of aortic lesions*

The hearts were flushed through the left ventricle, and the aortic root was obtained for analysis. The hearts were removed immediately after the mice were killed, rinsed in cold phosphate buffered saline to remove traces of blood, and placed in 4% paraformaldehyde overnight. The hearts were sliced with a scalpel on a plane parallel to the tips of the atria at the base of the aortic root, according to a procedure described by Paigen *et al.* [*17*]. The tissue was processed and embedded in OTC compound for histological sectioning by conventional methods. Fifteen alternate cryosections of 10-mm thickness were collected from the proximal aorta starting from the beginning of the aortic sinus and extending 300 μm distally. The sections were stained with Oil-Red-O and photographed at a magnification of 400 with an Olympus U-CMAD3 camera connected to an Olympus BX50 microscope and DXC-S500/OL digital capture (Olympus, Tokyo, Japan). The vessel structures were traced and areas calculated in the computer-digitized images with the Image J program (National Institutes of Health, MD). Sections from the aortic arch were used to quantify atherosclerosis (8 animals from each group). An observer who was blinded to all other data analyzed the lesions.

*Histological examination of lesion morphology*

Sections (10 μm) of OCT embedded tissue were acetone fixed, and endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. Serial sections were subjected to immunostaining with 2 μg/mL goat anti-COX-1 antibody (Santa Cruz Biotechnology, CA), 2 μg/mL goat anti-COX-2 antibody (Santa Cruz Biotechnology, CA), 5 μg/mL goat monoclonal antibodies to macrophages (MOMA2) antibody (AbD Serotec, Oxford, UK), followed by avidin-biotin rabbit anti-goat antibody (Nichirei, Tokyo, Japan). The slides were then incubated with 3,3-Diaminobenzidine tetrahydrochloride (Sigma, MO) and counterstained with hematoxylin (Wako Pure Chemical Industries, Osaka, Japan). Isotype-matched controls were stained in parallel and in all cases showed no significant reactivity (data not shown). Intensity of COX-1, COX-2, and MOMA2 staining was measured by determining the maximum density of DAB stained cells using the Image J program (National Institutes of Health, MD). Sections from the aortic arch were used to quantify atherosclerosis (8 animals from each group). A blinded observer analyzed the lesions.

*Statistical analysis*

Data are expressed as mean ± SEM. Statistical significance was calculated in the SPSS statistical package, version 11.0.1 J (SPSS, IL) for Windows. Student's *t* test was used for comparisons between two groups and Dunnett test was used to for comparisons among more than three groups. Probability values $<0.05$ were regarded as significant.

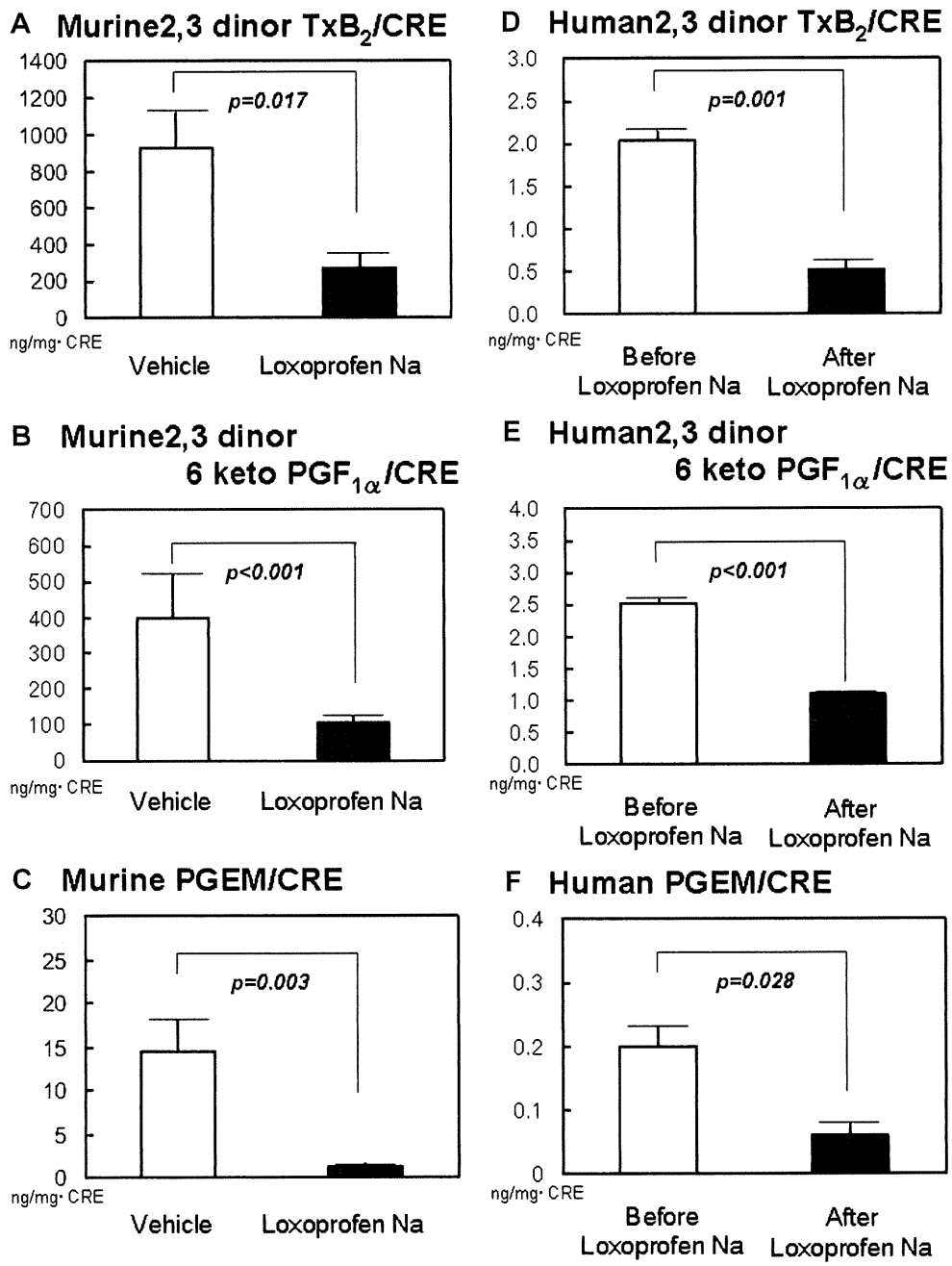


Fig. 1. Inhibition of prostaglandin production in healthy volunteers and apoE$^{-/-}$ mice by loxoprofen sodium. A, B, and C; Urinary prostaglandin metabolites in apoE$^{-/-}$ mice reconstituted with vehicle (open bars), with loxoprofen sodium (solid bars). From mice (15 to 16 weeks) given vehicle or loxoprofen sodium, urine samples for 24 h were collected in metabolic cages. Urinary metabolites of $TXB_2$ (A), $PGF_{1\alpha}$ (B), and $PGE_2$ (C) were analyzed by enzyme immune assay. D, E, and F, Urine was gathered from 3 healthy men for 24 h before and after receiving loxoprofen sodium 60 mg/body three times a day for 4 days. Urinary metabolites of $TXB_2$ (D), $PGF_{1\alpha}$ (E), and $PGE_2$ (F) were analyzed by enzyme immune assay. Data were expressed as the average (mean ± SEM) of the individual ($n = 8$) assays in each group. CRE indicates creatinine. Student $t$ test was used for comparisons between two groups and Dunnett test was used for comparisons between two groups.

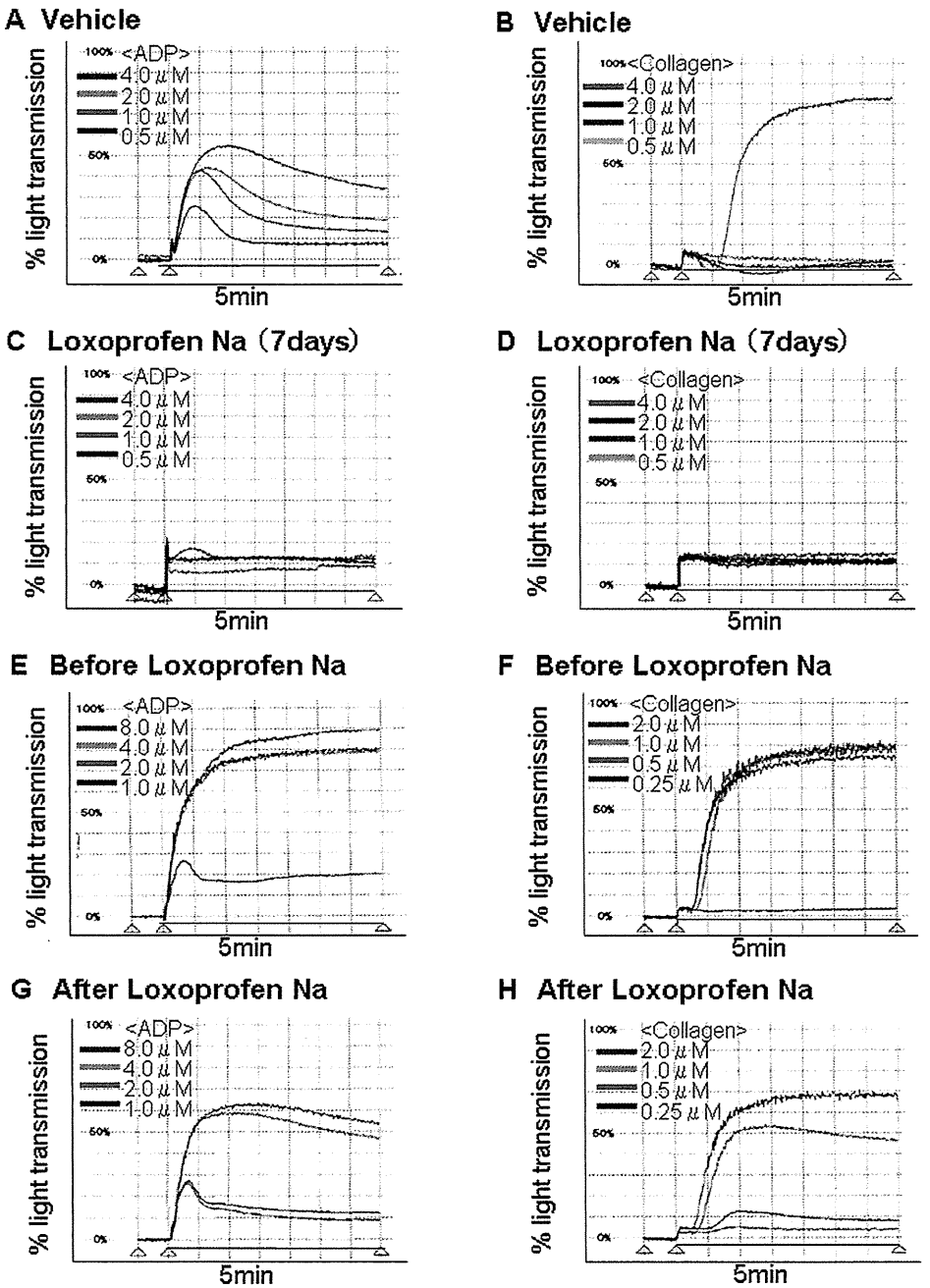


Fig. 2. Inhibition of platelet aggregation by loxoprofen sodium. Figures show representative % light transmission curves at each concentration of platelet aggregation stimulative agents. Platelet aggregation was stimulated by adenosine diphosphate (ADP) and collagen and was quantified with an MCM hematracer 313. Y-axis means % light transmission and X-axis means time. Black bar on X-axis indicates 5 min. A and B; mouse platelet aggregation with vehicle at day 7, C and D; mouse platelet aggregation with loxoprofen sodium at day 7, E and F; human platelet aggregation with vehicle at day 4, G and H; healthy human platelet aggregation with loxoprofen sodium at day 4.