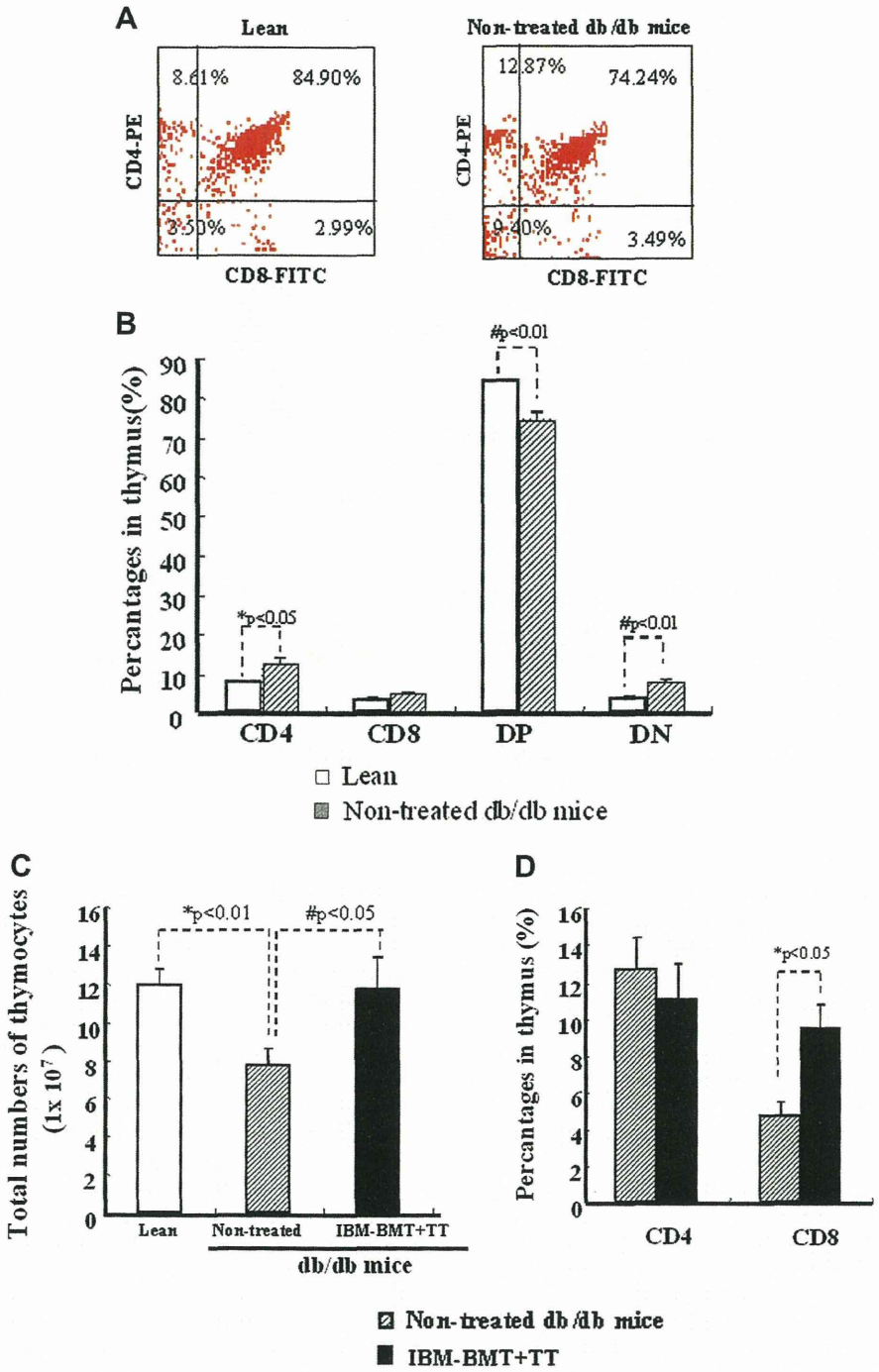

Fig. 5. Lymphocyte populations in the thymus and the ratios of CD4/CD8 in the peripheral blood and donor-derived cells. (A,B) Lymphocyte populations in the thymus by FACS, $*p < 0.0.1$, $\# p < 0.01$. (C) Total numbers of thymocytes $*p < 0.01$, $\#p < 0.05$. (D) Percentages of CD8- positive cells. $\#p < 0.05$, $n = 6$ in each group. (E) The ratios of CD4/CD8-positive cells in the peripheral blood. $*p < 0.05$, $\#p < 0.01$. The results are mean ± SE, $n = 6$ in each group. (F) Analyses of CD4, CD8, B220 and CD11b on donor-derived cells in recipient mice one month after IBM–BMT + TT.

mice [13]. In the present study, we have shown that improved hyperglycemia and insulinemia result from normalizing the imbalance of the lymphocyte subsets in the db/db mice treated with IBM–BMT + TT. The db/db mice treated with IBM–BMT alone showed decreased blood glucose levels (150 mg/dl) one week after IBM–BMT, but increased blood glucose levels (300 mg/dl) two weeks after the treatment. In contrast, the db/db mice treated with IBM–BMT + TT showed normal blood glucose levels even 7 weeks after the treatment. These data are inconsistent with a previous report: Cruzado JM et al. reported that db/db mice treated with BMT alone showed normoglycemia even 10 weeks after the treatment [44].

In the present study, the db/db mice treated with IBM–BMT + TT showed normalization of the percentages of DP, DN and CD4 cells in the thymus and also the normalization of CD4/CD8 ratios in the PB, which resulted in decreased plasma IL-6 and IL-1β levels, and

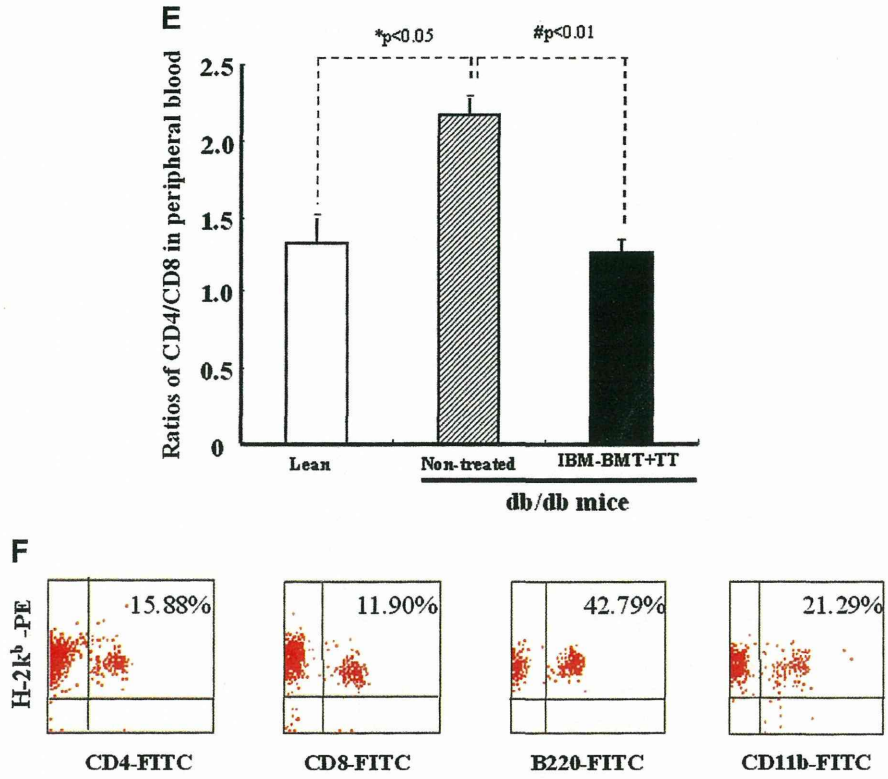

Fig. 5. (*continued*).

increased plasma adiponectin levels, followed by improved insulin sensitivity, and improved expression of AKT and pAKT on the liver and pancreas after IBM–BMT + TT. IBM–BMT + TT also led to the increased expression of HO-1 and pLKB1. The remarkable action of IBM–BMT + TT on adiponectin was associated with significant increases in pAKT and pAMPK expression, and also in the phosphorylation of insulin receptors; HO-1 increases the production of adiponectin, resulting in enhanced pLKB1-AKT-AMPK crosstalk. Thus, it seems likely that IBM–BMT + TT is a strategy that could potentially be therapeutically employed for diabetes mellitus and metabolic syndrome.

It is well known that obesity is accompanied by chronic low-grade inflammation of adipose tissue, which increases the production of inflammatory cytokines such as leptin, TNF-α, chemoattractant protein-1 (MCP-1) and IL-6 [45]. In addition, it has been shown that there are nutritional treatments that are potentially capable of modulating insulin resistance and inflammation. DM induces a variety of metabolic abnormalities because of insufficient insulin action. Abnormalities in glucose metabolism are manifested clinically as hyperglycemia after glucose ingestion. Hyperglycemia produces oxidative stress through elevated levels of ROS, which leads to beta cell damage and vascular dysfunction through a variety of mechanisms [46–48]. The normal phenotype should differ from the state of overwork when beta cells compensate for insulin resistance to keep glucose levels normal. When only mild hyperglycemia develops, beta cells are subjected to glucotoxicity. As hyperglycemia becomes more severe, so does glucotoxicity [49]. The hyperglycemic state leads to overworking of the pancreatic beta cells and, in the long term, hyperglycemia induces glucotoxicity and worsening of the impaired insulin secretion. The glucotoxicity-mediated pancreatic beta cell dysfunction is reversible to some degree [50,51].

Autologous bone marrow-derived rat MSCs i) promote PDX-1 and insulin expression in the islets, ii) alter T cell cytokine patterns, iii) preserve regulatory T cells in the PB and iv) induce sustained normoglycemia [52]. Bone marrow MSCs are self-renewing cells with the ability to differentiate into osteoblasts, chondrocytes and adipocytes under appropriate cell culture conditions. They can also differentiate into endothelial cells, hepatocytes and insulin-positive cells [53–55]. However, it remains to be elucidated how donor-derived MSCs can protect beta cells or can differentiate into beta cells.

Leptin injection induces a loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice [56]. In the present study, we have shown that fewer adipocytes are found in the bone of the db/db mice treated with IBM–BMT + TT than non-treated db/db mice. Progressive diabetic nephropathy in db/db mice was associated with increased numbers of kidney macrophages. Macrophage accumulation and activation correlated with prolonged hyperglycemia, glomerular immune complex deposition, and increased kidney chemokine production [57,58]. We have also found that more glycogen deposits are observed in the glomeruli in non-treated db/db mice than in lean mice, and that the deposits are improved as a result of IBM–BMT + TT (manuscripts in preparation). We are in the process of elucidating the exact mechanisms underlying these phenomena.

In conclusion, this is the first report indicating that increased insulin sensitivity and decreased blood glucose levels result from the normalized balance of lymphocyte subsets after IBM–BMT + TT in db/db mice. The novel effects of IBM–BMT + TT are that the treatment induces adiponectin secretion, followed by enhanced pLKB1-AKT-AMPK crosstalk, signaling pathway, insulin phosphorylation, and also HO-1. IBM–BMT + TT is a potential therapeutic intervention for metabolic disorders such as T2 DM, insulin-resistant diabetes and metabolic syndrome.

Acknowledgments

We would like to thank Mr. Hilary Eastwick-Field and Ms. K. Ando for their help in the preparation of the manuscript. This study was mainly supported by the 21st Century Center of Excellence (COE) program of the Ministry of Education, Culture, Sports, Science and Technology. This study was also supported by grants from Haiteku Research Center of the Ministry of Education, Health and Labour Sciences Research Grants, the Science Frontier program of the Ministry of Education, Culture, Sports, Science and Technology, the Department of Transplantation for Regeneration Therapy (sponsored by Otsuka Pharmaceutical Company, Ltd.), Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd., Japan Immunoresearch Laboratories Co., Ltd. (JIMRO), NIH grants DK068134, HL55601 and HL34300 (NGA).

References

[1] Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. Nature 2006;444:881–7.
[2] Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med 2009;15:914–20.
[3] Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006;444:860–7.
[4] Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. Arterioscler Thromb Vasc Biol 2005;25:2062–8.
[5] Clift RA, Thomas ED. Follow-up 26 years after treatment for acute myelogenous leukemia. N Engl J Med 2004;351:2456–7.
[6] Ikehara S, Ohtsuki H, Good RA, Asamoto H, Nakamura T, Sekita K, et al. Prevention of type I diabetes in nonobese diabetic mice by allogenic bone marrow transplantation. Proc Natl Acad Sci U S A 1985;82:7743–7.
[7] Ikehara S, Good RA, Nakamura T, Sekita K, Inoue S, Oo MM, et al. Rationale for bone marrow transplantation in the treatment of autoimmune diseases. Proc Natl Acad Sci U S A 1985;82:2483–7.
[8] Than S, Ishida H, Inaba M, Fukuba Y, Seino Y, Adachi M, et al. Bone marrow transplantation as a strategy for treatment of non-insulin-dependent diabetes mellitus in KK-Ay mice. J Exp Med 1992;176:1233–8.
[9] Gershwin ME. Bone marrow transplantation, refractory autoimmunity and the contributions of Susumu Ikehara. J Autoimmun 2008;30:105–7.
[10] Ikehara S. A novel method of bone marrow transplantation (BMT) for intractable autoimmune diseases. J Autoimmun 2008;30:108–15.
[11] Burt RK, Testori A, Craig R, Cohen B, Suffit R, Barr W. Hematopoietic stem cell transplantation for autoimmune diseases: what have we learned? J Autoimmun 2008;30:116–20.
[12] Ozawa K, Sato K, Oh I, Ozaki K, Uchibori R, Obara Y, et al. Cell and gene therapy using mesenchymal stem cells (MSCs). J Autoimmun 2008;30:121–7.
[13] Abraham NG, Li M, Vanella L, Peterson SJ, Ikehara S, Asprinio D. Bone marrow stem cell transplant into intra-bone cavity prevents type 2 diabetes: role of heme oxygenase-adiponectin. J Autoimmun 2008;30:128–35.
[14] Sonoda Y. Immunophenotype and functional characteristics of human primitive CD34-negative hematopoietic stem cells: the significance of the intrabone marrow injection. J Autoimmun 2008;30:136–44.
[15] Marmont AM. Will hematopoietic stem cell transplantation cure human autoimmune diseases? J Autoimmun 2008;30:145–50.
[16] Ratajczak MZ, Zuba-Surma EK, Wysoczynski M, Wan W, Ratajczak J, Wojakowski W, et al. Hunt for pluripotent stem cell – regenerative medicine search for almighty cell. J Autoimmun 2008;30:151–62.
[17] Hara M, Murakami T, Kobayashi E. In vivo bioimaging using photogenic rats: fate of injected bone marrow-derived mesenchymal stromal cells. J Autoimmun 2008;30:163–71.
[18] Rezvani AR, Storb RF. Separation of graft-vs.-tumor effects from graft-vs.-host disease in allogeneic hematopoietic cell transplantation. J Autoimmun 2008;30:172–9.
[19] Deane S, Meyers FJ, Gershwin ME. On reversing the persistence of memory: hematopoietic stem cell transplant for autoimmune disease in the first ten years. J Autoimmun 2008;30:180–96.
[20] Hosaka N, Nose M, Kyogoku M, Nagata N, Miyashima S, Good RA, et al. Thymus transplantation, a critical factor for correction of autoimmune disease in aging MRL/+mice. Proc Natl Acad Sci U S A 1996;93:8558–62.
[21] Ryu T, Hosaka N, Miyake T, Cui W, Nishida T, Takaki T, et al. Transplantation of newborn thymus plus hematopoietic stem cells can rescue supralethally irradiated mice. Bone Marrow Transplant 2008;41:659–66.
[22] Hosaka N, Ryu T, Miyake T, Cui W, Nishida T, Takaki T, et al. Treatment of autoimmune diseases in MRL/lpr mice by allogenic bone marrow transplantation plus adult thymus transplantation. Clin Exp Immunol 2007;147:555–63.
[23] Kushida T, Inaba M, Hisha H, Ichioka N, Esumi T, Ogawa R, et al. Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 2001;97:3292–9.
[24] Peterson SJ, Kim DH, Li M, Positano V, Vanella L, Rodella LF, et al. The L-4F mimetic peptide prevents insulin resistance through increased levels of HO-1, pAMPK, and pAKT in obese mice. J Lipid Res 2009;50:1293–304.
[25] Li M, Kim DH, Tsenovoy PL, Peterson SJ, Rezzani R, Rodella LF, et al. Treatment of obese diabetic mice with a heme oxygenase inducer reduces visceral and subcutaneous adiposity, increases adiponectin levels, and improves insulin sensitivity and glucose tolerance. Diabetes 2008;57:1526–35.
[26] Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature 1998;394:897–901.
[27] Martin-Romero C, Santos-Alvarez J, Goberna R, Sanchez-Margalet V. Human leptin enhances activation and proliferation of human circulating T lymphocytes. Cell Immunol 2000;199:15–24.
[28] Lord GM, Matarese G, Howard JK, Bloom SR, Lechler RI. Leptin inhibits the anti-CD3-driven proliferation of peripheral blood T cells but enhances the production of proinflammatory cytokines. J Leukoc Biol 2002;72:330–8.
[29] Fujita Y, Murakami M, Ogawa Y, Masuzaki H, Tanaka M, Ozaki S, et al. Leptin inhibits stress-induced apoptosis of T lymphocytes. Clin Exp Immunol 2002;128:21–6.
[30] Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372:425–32.
[31] Chua Jr SC, Chung WK, Wu-Peng XS, Zhang Y, Liu SM, Tartaglia L, et al. Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. Science 1996;271:994–6.
[32] Fantuzzi G, Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. J Leukoc Biol 2000;68:437–46.
[33] Matarese G, Moschos S, Mantzoros CS. Leptin in immunology. J Immunol 2005;174:3137–42.
[34] Fernandes G, Handwerger BS, Yunis EJ, Brown DM. Immune response in the mutant diabetic C57BL/Ks-dt+ mouse. Discrepancies between in vitro and in vivo immunological assays. J Clin Invest 1978;61:243–50.
[35] Kimura M, Tanaka S, Isoda F, Sekigawa K, Yamakawa T, Sekihara H. T lymphopenia in obese diabetic (db/db) mice is non-selective and thymus independent. Life Sci 1998;62:1243–50.
[36] Gainsford T, Willson TA, Metcalf D, Handman E, McFarlane C, Ng A, et al. Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. Proc Natl Acad Sci U S A 1996;93:14564–8.
[37] Thomas T, Gori F, Khosla S, Jensen MD, Burguera B, Riggs BL. Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. Endocrinology 1999;140:1630–8.
[38] Hathout EH, Thomas W, El-Shahawy M, Nahab F, Mace JW. Diabetic autoimmune markers in children and adolescents with type 2 diabetes. Pediatrics 2001;107:E102.
[39] Umpaichitra V, Banerji MA, Castells S. Autoantibodies in children with type 2 diabetes mellitus. J Pediatr Endocrinol Metab 2002;15(Suppl. 1):525–30.
[40] Gilliam LK, Brooks-Worrell BM, Palmer JP, Greenbaum CJ, Pihoker C. Autoimmunity and clinical course in children with type 1, type 2, and type 1.5 diabetes. J Autoimmun 2005;25:244–50.
[41] Songer T, Glazner J, Coombs LP, Cuttler L, Daniel M, Estrada S, et al. Examining the economic costs related to lifestyle and pharmacological interventions in youth with type 2 diabetes. Expert Rev Pharmacoecon Outcomes Res 2006;6:315–24.
[42] Reinehr T, Schober E, Wiegand S, Thon A, Holl R. Beta-cell autoantibodies in children with type 2 diabetes mellitus: subgroup or misclassification? Arch Dis Child 2006;91:473–7.
[43] Klingensmith GJ, Pyle L, Arslanian S, Copeland KC, Cuttler L, Kaufman F, et al. The presence of GAD and IA-2 antibodies in youth with a type 2 diabetes phenotype: results from the TODAY study. Diabetes Care; 2010.
[44] Flaquer M, Franquesa M, Barquinero J, Lloberas N, Gutierrez C, Torras J, et al. Bone marrow transplantation induces normoglycemia in a type 2 diabetes mellitus murine model. Transplant Proc 2009;41:2282–5.
[45] Fantuzzi G. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol 2005;115:911–9. quiz 20.
[46] Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes 1991;40:405–12.
[47] Li M, Peterson S, Husney D, Inaba M, Guo K, Terada E, et al. Interdiction of the diabetic state in NOD mice by sustained induction of heme oxygenase: possible role of carbon monoxide and bilirubin. Antioxid Redox Signal 2007;9:855–63.
[48] Li M, Peterson S, Husney D, Inaba M, Guo K, Kappas A, et al. Long-lasting expression of HO-1 delays progression of type I diabetes in NOD mice. Cell Cycle 2007;6:567–71.
[49] Weir GC, Marselli L, Marchetti P, Katsuta H, Jung MH, Bonner-Weir S. Towards better understanding of the contributions of overwork and glucotoxicity to the beta-cell inadequacy of type 2 diabetes. Diabetes Obes Metab 2009;11 (Suppl. 4):82–90.
[50] Kosaka K, Kuzuya T, Akanuma Y, Hagura R. Increase in insulin response after treatment of overt maturity-onset diabetes is independent of the mode of treatment. Diabetologia 1980;18:23–8.
[51] MacDonald MJ, Efendic S, Ostenson CG. Normalization by insulin treatment of low mitochondrial glycerol phosphate dehydrogenase and pyruvate carboxylase in pancreatic islets of the GK rat. Diabetes 1996;45:886–90.

[52] Boumaza I, Srinivasan S, Witt WT, Feghali-Bostwick C, Dai Y, Garcia-Ocana A, et al. Autologous bone marrow-derived rat mesenchymal stem cells promote PDX-1 and insulin expression in the islets, alter T cell cytokine pattern and preserve regulatory T cells in the periphery and induce sustained normoglycemia. J Autoimmun 2009;32:33–42.
[53] Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, et al. Liver from bone marrow in humans. Hepatology 2000;32:11–6.
[54] Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehninger G, Bornhauser M, et al. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. Stem Cells 2004;22:377–84.
[55] Li M, Inaba M, Guo KQ, Hisha H, Abraham NG, Ikehara S. Treatment of streptozotocin-induced diabetes mellitus in mice by intra-bone marrow bone marrow transplantation plus portal vein injection of beta cells induced from bone marrow cells. Int J Hematol 2007;86:438–45.
[56] Hamrick MW, Della-Fera MA, Choi YH, Pennington C, Hartzell D, Baile CA. Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice. J Bone Miner Res 2005;20:994–1001.
[57] Chow FY, Nikolic-Paterson DJ, Atkins RC, Tesch GH. Macrophages in streptozotocin-induced diabetic nephropathy: potential role in renal fibrosis. Nephrol Dial Transplant 2004;19:2987–96.
[58] Chow F, Ozols E, Nikolic-Paterson DJ, Atkins RC, Tesch GH. Macrophages in mouse type 2 diabetic nephropathy: correlation with diabetic state and progressive renal injury. Kidney Int 2004;65:116–28.

Transplant Immunology 24 (2010) 33–39

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Transplant Immunology

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A successful haploidentical bone marrow transplantation method in rabbits: Perfusion method plus intra-bone marrow–bone marrow transplantation

Yunze Cui [a,b,1], Shuji Nakamura [a,b,1], Ming Shi [a], Wei Feng [a,c], Wenhao Cui [a], Kequan Guo [a], Yuming Zhang [a], Susumu Ikehara [a,*]

[a] Department of Stem Cell Disorders, Kansai Medical University, Moriguchi City, Osaka 570-8506, Japan
[b] JIMRO Co., Ltd., Takasaki, Gunma, Japan
[c] Shanghai University of Traditional Chinese Medicine, Pudong, Shanghai, China

ARTICLE INFO

Article history:
Received 14 November 2009
Received in revised form 25 June 2010
Accepted 28 June 2010

Keywords:
Haploidentical bone marrow transplantation
Intra-bone marrow–bone marrow transplantation
Perfusion method
Tolerance induction
Rabbit

ABSTRACT

Graft versus host disease (GVHD), rejection, delayed immune reconstitution and infections have been significant hurdles to haploidentical BMT. In order to improve the outcome of the current haploidentical-related BMT, we performed a novel BMT method consisting of the perfusion method (PM) plus intra-bone marrow–bone marrow transplantation (IBM-BMT) in a rabbit model. The percentages of T cells in BMCs harvested by the PM and the conventional aspiration method (AM) were 6% and 14%, respectively ($p<0.01$). Conversely, the CFU-C counts of BMCs in the PM group were significantly higher than those in the AM group. When the BMCs were transplanted into lethally irradiated offspring rabbits by IBM-BMT, hemopoietic recovery in the PM group was faster than in the AM group. The cumulative incidence of acute GVHD was 25% in the PM group versus 75% in the AM group ($p<0.05$). In addition, the survival rate was 75% in the PM group versus 33% in the AM group ($p<0.05$). Thus, the new method is able to provide rapid hemopoiesis, reduce the cumulative incidence of acute GVHD, and achieve a higher survival rate. This novel strategy paves the way for new dimensions in haploidentical BMT.

1. Introduction

Allogeneic hemopoietic stem cell transplantation (HSCT) is one of the definitive therapies for advanced hemopoietic malignancies. The success of allogeneic HSCT depends entirely on the presence of a suitable donor with fully matched or acceptably mismatched human leukocyte antigen (HLA). In the absence of an HLA-matched sibling, patients need to have an HLA-matched-unrelated donor's BMCs or cord blood cells. However, the timing of transplantation is often influenced by the time-consuming procedure of a marrow donor program. In the case of cord blood cells, graft failure is sometimes induced by the insufficient concentration of the hemopoietic stem cells (HSCs). In the case of BMCs, to prevent acute GVHD, T-cell-depleted or $CD34^+$-selected HSCT has been extensively attempted during the past decade. However, transplantation frequently failed due to infection and leukemia relapse [1–11]. Recently, we developed a novel BMT method, which combines an advanced BMC-harvesting method (PM) with IBM-BMT [12]. Compared with the conventional AM, the PM, which has been performed on the long bones (humerus, femur and tibia) and also the iliac bones of cynomolgus monkeys, allows BMCs to be collected with minimal contamination with peripheral T cells [13,14]. As a consequence, PM can significantly reduce the risk of GVHD. In the case of IBM-BMT, donor BMCs are directly injected into the recipient bone marrow cavity. Therefore, injected allogeneic donor cells are able to interact efficiently with donor-derived stromal cells, including mesenchymal stem cells (MSCs), which cause the proliferation, differentiation, and even maintenance of HSCs [15,16]. In the case of conventional intravenous BMT (IV-BMT), the majority of the BMCs (both HSCs and MSCs) are trapped in the lung [17–19] and liver [20], where they are killed by the radio-resistant host cells. Compared with IV-BMT, this novel method generates an earlier engraftment of hematolymphoid cells of donor-origin [21]. Thus, we succeeded in treating the intractable autoimmune diseases in chimeric-resistant *MRL/lpr* mice by IBM-BMT. Following these results, in order to diminish the hurdles to allogeneic BMT, PM + IBM-BMT was performed on haploidentical-related rabbits. Due to their higher radiosensitivity [22,23] and their resistance to inbreeding [24], rabbits are well-recognized as being difficult animals on which to perform allogeneic BMT. However, the genetic heterogeneity of a rabbit population makes the rabbit much more akin to human beings in terms of their biological individuality than the pure-bred homogeneous strains of mice usually used in these studies. Thus, we used the rabbit system to gauge the effectiveness of this novel BMT method. In this report, we demonstrate that PM + IBM-BMT is able to allow a wide spectrum of donor-matching, prevent the development of GVHD, improve the survival rate, and generate a persistent and stable chimera for at least 2 years.

* Corresponding author. Department of Stem Cell Disorders, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka 570-8506, Japan. Tel.: +81 6 6993 9625; fax: +81 6 6993 9627.
E-mail address: ikehara@takii.kmu.ac.jp (S. Ikehara).
[1] The two authors contributed equally to this work.

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doi:10.1016/j.trim.2010.06.014

Accordingly, this novel approach might deliver the expected innovative therapies for the various otherwise intractable diseases in humans, including autoimmune diseases, genetic disorders, and leukemia.

2. Materials and methods

2.1. Animals

Specific pathogen-free Japanese white (JW) rabbits (recipients: 16- to 20-week-old male offspring littermates, donors: 60- to 118-week-old maternal rabbits) were obtained from Oriental BioService, Inc. (Kyoto, Japan), and maintained under conventional conditions in our animal facility throughout the study. After receiving the rabbits, their health was examined at least one week before initiating the experiments. The experimental protocol was reviewed and approved by the Animal Experimentation Committee, Kansai Medical University.

2.2. Harvesting of BMCs by PM

Donor JW rabbits were intramuscularly anesthetized using Ketalar (13 mg/kg), and analgesic, Xylazine (9 mg/kg), before the operation. BMCs were harvested from the long bones such as humerus or femur, as described previously [13]. In summary, one bone marrow puncture needle (trans-needle; JIMRO Co., Ltd., Gunma, Japan) [12] was inserted into the proximal end of a long bone, and the other needle was inserted into the distal end. The needle at the proximal end was connected to a syringe (50 ml), containing 5 ml of heparin (10 U/ml in saline; Novo Nordisk, Copenhagen, Denmark), and the needle at the distal end was connected to a syringe containing 30 ml of phosphate-buffered saline (PBS). PBS was poured gently into the bone marrow cavity to harvest the BMCs. In some experiments, BMCs were harvested from the long bone or the iliac bone by the AM using the trans-needle.

2.3. Preparation of BMCs

BMCs harvested by the PM or AM were centrifuged, resuspended, and the volume was then minimized in order to inject them into the medullary cavity. The injection volume of BMCs harvested using the PM could be adjusted within 1 ml. However, the volume harvested using the AM was larger due to the large amount of contamination with red blood cells (RBCs).

2.4. Procedure for IBM-BMT

Gamma-irradiation was delivered by Gammacell 40 Exactor (MDS Nordion, Kanata, ON, Canada) with two ^{137}Cs sources at the dose rate of 1.05 Gy/min. Recipient rabbits were lethally irradiated with fractionated total-body irradiation (6 Gy twice with a 6 h interval) 1 day before BMT. After irradiation, rabbits were divided into a PM and an AM group.

After administering Ketalar and Xylazine, a trans-needle was inserted into the humerus. After inserting the needle, whole BMCs (1×10^8/kg) harvested using the PM were slowly injected into the bone marrow cavity. Whole BMCs (1×10^8/kg) harvested using the AM were, because of the larger volume, injected into the bilateral humeri.

A dose of 0.1 ml/kg orbifloxacin injection-5% (Dainippon Sumitomo Co., Ltd., Osaka, Japan) was subcutaneously administered the day before BMT and on each of the subsequent 30 days to prevent infection.

Recipient rabbits were also concomitantly administered FK506 (Astelas Co., Ltd., Tokyo, Japan) and predonine (Shionogi & Co., Ltd., Osaka, Japan) as a prophylaxis against GVHD. Intramuscular administration of FK506 was initiated the day before BMT (0.5 mg/kg/day). Administration of predonine was initiated on day 7 after BMT (0.5 mg/kg/day). A reduction in doses of both drugs was initiated on day 21 and both drugs were fully withdrawn by day 32.

2.5. T cell subsets of donor BMCs and recipient peripheral blood cells

Before grafting, white blood cells (WBCs) and RBCs in donor BMCs were counted using an SF-3000 auto-analyzer (Sysmex, Kobe, Japan). The cells were stained with fluorescein isothiocyanate-conjugated anti-rabbit CD4 and CD8 monoclonal antibodies (mAb; DS Pharma Biomedical, Oxford, UK). The numbers of $CD4^+$ and $CD8^+$ T cells were analyzed using FACScan (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The peripheral blood WBCs, platelets (Plts), $CD4^+$ and $CD8^+$ T cells of recipient rabbits were examined every 3 days for 3 months after BMT.

2.6. CFU-C assay

CFU-C of BMCs was assayed as described previously [13]. In summary, 10^4 BMCs were plated in 12-well plates (MP Biomedicals, Solon, OH, USA) in a volume of 1 ml of the optimal concentration of cytokine-containing MethoCult GF H4434 (Stem Cell Technologies, Vancouver, BC, Canada). CFU-C was measured on day 14 of culture.

2.7. Mitogen response and mixed lymphocyte reaction

In order to analyze lymphocyte function and tolerance, peripheral blood was obtained from the recipient rabbits 3 months after BMT, as well as donor and third party rabbits (JW and Dutch). Lymphocytes were then isolated by Lymphoprep density solution (1.077 g/ml; Nycomed, Oslo, Norway). Mixed lymphocyte reaction (MLR) and mitogen response were performed as described previously [25].

2.8. Transplantation of skin grafts

Skin grafts from donor and third party JW rabbits were transplanted to the recipient rabbits 3 months after BMT. 5×5 cm of the full-thickness skin grafts were taken from donor and third party rabbits and were kept in dishes with PBS at room temperature. Subsequently, recipient rabbits were anesthetized and sections of the dorsal skin were gently removed. Prepared skin grafts were then sutured using 3-0 nylon to the areas where skin had been removed. A tie-over dressing was used to prevent the detachment of the grafted skin by exercise. The initial examination was carried out on day 7, and daily examination was continued for 3 weeks. The skin graft was evaluated as rejected when the necrotic and escharotic lesion exceeded 50% of the skin graft.

2.9. Assessment of GVHD

Rabbits were weighed every 3 days and assessed daily for clinical signs of GVHD. Autopsies were carried out when the recipient rabbits had died. The histological characteristics of GVHD were evaluated in the skin, liver, intestine, and hematolymphoid organs (spleen, lymph nodes, thymus, and bone marrow). Clinical and histological criteria of GVHD were as described previously [26].

2.10. Chimerism using sex determining region of Y (SRY)

Genomic DNA was extracted from the peripheral blood samples taken from the male rabbits and donors as a reference as well as the recipients at 3 months after BMT using Wizard Genomic DNA Purification Kit (Promega KK, Tokyo, Japan). Subsequently, 2 μg genomic DNA was digested by EcoR I and Hinc II (TaKaRa, Otsu, Japan) in order to refine the original genomic DNA.

RT-PCR amplification was performed using a LightCycler Quick System 350S (Roche Diagnostics, Basel, Switzerland). Reactions were set

up in 50 μl volumes of FastStart DNA Master HybProbe, consisting of 30 ng of the refined genomic DNA, 4 mM of $MgCl_2$, 100 pM of each primer, and 200 pM of HybriProbe. The primer and fluorescently labeled probe were as follows: 5′-CGAAACTCAGACATCAGC-3′ (forward primer) 5′-CTTCATTCCCGTGCAAGTA-3′ (reverse primer), 5′-GAGAAATACCCG-GACTACAAGTGCAG-3′-FITC, and LC Red 640-5′-CTCGTCGGAAGGT-TAAAATCCTACAG-3′P (NIHON GENE RESEARCH LABORATORIES, Sendai, Japan).

Thermal cycle conditions consisted of 95 °C for 10 min, then 50 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 10 s, followed by a final cooling step of 30 s at 40 °C. The specific melting peak of SRY can be detected at 86.5 °C. Amplified genomic DNA was digested by EcoR I and Hinc II. Subsequently, 30 ng of the amplified genomic DNA was quantitated using a Fluorescent DNA Quantitation Kit (BIO-RAD, Hercules, CA, USA). The percentage of SRY in the genomic DNA was calculated.

2.11. Statistical analyses

Statistical differences of the survival rates and probability of GVHD were analyzed by a log-rank test, and the results of other experiments are represented as means ± SD. The Student's 2-tailed *t*-test was used to determine the statistical significance. A p value <0.05 was considered to represent a significant difference.

3. Results

In our preliminary experiments, we first carried out BMT using various methods among complete MHC-disparate combinations in rabbits. However, less than 20% of recipients only survived and were reconstituted with donor-derived hemopoietic cells, even when using 6 Gy×2 followed by PM + IBM-BMT (radiation dose of 6 Gy×2 is critical for rabbits due to their radiosensitivity and vulnerability to infection under this condition). In addition, all the rabbits that had undergone conventional AM + IV-BMT died of acute GVHD within 1 month. Taken together, we compared survival rates between the AM + IBM-BMT and the PM + IBM-BMT using the haploidentical BMT model.

3.1. Characterization of BMCs harvested by AM or PM

We first compared the degrees of contamination with peripheral T cells of the BMCs harvested by the AM and the PM. As shown in Table 1, more than 30% of the mononuclear cells in the peripheral blood were T cells ($CD4^+ + CD8^+$), and the BMCs harvested by the AM contained more than 14% T cells. In contrast, less than 6% of the BMCs harvested by the PM were T cells (AM versus PM: $p<0.01$). In addition, RBC/WBC ratios were significantly lower in the BMCs harvested by the PM than by the AM. These results show that the PM allows us to harvest BMCs with the lower contamination of the peripheral blood, including T cells and RBCs.

Subsequently, we performed the CFU-C assay to examine the concentration of progenitor cells in the BMCs. The BMCs harvested by the PM generated a significantly higher number of CFU-C than those harvested by the AM ($28.66 \pm 0.49/10^4$ cells versus $19.11 \pm 0.45/10^4$ cells, $p<0.01$) (Table 1). The results indicate that a higher concentration of progenitor cells can be obtained by the PM than by the AM.

Table 1
Characterization of BMCs harvested by PM or AM. The numbers of $CD4^+$ and $CD8^+$ T cells in BMCs harvested by the PM were significantly lower than in BMCs harvested by the AM. The CFU-C assays showed that the concentration of progenitor cells in BMCs (10^4 cells/well) harvested by the PM was significantly higher than that in BMCs (10^4 cells/well) harvested by the AM. The data are expressed as the mean ± SD. Statistical analyses were performed by *t*-test: *$p<0.01$ (PM versus AM).

	BMCs by PM	BMCs by AM	Peripheral blood
CD4 (%)	3.68 ± 0.32* (n=8)	8.74 ± 0.99* (n=8)	21.25 ± 3.69 (n=6)
CD8 (%)	1.73 ± 0.17* (n=8)	5.92 ± 1.18* (n=8)	9.71 ± 2.17 (11=6)
RBC/WBC	18.0 ± 1.20* (n=8)	425.5 ± 95.5* (n=8)	725.02 ± 0.03 (n=6)
CFU-C ($/10^4$ cells)	28.66 ± 0.49* (n=5)	19.11 ± 0.45* (n=5)	

3.2. Recovery of hemopoiesis

The numbers of WBCs, Plts, $CD4^+$ and $CD8^+$ T cells in the peripheral blood of each recipient were analyzed every 3 days for 3 months after BMT. Fig. 1 shows the engraftment of WBCs (A) and Plts (B) of the recipients. In the acute stage after BMT, a faster increase in WBC counts was observed in the PM group than in the AM group. On day 7, the number of WBCs in the PM group reached 10^3/μl, which was higher than that in the AM group, whereas the number of WBCs in the AM group reached 10^3/μl on day 21. The number of WBCs in the PM group was significantly higher than in the AM group within 45 days.

Similarly, a faster engraftment of Plts was seen in the PM group than in the AM group; the number of Plts in the PM group reached 5×10^4/μl on day 7 (Fig. 1 and Table 2).

There was no significant difference in the numbers of either $CD4^+$ or $CD8^+$ T cells in the peripheral blood between the two groups (Fig. 2). The numbers of $CD4^+$ and $CD8^+$ T cells in the peripheral blood reverted to the baseline level by 3 months after BMT (Fig. 2). These results show that the PM is able to restore hemopoiesis quickly in the recipients.

3.3. Immune reconstruction and tolerance induction

Immunological functions and tolerance state of the recipient rabbits were examined by mitogen response, MLR and skin grafting. As shown in Fig. 3A, in terms of mitogen response, the peripheral blood lymphocytes of recipients showed sufficient responses to Con A, PHA, and LPS, the levels being comparable to those of the untreated JW rabbits.

We further investigated the induction of tolerance in the recipients. As shown in Fig. 3B, the peripheral blood lymphocytes obtained from both the PM group and the AM group, which survived more than 3 months after IBM-BMT without showing any clinical signs of GVHD, exhibited tolerance to both host-type and donor-type MHC determinants, whereas they showed a significant response to the third party MHC determinants (both JW and Dutch rabbits) in MLR.

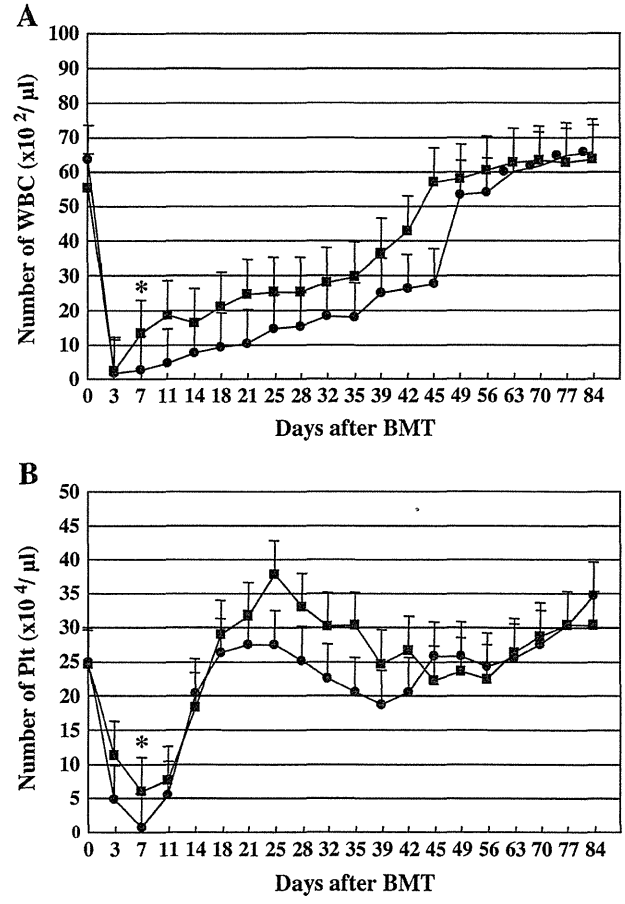

Fig. 1. Recovery of WBC and Plt counts after IBM-BMT. The numbers of peripheral blood WBCs (A) and Plts (B) were analyzed by an SF-3000 auto-analyzer every 3 days for 3 months after BMT. The recovery of WBC and Plt counts was accelerated in the PM group during the acute phase after BMT. Both WBC and Plt counts in the PM group (solid square) were significantly higher than those in the AM group (solid circle) on day 7 ($p<0.002$). The data are expressed as the mean ± SD.

Table 2
Outcomes of haploidentical BMT-treated rabbits. One of 12 rabbits that had shown grade II GvHD recovered and survived more than 2 years.

	GVHD	Engraftments (days)		Chimerism (>97% donor-derived)	Skin grafts		Survival rates (%)	Causes of death
	Grading	WBC ($>10\times10^2$/μl)	PLT ($>5\times10^4$/μl)		Donor	Third party		
AM	8/12 (≥III) 1/12 (II)*	21	11	4/4	Accepted 4/4	Rejected 4/4	4/12 (33.3%)	GVHD (8)
PM	2/12 (≥III) 1/12 (II)*	7	7	9/9	Accepted 9/9	Rejected 9/9	9/12 (75%)	GVHD (2) TAM (1)

Regarding skin grafting, the donor skins were fully accepted 5 months or later after transplantation, whereas the third party skins were rejected (mean survival time: 10.5 ± 1.6 days, $n=13$). No contradictory results regarding the immunological recognition and reactivity between MLR and skin grafting were observed. These findings indicate that co-operation was successfully achieved among newly-developed T cells, B cells and antigen-presenting cells in the recipient rabbits treated with IBM-BMT.

3.4. Reduction of acute GVHD by PM

Each rabbit was assessed daily for clinical signs of GVHD and weighed every 3 days. Based on the clinical signs and histological findings, the cases of death due to regimen-related toxicity within 1 week were excluded. As shown in Table 2 and Fig. 4, 3 of 12 recipient rabbits in the PM group developed acute GVHD (25%): one showed grade II GVHD and 2 developed grade III or higher GVHD. The AM group showed a higher incidence of acute GVHD: 9 of 12 recipients developed acute GVHD (75%); one showed grade II GVHD and 8 developed grade III or higher GVHD. GVHD was diagnosed on the basis of the clinical symptoms, such as weight loss, hair loss, anorexia, and diarrhea as well as histological examination after autopsy. The characteristics of the histological findings are shown in Fig. 5: moderate lymphocyte infiltration into the bile ducts and moderate damage of the bile ducts in the liver (C), and moderate apoptosis of the epithelial cells and inflammation in the lamina propria of the small intestine (D).

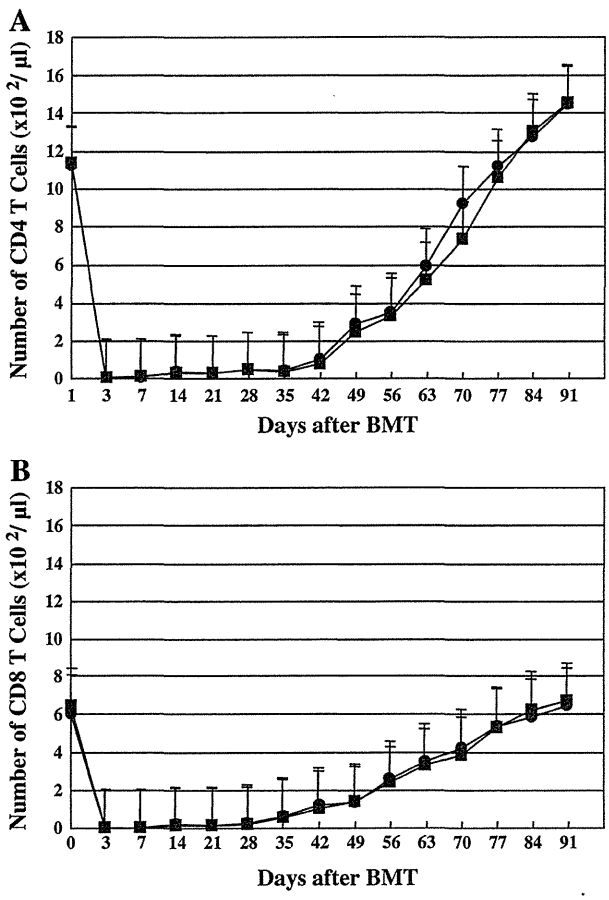

Fig. 2. Recovery of numbers of $CD4^+$ and $CD8^+$ T cells after IBM-BMT. The numbers of $CD4^+$ (A) and $CD8^+$ (B) T cells were analyzed using a FACScan every 3 days for 3 months after IBM-BMT. The numbers of $CD4^+$ and $CD8^+$ T cells were not significantly different between the PM group (solid square) and AM group (solid circle). The data are expressed as the mean ± SD.

3.5. Survival rate and chimerism

Follow-up investigation was continued for 5 or more months after BMT in all recipients. As shown in Fig. 6, a significantly-improved survival rate was observed in the PM group, compared with that in the AM group (75% versus 33%), in contrast to the incidence of GVHD.

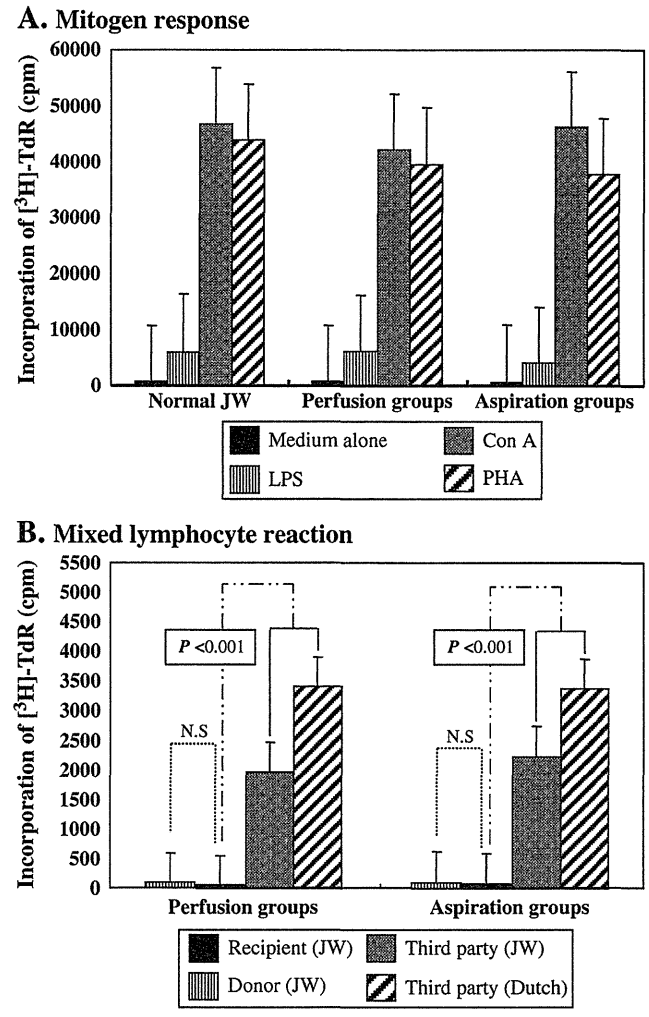

Fig. 3. Mitogen responses and MLR in experimental groups. Mitogen responses (A) and MLR (B) were performed 3 months after BMT. Responder cells (2×10^5 WBCs in the peripheral blood) were cultured with either mitogen (Con A, PHA or LPS) or 15 Gy-irradiated stimulator cells (2×10^5). A: there were no significant differences in mitogen response among the BMT groups and normal rabbits. B: the responder cells from both the PM group and the AM group showed tolerance to both donor (mother) and recipient (auto) MHC determinants. In contrast, the responder cells showed a significant response to the third party MHC determinants (JW and Dutch rabbits). The experiments were carried out twice. The data are expressed as the means ± SD. $p<0.001$, NS: no significant difference.

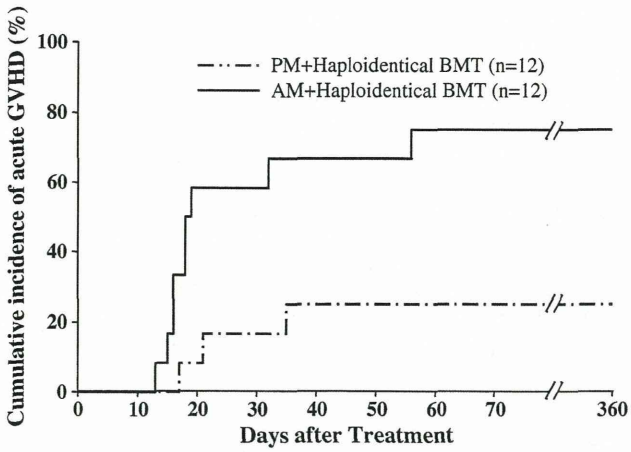

Fig. 4. Cumulative incidence of acute GVHD in PM group and AM group. All the rabbits were lethally irradiated with fractionated total-body irradiation (6 Gy twice with a 6 h interval) 1 day before BMT. Whole BMCs (1×10^8/kg) were transplanted by IBM-BMT. Cumulative incidence of acute GVHD in the PM group and the AM group was observed up to 360 days: 25% and 75%, respectively ($p<0.05$). Statistical analyses were carried out by a log-rank test.

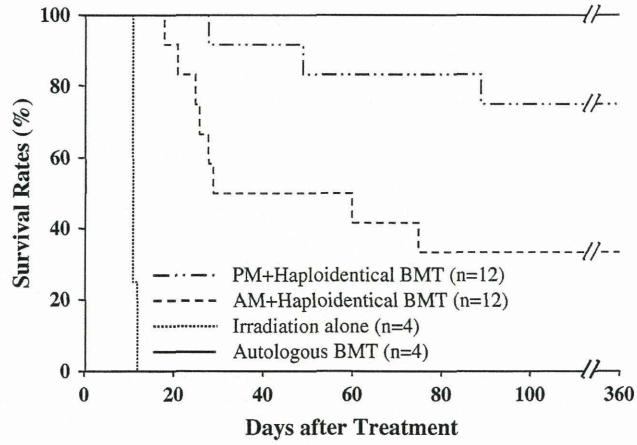

Fig. 6. Survival rates in PM group and AM group. After IBM-BMT, all the rabbits were observed up to 360 days. Survival rates of the PM group and the AM group were 75% and 33%, respectively ($p<0.05$). In the autologous IBM-BMT group, all the rabbits survived. In contrast, all the rabbits that had undergone irradiation alone died within 12 days. Statistical analyses were carried out by a log-rank test.

The chimerism status of the peripheral blood of the recipients was assessed at 3 months after BMT using a quantitative RT-PCR amplification of SRY. Less than 3% recipient-type SRY was observed in all the surviving recipients. The results demonstrate that the hemopoietic cells of the recipients were almost totally replaced with donor-derived cells (>97%).

4. Discussion

Haploidentical-related BMT must be able to overcome the various existing hurdles to HSCT, including donor issues and the time-consuming coordination process in the case of classical BMT or the higher rejection rate and protracted hemopoietic recovery in the case of cord blood transplantation. In haploidentical-related BMT, a family member is usually able to function as donor, thereby resolving the donor issue. However, severe acute and chronic GVHD still constitute barriers to its practical realization.

Although the pathogenesis of GVHD is complicated, it is widely accepted that donor T cells recognize and respond to the antigenic differences of HLA between donors and recipients. The donor T cells

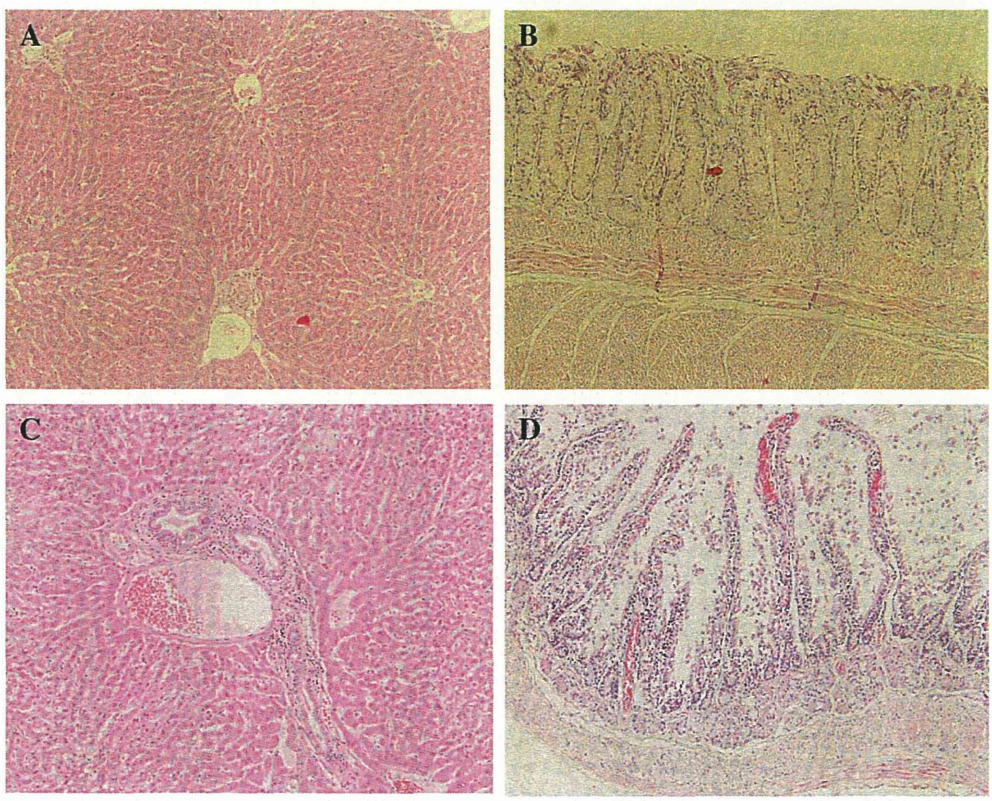

Fig. 5. Histological findings of acute GVHD in PM Group. Histological examination for acute GVHD was performed using the liver and small intestine obtained from autopsy rabbits. A: normal liver. B: normal small intestine. C: liver with GvHD; moderate lymphocyte infiltration into the bile ducts and moderate bile duct damage in the liver. D: small intestine with GvHD; moderate apoptosis in the epithelium and moderate inflammation in the lamina propria. Hematoxylin–eosin; original magnification ×100.

are activated and undergo clonal T cell expansion. As a consequence, the cytolytic mechanism with the activated T cells directly influences the functions of host tissues, while various cytokines produced by the activated T cells indirectly contribute to the clinical manifestations of GVHD [27–29].

Over the past decade, in order to prevent severe GVHD, BMT has been carried out using T-cell-depleted BMCs or $CD34^+$-selected HSCs from haploidentical donors. In 2008, a clinical report from the European Blood and Marrow Transplant Group showed that the survival rate and incidence of relapse were $48\% \pm 10\%$ and $21\% \pm 4\%$ in 173 acute myeloid leukemia patients transplanted with T-cell-depleted haploidentical BMCs [30]. Stern et al. also reported that the survival rate and relapse incidence were $50.6\% \pm 7.6\%$ and $22.7\% \pm 6\%$ in 47 acute leukemia patients transplanted with T-cell-depleted haploidentical BMCs donated by the mother [31]. Thus, these results were not sufficiently positive to allow clinical application of haploidentical BMT.

Our previous studies in mice demonstrated that the graft-facilitating cells in the donor bone marrow include $CD8^+$ T cells and stromal cells, which were required for the engraftment of HSCs in the recipients, especially when employing a nonmyeloablative conditioning regimen [32]. Martin et al. reported similar results: donor-derived $CD8^+$ T cells were essential for the engraftment of the donor hemopoietic cells in mice, and the addition of a small number of donor $CD8^+$ T cells to T-cell-depleted donor BMCs allowed the engraftment of donor hemopoietic cells in the recipients [33]. The graft-facilitating effect of $CD8^+$ T cells in the donor BMCs could be attributable to their cytotoxic or suppressive activity against the recipient's $CD8^+$ and/or $CD4^+$ T cells, which are responsible for the graft rejection [34,35]. The whole BMCs contain not only HSCs but also MSCs, the latter being essential for organizing the hemopoietic microenvironment in which HSCs grow and differentiate. From this point of view, T-cell-depletion and $CD34^+$ cell selection might eliminate the graft-facilitating cells, such as $CD8^+$ T cells and MSCs/stromal cells.

It is well known that immunosuppressants are essential in HLA-haploidentical BMT using unmanipulated grafts in order to prevent GVHD. Drobyski et al. reported that the overall survival rate and cumulative incidence of GVHD in haploidentical BMT were 21% and 46% even when the patients were given the combination of cytosine arabinoside, cyclophosphamide, methylprednisolone and antithymocyte globulin [36]. More intensified immunosuppressants were used by Ogawa et al. as the prophylaxis against GVHD in patients with haploidentical BMT, which resulted in improving the survival rate and cumulative incidence of GVHD (49.9% and 37.9%, respectively) [37]. However, the adverse effects of immunosuppressants were obvious: delayed immune reconstitution and incomplete T cell recovery, both of which increase the incidence of infection.

Using monkeys, we developed the novel PM for harvesting BMCs in order to reduce the degree of contamination with peripheral T cells [13]. In the present study, we have also proved in rabbits that the PM is able to reduce the amount of contamination with peripheral T cells, compared with the AM (Table 1). In addition, the PM allowed us to obtain a higher concentration of progenitor cells in comparison with the AM ($28.66 \pm 0.49/10^4$ cells versus $19.11 \pm 0.45/10^4$ cells, $p<0.01$).

In the present study, when BMCs harvested by the PM were transplanted using IBM-BMT, the cumulative incidence of acute GVHD was 25% and the survival rate was 75%. In contrast, the cumulative incidence of acute GVHD in the AM group was 75%, and the survival rate was 33%.

In the acute stage after BMT, we observed a more rapid increase in the number of WBCs in the PM group than in the AM group (Fig. 1). One week after BMT, the number of WBCs in the PM group reached $10^3/\mu l$. However, it took 21 days for the WBCs to reach the same level in the AM group. Similar results were observed with Plt reconstitution. The rapid reconstitution of WBCs and Plts using the PM is so meaningful that the amyelonic period could be shortened. As a consequence, the PM reduces the various complications of BMT, such as infection and lethal hemorrhage. In addition, successful BMT can be achieved by the short-term administration of low doses of conventional immunosuppressants, such as FK506 and predonine.

In summary, this novel BMT method enables the following remarkable characteristics: rapid reconstructing of donor hematopoietic cells [21,38], efficient grafting, use of mild conditioning regimens, avoidance of the long-term use of immunosuppressants [39,40], donor-specific tolerance [41–43], and persistent and stable chimerism. Furthermore, this method is flexible enough to allow the wide spectrum of donor-matching.

Taken together, haploidentical-related BMT was successfully conducted with PM + IBM-BMT in rabbits. This method will facilitate the practical application of haploidentical-related BMT and expand the indications for BMT.

Acknowledgements

We thank Y. Tokuyama, K. Hayashi, and A. Kitajima for their expert technical assistance, and we also thank Mr. Hilary Eastwick-Field and Ms. K. Ando for their help in the preparation of the manuscript.

These studies were mainly supported by the 21st Century Center of Excellence (COE) program of the Ministry of Education, Culture, Sports, Science and Technology. Supported also by a grant from Haiteku Research Center of the Ministry of Education, Health and Labor Sciences Research Grants, a grant from the Science Frontier program of the Ministry of Education, Culture, Sports, Science and Technology, and a grant from the Department of Transplantation for Regeneration Therapy (sponsored by Otsuka Pharmaceutical Co., Ltd.), a grant from Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd., and a grant from JIMRO Co., Ltd.

References

[1] Beatty PG, Clift RA, Mickelson EM, et al. Marrow transplantation from related donors other than HLA-identical siblings. N Engl J Med 1985;26(313):765–71.
[2] Szydlo R, Goldman JM, Klein JP, et al. Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. J Clin Oncol 1997;15:1767–77.
[3] Vallera DA, Soderling CC, Carlson GJ, et al. Bone marrow transplantation across major histocompatibility barriers in mice. Effect of elimination of T cells from donor grafts by treatment with monoclonal Thy-1.2 plus complement or antibody alone. Transplantation 1981;31:218–22.
[4] Kolb HJ, Rieder I, Rolt H, et al. Antilymphocytic antibodies and marrow transplantation. VI. Graft-vs-host tolerance in DLA-incompatible dogs after in vitro treatment of bone marrow with absorbed antithymocyte globulin. Transplantation 1979;27:242–5.
[5] Reisner Y, Kapoor N, Kirkpatrick D, et al. Transplantation for severe combined immunodeficiency with HLA-A, B, D, DR incompatible parental marrow cells fractionated by soybean agglutinin and sheep red blood cells. Blood 1983;61: 341–8.
[6] O'Reilly RJ, Collins NH, Kernan N, et al. Transplantation of marrow-depleted T cells by soybean lectin agglutination and E-rosette depletion major histocompatibility complex-related graft resistance in leukemic transplant recipients. Transplant Proc 1985;17:455–9.
[7] Cottler-Fox M, Cipolone K, Yu M, et al. Positive selection of $CD34^+$ hematopoietic cells using an immunoaffinity column results in T cell depletion equivalent to elutriation. Exp Hematol 1995;23:320–2.
[8] Urbano-Ispizua A, Rozman C, Martinez C, et al. Rapid engraftment without significant graft-versus-host disease after allogeneic transplantation of $CD34^+$ selected cells from peripheral blood. Blood 1997;89:3967–73.
[9] Shpall EJ, Jones RB, Bearman SI, et al. Transplantation of $CD34^+$ hematopoietic progenitor cells. J Hematother 1994;3:145–7.
[10] Peters C, Matthes-Martin S, Fritsch G, et al. Transplantation of highly purified peripheral blood $CD34^+$ cells from HLA-mismatched parental donors in 14 children: evaluation of early monitoring of engraftment. Leukemia 1999;13: 2070–8.
[11] Handgretinger R, Schumm M, Lang P, et al. Transplantation of megadoses of purified haploidentical stem cells. Ann NY Acad Sci 1999;872:351–62.
[12] Inaba M, Adachi Y, Hisha H, et al. Extensive studies on perfusion method plus intra-bone marrow–bone marrow transplantation using cynomolgus monkeys. STEM CELL 2007;25:2098-103.
[13] Kushida T, Inaba M, Ikebukuro K, et al. A new method for bone marrow cell harvesting. Stem Cells 2000;18:453–6.
[14] Kushida T, Inaba M, Ikebukuro K, et al. Comparison of bone marrow cells harvested from various bones of cynomolgus monkeys various ages by perfusion or

aspiration methods: a preclinical study for human BMT. Stem Cells 2002;20: 155–62.
[15] Hashimoto F, Sugiura K, Inoue K, et al. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vitro. Blood 1997;89:49–54.
[16] Sugiura K, Hisha H, Ishikawa J, et al. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vitro. Stem Cells 2001;19: 46–58.
[17] van Hennik PB, de Koning AE, Ploemacher RE. Seeding efficiency of primitive human hematopoietic cells in nonobese diabetic/severe combined immune deficiency mice: implications for stem cell frequency assessment. Blood 1999;94:3055–306.
[18] Cashman JD, Eaves CJ. High marrow seeding efficiency of human lymphomyeloid repopulating cells in irradiated NOD/SCID mice. Blood 2000;96:3979–81.
[19] Panoskaltsis-Mortari A, Price A, Hermanson JR, et al. In vivo imaging of graft-versus-host-disease in mice. Blood 2004;103:3590–8.
[20] Zhang Y, Yasumizu R, Sugiura K, et al. Fate of allogeneic or syngeneic cells in intravenous or portal vein injection: possible explanation for the mechanism of tolerance induction by portal vein injection. Eur J Immunol 1994;24:1558–65.
[21] Kushida T, Inaba M, Hisha H, et al. Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 2001;97:3292–9.
[22] Porter KA. Effect of homologous bone marrow injections in X-irradiated rabbits. Br J Exp Pathol March 1957;30:401–12.
[23] Porter KA, Murray JE. Long-term study of X-irradiated rabbits with bone-marrow homotransplants. J Natl Cancer Inst 1958;20:189–205.
[24] Chai CK. Effect of inbreeding in rabbits: skeletal variation and malformations. J Hered 1970;61:3–8.
[25] Cui WH, Hosaka N, Miyake T, et al. Analysis of tolerance induction using triple chimeric mice: major histocompatibility complex-disparate thymus, hemopoietic cells, and microenvironment. Transplantation 2008;85:1151–8.
[26] Markus PM, Cai X, Ming W, et al. Prevention of graft-versus-host disease following allogeneic bone marrow transplantation in rats. Transplantation 1991;52:590–4.
[27] Antin JH, Ferrara JL. Cytokine dysregulation and acute graft-vs-host disease. Blood 1992;80:2964–8.
[28] Baker KS, Allen RD, Roths JB, et al. Kinetic and organ-specific patterns of cytokine expression in acute graft-vs-host disease. Bone Marrow Transplant 1995;15: 595–603.
[29] Roy J, Blazar BR, Ochs L, et al. The tissue expression of cytokines in human acute cutaneous graft-vs-host disease. Transplantation 1995;60:343–8.
[30] Ciceri F, Labopin M, Aversa F, et al. A survey of fully-haploidentical hematopoietic stem cells transplantation in adults with high-risk acute leukemia: a risk factor analysis of outcomes for patients transplanted in remission. Blood 2008;112: 3574–81.
[31] Stern M, Ruggeri L, Mancusi A, et al. Survival after T cell-depleted haploidentical stem cell transplantation is improved using the mother as donor. Blood 2008;112: 2990–5.
[32] Takeuchi K, Inaba M, Miyashima S, et al. A new strategy for treatment of autoimmune disease in chimeric resistant MRL/lpr mice. Blood 1998;91:4616–23.
[33] Martin PJ. Donor CD8 cells prevent allogeneic marrow graft rejection in mice: potential implications for marrow transplantation in humans. J Exp Med 1993;178:703–12.
[34] Tomita Y, Mayumi H, Eto M, et al. Importance of suppressor T cells in cyclophosphamide-induced tolerance to the non-H-2-encoded alloantigens. J Immunol 1990;144:463–73.
[35] Asiedu C, Meng Y, Wang W, et al. Immunoregulatory rule of CD8α in the effect. Transplantation 1999;67:372–80.
[36] Drobyski WR, Klein J, Flomenberg N, et al. Superior survival associated with transplantation of matched unrelated versus one-antigen-mismatched unrelated or highly human leukocyte antigen-disparate haploidentical family donor marrow grafts for the treatment of recipients of alternative donor grafts. Blood 2002;99: 806–14.
[37] Ogawa H, Ikegame K, Kaida K, et al. Unmanipulated HLA 2–3 antigen-mismatched (haploidentical) bone marrow transplantation using only pharmacological GVHD prophylaxis. Exp Hematol 2008;36:1–8.
[38] Li Q, Hisha H, Yasumizu R, et al. Analysis of very early hemopoietic regeneration after bone marrow transplantation: comparison of intravenous and intrabone marrow routes. Stem Cells 2007;25:1186–94.
[39] Wang JF, Kimura T, Asada R, et al. SCID-repopulating cell activity of human cord blood-derived $CD34^-$ cells assured by intra-bone marrow injection. Blood 2003;101:2924–31.
[40] Guo KQ, Inaba M, Li M, et al. Long-term donor-specific tolerance in rat cardiac allografts by intra-bone marrow injection of donor bone marrow cells. Transplantation 2008;85:93–101.
[41] Esumi T, Inaba M, Ichioka N, et al. Successful allogeneic leg transplantation in rats in conjunction with intra-bone marrow injection of donor bone marrow cells. Transplantation 2003;76:1543–8.
[42] Ikehara S. New strategies for BMT, organ transplantation, and regeneration therapy. Hematology 2003;8:77–81.
[43] Taira M, Inaba M, Baba S, et al. Treatment of streptozotocin-induced diabetes mellitus in rats by transplantation of islet cells from two MHC-disparate rats in combination with intra-bone marrow injection of allogeneic bone marrow cells. Transplantation 2005;79:680–7.

STEM CELLS AND DEVELOPMENT
Volume 19, Number 11, 2010

DOI: 10.1089/scd.2009.0370

Signaling from Fibroblast Growth Factor Receptor 2 in Immature Hematopoietic Cells Facilitates Donor Hematopoiesis After Intra-Bone Marrow–Bone Marrow Transplantation

Akio Shigematsu,[1,2,*] Ming Shi,[1,3,*] Mitsuhiko Okigaki,[4,*] Yasushi Adachi,[1,*] Naoko Koike,[1] Jishan Che,[4] Masayoshi Iwasaki,[1] Hiroaki Matsubara,[4] Masahiro Imamura,[2] and Susumu Ikehara[1,3]

Fibroblast growth factor (FGF) and FGF receptor (FGFR) are expressed in various cells including endothelial progenitor cells and hematopoietic cells. The interaction between FGF and FGFR is associated with the proliferation, migration, and survival of these cells. In this report, we examined the effects of FGFR2 signaling on hematopoiesis in immature hematopoietic cells, using mutant mice in which a constitutively active form of FGFR2 mutant was caused to be overexpressed by the Tie2 promoter (FGFR2 Tg mice). Under normal conditions, hematopoiesis of FGFR2 Tg mice and wild type (Wt) mice do not differ significantly, except for the weight and cell numbers of the thymus. However, the c-kit$^+$Sca-1$^+$lineage$^-$ bone marrow cells (BMCs) of FGFR2 Tg mice facilitate the formation of colony-forming units of culture. When these BMCs were transplanted into the recipient bone marrow (intra-bone marrow–bone marrow transplantation), there was better reconstitution of donor hematopoietic cells. In the in vitro experiment, the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs from FGFR2 Tg mice showed fewer apoptotic cells than those from Wt mice. These results suggest that the antiapoptotic effect of FGFR2 signaling facilitates the hematopoiesis of FGFR2 Tg mice.

Introduction

Fibroblast growth factor (FGF) belongs to a family of heparin-binding polypeptides and shows multiple functions, including effects on cell proliferation, differentiation, survival, and motility [1,2]. Twenty-four members of the FGF family have been identified, ranging in molecular mass from 17 to 34 kDa and share 13%–71% amino acid identity [3,4]. To date, 4 kinds of FGF receptors (FGFR) have been reported [5]. Since the expression of FGFR is widely distributed on various cells, FGF signaling plays an important role in development and morphogenesis as well as in physiological and pathological situations such as wound healing, neovascularization, tumor growth, and tumor progression [6–8]. FGF signaling also facilitates hematopoiesis. FGF acts not only directly on the hematopoietic stem cells (HSCs) and immature hematopoietic progenitor cells (IHPCs) but also indirectly on them through bone marrow stromal cells [9–11].

Tie2 is a receptor tyrosine kinase expressed in both HSCs/IHPCs and endothelial cells. The interaction of Tie2 with its ligand, angiopoietin-1 (Ang-1), induces the cobblestone formation of HSCs in vitro and maintains the long-term repopulating activity of HSCs in vivo [12–14]. Arai et al. suggested that the Tie2/Ang-1 signaling pathway plays a critical role in maintaining HSCs in a quiescent state in the bone-marrow niche [14,15].

Recently, we established mutant mice in which a constitutively active form of FGFR2 mutant was caused to be overexpressed using the Tie2 promoter (FGFR2 Tg mice) [16]. The mice showed decreased infarct size and improved cardiac performance compared with wild type (Wt) mice when acute cardiac ischemia was induced [16].

In the present study, we show that the c-kit$^+$Sca-1$^+$lineage$^-$ bone marrow cells (BMCs) from FGFR2 Tg mice facilitate the formation of day-14 colony-forming units of culture (CFU-C), and that these BMCs facilitate better engraftment of donor

[1]First Department of Pathology, [3]Department of Stem Cell Disorders, Kansai Medical University, Moriguchi City, Osaka, Japan.
[2]Department of Hematology and Oncology, Hokkaido University Graduate School of Medicine, Sapporo City, Japan.
[4]Department of Cardiovascular Medicine, Kyoto Prefectural University School of Medicine, Kyoto, Japan.
*These authors contributed equally to this study.

hematopoietic cells than those from Wt mice by intra-bone marrow–bone marrow transplantation (IBM-BMT).

Materials and Methods

Mice

FGFR2 Tg mice were prepared as described previously [16]. C57BL/6 mice congenic for the ly5 locus (B6-ly5.1 mice) were bred and maintained at the animal center of Kansai Medical University (Moriguchi City, Osaka, Japan). The background mice of the FGFR2 Tg mice are C57BL/6 mice (B6 mice) (ly5.2). Therefore, B6 mice were purchased from Japan SLC Inc. and used as Wt mice.

Reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-CD45.1 (ly5.1) antibody (Ab), FITC-conjugated anti-c-kit Ab, FITC-conjugated anti-Mac-1 Ab, phycoerythrin (PE)-conjugated anti-CD3 Ab, PE-conjugated anti-Gr-1 Ab, PE-conjugated anti-B220 Ab, PE-conjugated anti-Sca-1 Ab, PE-conjugated anti-CD45 Ab, biotin-conjugated anti-CD45.2 (ly5.2) Ab, biotin-conjugated anti-B220 Ab, biotin-conjugated anti-Mac-1 Ab, biotin-conjugated anti-Ter119 Ab, biotin-conjugated anti-Gr-1 Ab, biotin-conjugated anti-CD3 Ab, biotin-conjugated anti-NK1.1 Ab, biotin-conjugated anti-CD11c Ab, Per-CP Cy5.5-conjugated anti-CD3 Ab, antiproliferating cell nuclear antigen (PCNA) Ab, and Per-CP Cy5.5-conjugated avidin were purchased from BD Biosciences. Ab and allophycocyanin (APC)-conjugated anti-B220 Ab was obtained from Caltag, and PE-conjugated anti-Tie2 Ab was obtained from eBioscience. Annexin V-FITC apoptosis detection kit, containing FITC-conjugated annexin V, was obtained from BioVision. Tetracolor-one was obtained from Nacalai tesque. A permeabilization reagent, IntraPrep™, was obtained from Immunotech phosphatidyl inositol (PI)-3-kinase inhibitor (LY294002) was obtained from Sigma Chemical Co.

Detection of mRNA of FGFR2 by reverse transcription (RT)-polymerase chain reaction and real-time RT-polymerase chain reaction

RNA preparation from the BMCs of Wt mice or FGFR2 Tg mice, cDNA synthesis, and polymerase chain reaction (PCR) were carried out as described previously [17]. Primers for the detection of mRNAs in this experiment were glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Toyobo) and FGFR2 [16]. PCR products were separated on a 1.2% agarose gel (Gibco BRL) and viewed by ethidium bromide (Nakarai) staining.

We also performed real-time PCR using cDNA with OPTICON2 (MJ Research), QuantiTect SYBR Green PCR kit (Qiagen), GAPDH-specific primers (Qiagen), and the primers for FGFR2 [16] to estimate the expression of FGFR2 mRNA accurately, as described previously [18].

Analyses of peripheral blood and organs

Blood samples obtained from 8- to 13-week-old FGFR2 Tg mice and Wt mice were analyzed using an automated hematology analyzer (SE 9000; Sysmex). The individual mice organs were weighed and fixed with 15% buffered formalin (Wako) for microscopical examination. A single-cell suspension was prepared from the spleen, bone marrow, and thymus of the mice. The cells were then stained with FITC-conjugated anti-Mac-1 Ab, PE-conjugated anti-Gr-1 Ab, Per-CP Cy5.5-conjugated anti-CD3 Ab, and APC-conjugated anti-B220 Ab; FITC-conjugated anti-CD8 Ab, PE-conjugated anti-CD4 Ab, and PerCP Cy5.5-conjugated anti-CD3 Ab; or FITC-conjugated anti-NK1.1 Ab. The stained cells were analyzed using the Becton Dickinson LSR flow cytometer (Becton Dickinson).

Preparation of BMCs

BMCs were harvested from the femoral and tibial bones of 8- to 12-week-old FGFR2 Tg mice (ly5.2) or Wt mice (ly5.2) and suspended in phosphate-buffered saline (PBS). The BMCs were then filtered through a 70-μm nylon wool mesh (Becton Dickinson Labware) and centrifuged at 1,500 rpm for 7 min at 4°C. After centrifugation, the BMCs were resuspended in PBS.

Preparation of lineage$^+$ and c-kit$^+$Sca-1$^+$lineage$^-$ BMCs

BMCs from Wt mice or FGFR2 Tg mice were incubated with a biotin-conjugated cocktail of lineage antibodies including anti-Mac-1, anti-Gr-1, anti-NK1.1, anti-B220, anti-Ter119, anti-CD3, and anti-CD11c. The cells were then treated with magnetic beads conjugated with streptavidin (Dynabeads M-280; Dynal AS). The positively selected cells were used as lineage$^+$ BMCs, and the negatively selected cells were incubated with FITC-conjugated c-kit Ab, PE-conjugated Sca-1 Ab, and Red PE-Cy5-conjugated streptavidin. The c-kit$^+$Sca-1$^+$lineage$^-$ BMCs were isolated using a fluorescence-activated cell sorter (FACS) (EPICS ALTRA; Beckman Coulter).

CFU-C assays

The colony-forming ability of the BMCs (CFU-C) was assayed as described previously [19]. Briefly, whole BMCs (10^4 cells/mL/well) or c-kit$^+$Sca-1$^+$lineage$^-$ BMCs (50 cells/mL/well) were plated in Petri dishes (Becton Dickinson) in 10 mL of Methocult GF H3434 (StemCell Technologies, Inc.). The colonies were counted 7 and 14 days later.

Examination of long-term facilitation after IBM-BMT

Recipient mice (ly5.1-B6) were exposed to ^{137}Cs gamma irradiation at 0.96 Gy/min. The exposure dose was 8.0 Gy. The c-kit$^+$Sca-1$^+$lineage$^-$ BMCs were injected directly into the bone marrow cavity of the tibial bones of the recipient mice (IBM-BMT), as previously described [20]. Briefly, the region from the groin to the knee joint was shaved, and an ~5-mm incision was made on the thigh. The knee was flexed to 90°, the proximal side of the tibia was drawn to the anterior, and a 26-gauge needle was inserted into the bone marrow cavity. Using a microsyringe (50 μL; Hamilton Company), the donor c-kit$^+$Sca-1$^+$lineage$^-$ BMCs (3×10^4 cells/10 μL) were injected via the needle into the bone marrow cavity.

Blood was collected from recipient mice at 4 weeks and 6 months after IBM-BMT, and the ly5 (CD45) haplotypes of the leukocytes were then analyzed by flow cytometry. In this

analysis, blood was suspended in PBS supplemented with 2% fetal bovine serum and incubated with FITC-conjugated anti-CD45.1 (ly5.1) Ab; PE-conjugated anti-Gr-1, anti-CD3, anti-B220, or anti-CD45 Abs; and biotin-conjugated anti-CD45.2 (ly5.2) Ab, followed by staining with Red PE-Cy5-avidin. The stained cells were analyzed by FACScan (Becton Dickinson).

Assay for live cells, proliferating cells, and apoptotic cells

BMCs (2×10^6/mL) from Wt mice and FGFR2 Tg mice were stained with FITC-conjugated anti-c-kit Ab; PE-conjugated anti-Sca-1 Ab; and biotin-conjugated lineage antibodies, including anti-CD3, anti-NK1.1, anti-Gr-1, anti-Mac-1, anti-Ter119, anti-CD11c, and biotin-labeled anti-B220, followed by staining with PE-Cy5.5-coupled avidin. The c-kit$^+$Sca-1$^+$lineage$^-$ BMCs were collected by a cell sorter. Sorted cells were cultured in a CO_2 incubator for 1 day, and the numbers of live cells, proliferating cells, and apoptotic cells were estimated. Tetracolor-one was used to determine the number of live cells.

The percentage of proliferating cells in c-kit$^+$Sca-1$^+$lineage$^-$ BMCs was determined by detecting the cells expressing PCNA. The cultured cells were stained with PE-labeled anti-c-kit Ab; APC-labeled anti-Sca-1 Ab; biotin-conjugated lineage antibodies, including anti-Gr-1, anti-Mac-1, anti-CD3, anti-B220, anti-NK1.1, and anti-CD11c, followed by PerCP Cy5.5-avidin. Next, the cells were fixed with reagent 1 of IntraPrep for 15 min at room temperature. After washing the cells, they were incubated in reagent 2 of IntraPrep and permeabilized for 5 min at room temperature. FITC-conjugated anti-PCNA Ab or isotype-matched control was then added to the cells with reagent 2. After incubation for 20 min, the cells were washed, resuspended in PBS, and analyzed by FACSCaliber (BD Biosciences).

Apoptotic cells were detected using the annexin V-FITC apoptosis detection kit. The cultured cells were stained with FITC-conjugated annexin V; PE-conjugated anti-c-kit Ab; APC-conjugated anti-Sca-1 Ab; biotin-conjugated lineage antibodies, such as anti-Gr-1, anti-Mac-1, anti-CD3, anti-B220, anti-NK1.1, and anti-CD11c in the annexin buffer; followed by PerCP Cy5.5-avidin. The stained cells were analyzed by FACSCaliber. In the culture using PI-3-kinase inhibitor, BMCs (2×10^6/mL) were cultured with or without the inhibitor (LY294002) for 12 h and the percentage of apoptotic cells in the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs calculated.

Statistical analyses

Differences between groups were evaluated by the Student's *t*-test. *P* values <0.05 were considered to be statistically significant.

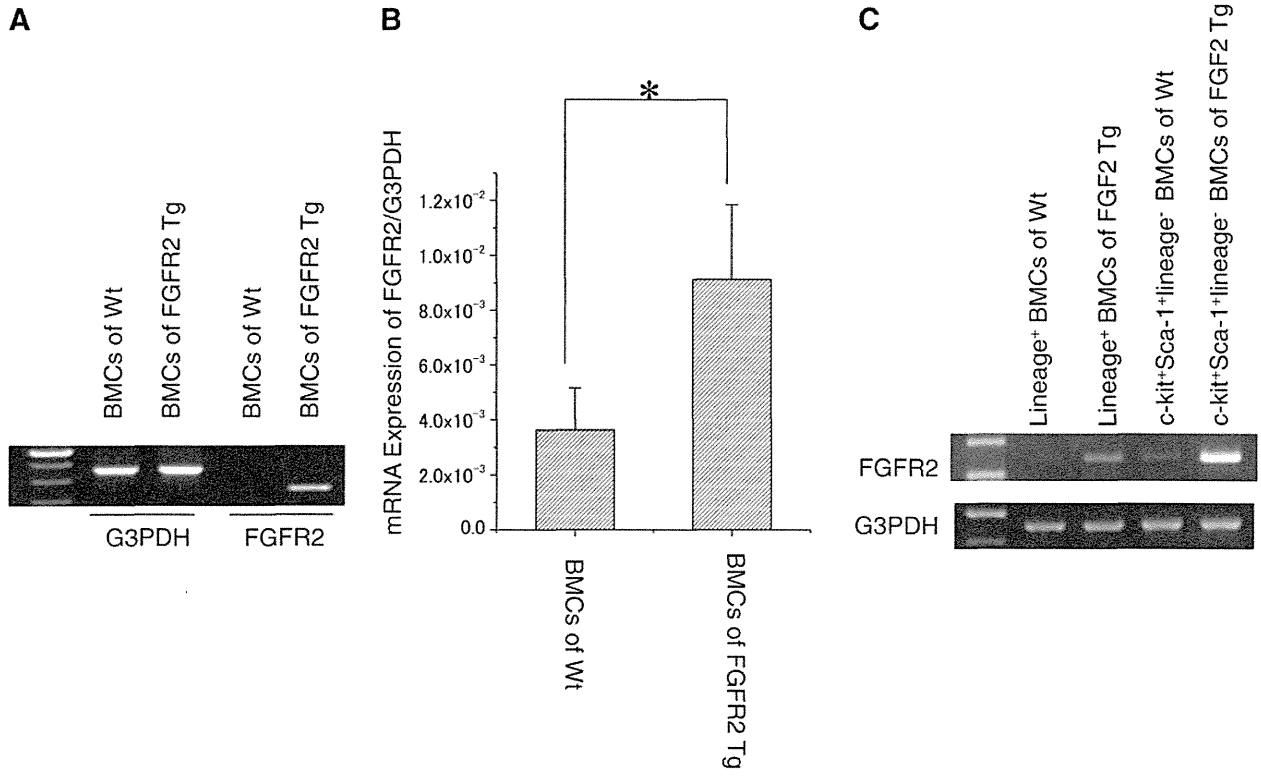

FIG. 1. mRNA expression of FGFR2 in BMCs of FGFR Tg mice and Wt mice. RNA was extracted from the BMCs of mice, followed by preparation of cDNA. RT-PCR **(A)** and real-time RT-PCR **(B)** were performed using the cDNA, as described in the Materials and Methods section. We obtained lineage$^+$ cells and purified the c-kit$^+$Sca-1$^+$lineage$^-$ cells from the BMCs of Wt mice and FGFR2 Tg mice using magnetic beads and a cell sorter, as described in the Materials and Methods section. RT-PCR was performed to detect the mRNA expression of FGFR2 in lineage$^+$ BMCs and c-kit$^+$Sca-1$^+$lineage$^-$ BMCs (**C**). *, $p<0.05$. BMCs, bone marrow cells; FGFR, fibroblast growth factor receptor; PCR, polymerase chain reaction; Wt, wild type.

Results

Expression of FGFR2 mRNA

FGFR2 expression in the heart, aorta, lungs, and endothelial cells of FGFR2 Tg mice was higher than in Wt mice, as previously described [16]. Since this study aimed to examine the effects of overexpression of FGFR2 on the immature hematopoietic cells expressing Tie2, we first examined the mRNA expression of BMCs in both types of mice. As shown in Fig. 1A and B, mRNA expression of FGFR2 in the BMCs of FGFR2 Tg mice was higher than in Wt mice. Next, we studied mRNA expression of FGFR2 in the lineage$^+$ cells and c-kit$^+$Sca-1$^+$lineage$^-$ cells obtained from the bone marrow of Wt mice and FGFR2 Tg mice. As shown in Fig. 1C, mRNA of FGFR2 was more highly expressed in the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs than in the lineage$^+$ BMCs in FGFR Tg mice. In Wt mice, mRNA of FGFR2 was expressed in c-kit$^+$Sca-1$^+$lineage$^-$ BMCs but not in lineage$^+$ BMCs. These results suggest that FGFR2 is highly expressed not only in the heart, aorta, lungs, and endothelial cells but also in the BMCs of FGFR2 Tg mice, and that FGFR2 is more highly expressed in immature hematopoietic cells than in lineage$^+$ cells of both Wt and FGFR2 Tg mice.

Hematopoiesis in FGFR2 Tg mice under normal conditions

To compare the hematopoietic function of FGFR2 Tg mice and normal mice (Wt mice), we first analyzed complete blood counts (CBCs), including white blood cell (WBC) numbers, populations of WBCs, red blood cell numbers, and platelet numbers using SE 9000. There were no significant differences in the CBCs of FGFR2 Tg mice and Wt mice (data not shown). We also examined the weights of the spleen, liver, thymus, kidneys, and heart, and the percentages of CD4$^+$ T cells, CD8$^+$ T cells, B cells, NK cells, Mac-1$^+$ cells, and Gr-1$^+$ cells in the spleen and the thymus. There were no significant differences in the cell populations or weights of these organs, except for the thymus. The thymus in FGFR2 Tg mice was significantly heavier than in Wt mice (88.3 ± 8.1 mg vs. 56.3 ± 14.8 mg). However, there were no significant differences in the populations of CD4$^-$CD8$^-$, CD4$^+$CD8$^-$, CD4$^-$CD8$^+$, or CD4$^+$CD8$^+$ cells between Wt and FGFR2 Tg mice. These results suggest that normal hematopoiesis occurs in FGFR2 Tg mice under normal conditions.

CFU-C assays

The hematopoietic function of FGFR2 Tg mice in comparison with Wt mice was examined using the day-7 CFU-C assays, because this assay shows the hematopoietic activity of hematopoietic progenitors [21]. The day-7 CFU-C assays using whole BMCs showed greater number of colonies in FGFR2 Tg mice than in Wt mice (Fig. 2A). However, there was statistically no significant difference in CFU-C counts between Wt and FGFR2 Tg mice. Therefore, the assays were performed with the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs from Wt mice or FGFR2 Tg mice purified using magnetic beads and a cell sorter, because the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs are very immature HPCs and HSCs [22].As shown in Fig. 2B and C, the numbers of day-7 and day-14 CFU-Cs in FGFR2 Tg mice were significantly higher than in Wt mice. These results suggest that hematopoiesis in FGFR2 Tg mice is regulated

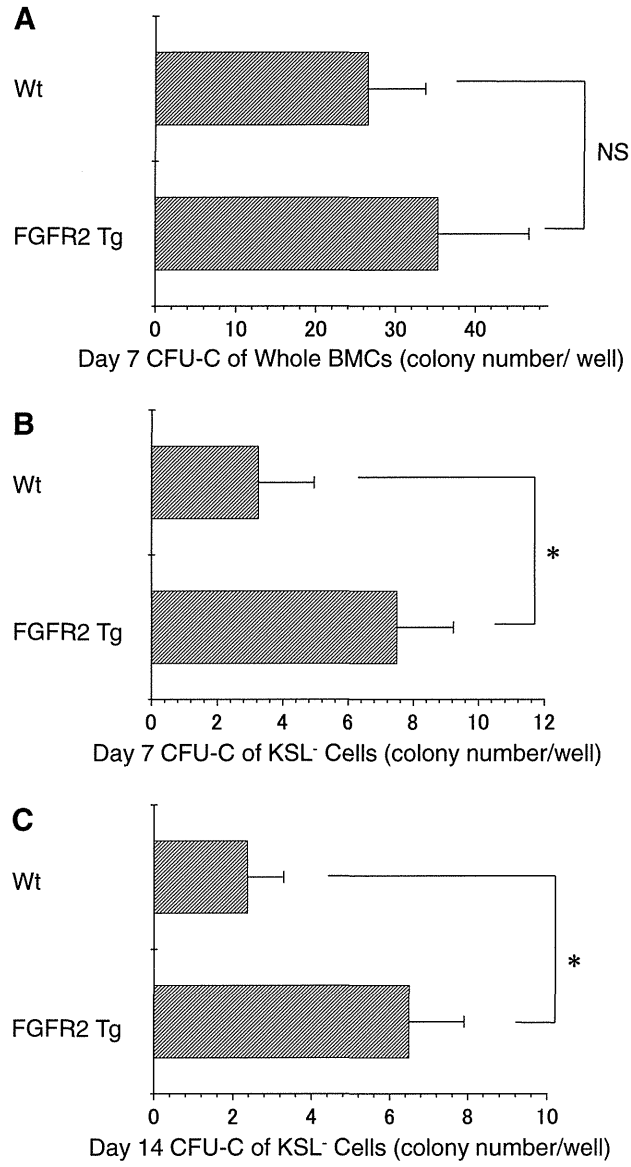

FIG. 2. The c-kit$^+$Sca-1$^+$lineage$^-$ BMCs of FGFR2 Tg mice facilitate the formation of CFU-C. BMCs prepared from Wt mice and FGFR2 Tg mice, as described in the Materials and Methods section, were used to perform the day-7 CFU-C assays ($n = 4$) **(A)**. The c-kit$^+$Sca-1$^+$lineage$^-$ cells were prepared from the BMCs of Wt mice and FGFR2 Tg mice using magnetic beads and a fluorescence-activated cell sorter. The day-7 and day-14 CFU-C assays were carried out using these cells, as described in the Materials and Methods section ($n = 7$) **(B, C)**. BMCs, bone marrow cells; CFU-C, colony-forming units of culture; FGFR, fibroblast growth factor receptor; Wt, wild type; KSL, c-kit$^+$Sca-1$^+$lineage$^-$; NS, not significant. *, $p < 0.05$.

normally under normal conditions, but that c-kit$^+$Sca-1$^+$lineage$^-$ BMCs in FGFR Tg mice show high hematogenic function when rapid hematopoiesis is required.

The percentages of c-kit$^+$Sca-1$^+$lineage$^-$ BMCs in Wt and FGFR2 Tg mice were 0.119 ± 0.017 and 0.126 ± 0.012, respectively, consistent with previous data [22]. The number of day-7 CFU-C using whole BMCs in 1 leg was $5{,}565 \pm 1{,}519$ in

Wt mice and 7,067 ± 2,262 in FGFR2 Tg mice. The numbers of day-7 CFU-C using c-kit$^+$Sca-1$^+$lineage$^-$ BMCs in 1 leg were 3,962 ± 2,081 and 9,469 ± 2,187, while the values for day-14 CFU-C using c-kit$^+$Sca-1$^+$lineage$^-$ BMCs in 1 leg were 2,786 ± 1,160 and 8,116 ± 1,909 in Wt mice and FGFR2 Tg mice, respectively. Comparing CFU-C in 1 leg, the numbers were greater in FGFR2 Tg than in Wt mice.

Reconstitution of donor hematopoietic cells after IBM-BMT

Next, we examined the hematopoietic function of c-kit$^+$Sca-1$^+$lineage$^-$ BMCs from FGFR2 Tg mice in vivo. Irradiated B6-ly5.1 mice were transplanted with c-kit$^+$Sca-1$^+$lineage$^-$ BMCs from FGFR2 Tg mice or Wt mice by IBM-BMT. Peripheral blood was collected from the recipient mice 4 weeks later. Mice transplanted with the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs from FGFR2 Tg mice showed a higher reconstitution of donor-type hematopoietic cells, as shown in Fig. 3. Donor hematopoietic cells were dominant not only in total WBCs (CD45$^+$ cells) but also in various lineages in the peripheral blood. A similar result was obtained when the mice were analyzed 6 months after IBM-BMT (data not shown).

Reduction of apoptosis in c-kit$^+$Sca-1$^+$lineage$^-$ BMCs in FGFR2 Tg mice

We examined the mechanisms underlying the better reconstitution of the hematopoietic cells of FGFR2 Tg mice. First, we cultured purified c-kit$^+$Sca-1$^+$lineage$^-$ BMCs in RPMI 1640 containing 10% fetal calf serum for 24 h and estimated the number of live cells using tetracolor-one, since the chromogenic reaction of tetracolor-one positively correlates with cell numbers [23]. As shown in Fig. 4A, the number of live cells in FGFR2 Tg mice was significantly higher than in Wt mice, suggesting that FGFR2 signaling can accelerate cell proliferation, or can suppress apoptosis, or both. The percentages of proliferative cells and apoptotic cells in c-kit$^+$Sca-1$^+$lineage$^-$ BMCs were determined 1 day after culture. Proliferative cells were detected by examining the cells expressing PCNA, since PCNA is reported to express in proliferating cells [24]. Apoptotic cells were detected using annexin V, which can bind to the phosphatidylserine expressed on the surface of apoptotic cells [25]. As shown in Fig. 4B, the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs from FGFR2 Tg mice showed more PCNA$^+$ cells than those from Wt mice. However, there was statistically no significant difference in PCNA$^+$ cells between FGFR2 Tg mice and Wt mice. In contrast, there were significantly fewer apoptotic c-kit$^+$Sca-1$^+$ lineage$^-$ BMCs in FGFR2 Tg mice than in Wt mice, which suggests that the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs of FGFR2 mice are resistant to apoptosis, resulting in better hematopoietic activity compared with those of Wt mice. We also cultured the BMCs of FGFR2 mice with or without PI-3-kinase inhibitor for 12 h and analyzed the percentages of apoptotic cells. The percentages of apoptotic cells in the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs increased in the culture condition with the

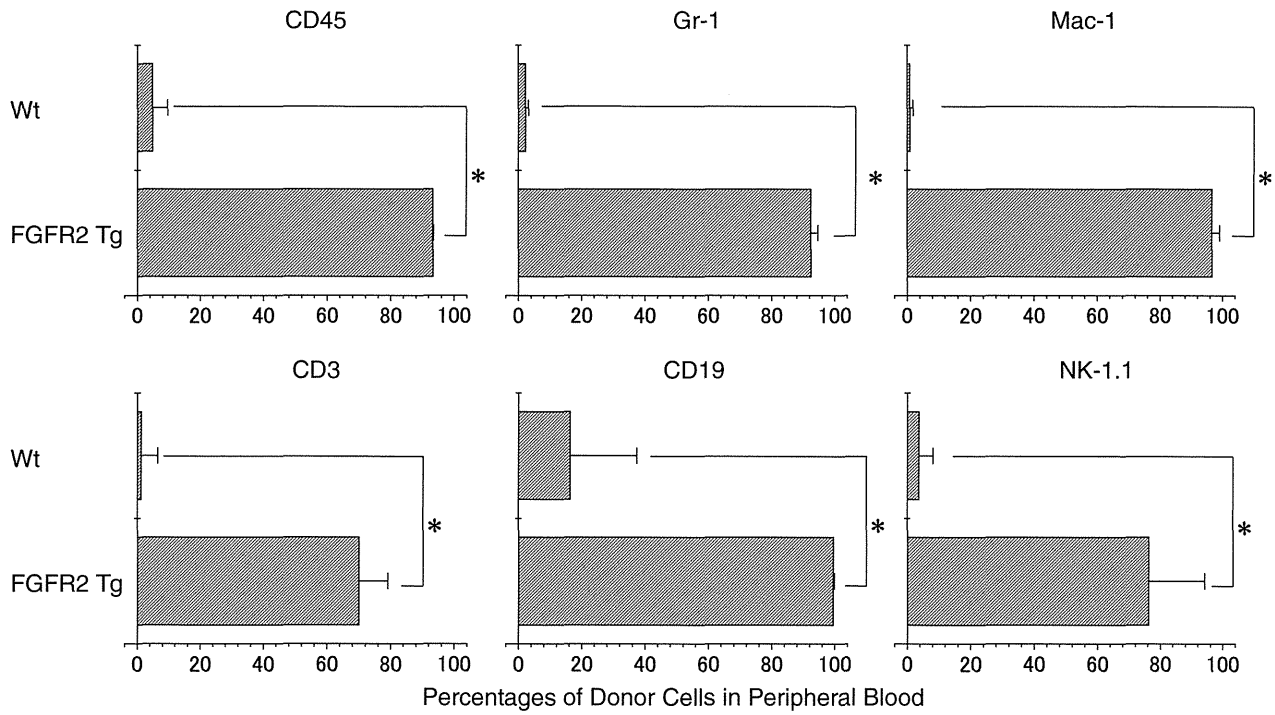

FIG. 3. The c-kit$^+$Sca-1$^+$lineage$^-$ BMCs from FGFR2 Tg mice facilitate dominant donor hematopoiesis. c-kit$^+$Sca-1$^+$lineage$^-$ cells were prepared from the BMCs of Wt mice and FGFR2 Tg mice using magnetic beads and a fluorescence-activated cell sorter, and transplanted into irradiated B6-ly5.1 mice. One month after the transplantation, peripheral blood was obtained from the mice. Donor hematopoietic cells were detected with expression of both CD45.2 (ly5.2) and CD45. Percentages of donor nuclear cells and various donor-derived lineage cells were examined in the peripheral blood. $n = 4$. Representative data are shown from 2 independent experiments. BMCs, bone marrow cells; FGFR, fibroblast growth factor receptor; Wt, wild type. *, $p < 0.05$.

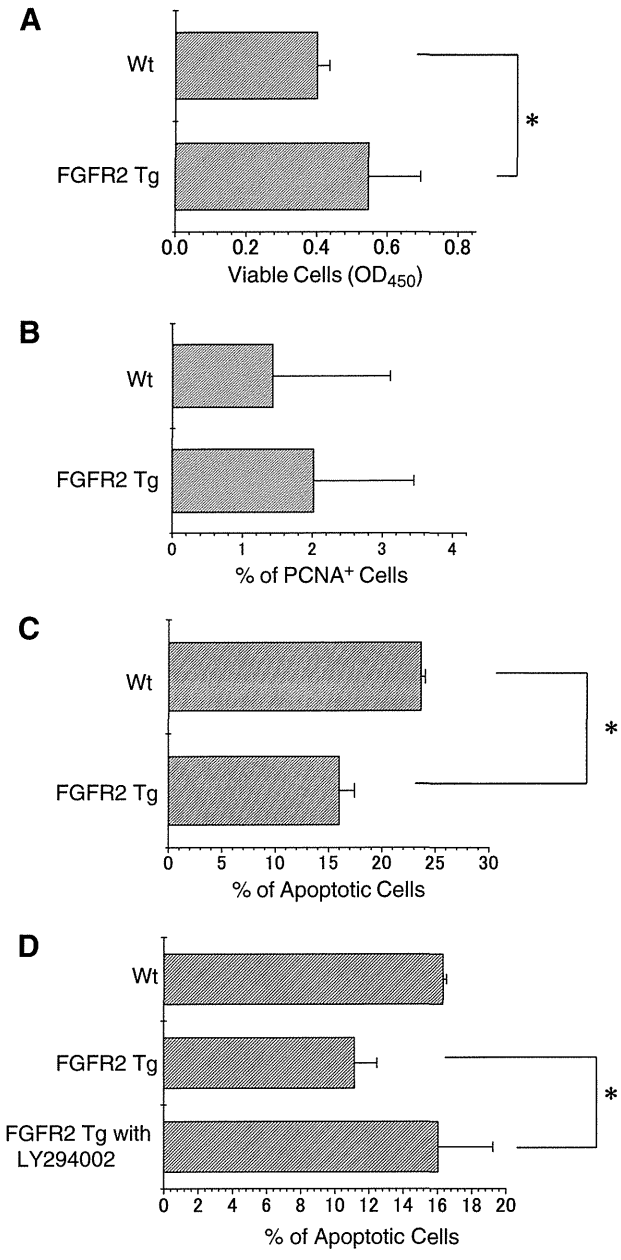

FIG. 4. The c-kit$^+$Sca-1$^+$lineage$^-$ BMCs from FGFR2 Tg mice are more resistant to apoptosis than those from Wt mice. **(A)** c-kit$^+$Sca-1$^+$lineage$^-$ cells were prepared from the BMCs of Wt mice and FGFR2 Tg mice using magnetic beads and a fluorescence-activated cell sorter. The cells were cultured for 24 h and the number of live cells examined with tetracolorone. **(B, C)** BMCs from Wt mice and FGFR2 Tg mice were cultured for 24 h. c-kit/Sca-1/Lin/PCNA (B) or c-kit/Sca-1/Lin/annexin V (C) were stained to examine the expression of PCNA (B) or annexin V (C) in c-kit$^+$Sca-1$^+$lineage$^-$ BMCs. BMCs of Wt mice and FGFR2 Tg mice were also cultured with or without PI-3-kinase (LY294002, 25 μM) for 12 h. The percentages of annexin V$^+$ cells were determined to detect apoptotic cellsin the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs. Representative data are shown from 2 independent experiments. $n = 4$. BMCs, bone marrow cells; FGFR, fibroblast growth factor receptor; PCNA, proliferating cell nuclear antigen; Wt, wild type; PI, phosphatidyl inositol. *, $p < 0.05$.

PI-3-kinase inhibitor, suggesting that this inhibitor can disable the function of FGFR2 signaling, as we previously described [16].

Discussion

In this study, we have shown that the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs of FGFR2 Tg mice have greater resistance to apoptosis and thereby facilitate hematopoiesis after IBM-BMT.

IBM-BMT has various benefits compared with conventional BMT. For instance, IBM-BMT can facilitate donor hematopoiesis and reduce the severity of graft-versus-host disease [20,26–28], based on which it has recently been attempted in humans [29]. The interaction of HSCs and stromal cells is crucial for the maintenance and proliferation of hematopoiesis [30,31]. The stromal cells form a niche that an support and regulate the maintenance, proliferation, and differentiation of HSCs. IBM injection of donor BMCs is an ideal method for setting them within the niche, and was therefore performed here.

In the present study, there were no significant differences in the CBCs, percentages of respective fractions of the peripheral blood, cell numbers of the spleen, cell numbers of the BM, or populations of the BM between Wt mice and FGFR2 Tg mice, even though FGFR2 was constitutively activated in the cells expressing Tie2, which is expressed on immature hematopoietic cells. This suggests that hematopoiesis in FGFR2 Tg mice is regulated normally. In the day-7 CFU-C assays, whole BMCs from FGFR2 Tg mice formed greater number of colonies, but there were no significant differences between the BMCs of Wt mice and FGFR2 Tg mice (Fig. 2).The c-kit$^+$ Sca-1$^+$lineage$^-$ BMCs from FGFR2 Tg mice formed significantly more colonies (Fig. 3) and also showed better hematopoietic reconstitution ability (data not shown) than those from Wt mice. These results suggest that the c-kit$^+$Sca-1$^+$ lineage$^-$ BMCs from FGFR2 Tg mice have better reconstitution capacity not only in the short term but also in the long term. The proliferation assay and the assay for apoptosis revealed that the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs of FGFR2 Tg mice are more resistant to apoptosis than those of Wt mice.

This study also showed that signaling through PI-3-kinase is crucial for the antiapoptotic effects of FGFR2 Tg mice, since these effects were suppressed by the PI-3-kinase inhibitor LY294002, as we previously described [16]. The effects of FGFR on cell proliferation are controversial. It has been reported that FGFR can transmit the signal for cell proliferation via the mitogen-activated protein (MAP)-kinase pathway but can transmit negative signals for cell proliferation through Sprouty, which inhibits the FGFR-stimulated rat sarcoma/microtubule affinity regulating kinase (Ras/MARK) pathway [32,33]. Consistent with the above results, this suggests that the mechanisms underlying the resistance to apoptosis in BMCs are similar to those in endothelial cells of FGFR2 Tg mice.

Our data suggest that the overexpression of FGFR2 is useful for BMT. However, its application in human beings requires a more efficient method for the transfection of the FGFR2 gene construct to the HSCs safely and securely. We have shown that the expression of FGFR2 in c-kit$^+$Sca-1$^+$lineage$^-$ BMCs is higher than in lineage$^+$ BMCs in FGFR2 Tg mice, and that FGFR2 is expressed in c-kit$^+$Sca-1$^+$lineage$^-$ BMCs even in Wt mice. Thus, the stimulation of FGFR2 has

some effect on the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs even in normal mice. It has been reported that FGFs can sustain the proliferation of hematopoietic progenitor cells, maintaining their primitive phenotype [34,35], and can induce myelopoiesis [36], megakaryocytopoiesis [37], and erythropoiesis [38]. Therefore, if the expression of FGFR2 on HSCs can be upregulated in one way or another, FGFs could be used more effectively to accelerate hematopoiesis.

Tie2 is expressed not only on endothelial cells but also on c-kit$^+$Sca-1$^+$lineage$^-$ BMCs [14]. In the FGFR2 Tg mice, the promoter of Tie2 regulates the expression of FGFR2: the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs expressing Tie2 produce constitutively activated FGFR2. Even though the percentages of Tie2$^+$ cells in the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs of Wt mice and FGFR2 Tg mice did not differ significantly, the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs from FGFR2 Tg mice were better facilitated than those from Wt mice or the whole BMCs from FGFR2 Tg mice. This could be attributed to the expression of FGFR2, which suppresses apoptosis, resulting in better reconstitution of c-kit$^+$Sca-1$^+$lineage$^-$ BMCs of FGFR2 Tg mice than those of Wt mice.

In this study, we have shown that the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs of FGFR2 mice are more resistant to apoptosis than those of Wt mice. However, hematopoiesis in FGFR2 Tg mice is normally regulated under normal conditions. When rapid hematopoiesis is required, the c-kit$^+$Sca-1$^+$lineage$^-$ BMCs of FGFR2 Tg mice show high hematogenic function, resulting in rapid recovery of donor hematopoietic cells after BMT. Since these phenomena are convenient and suitable for BMT, an efficient and safe method to transfect the FGFR2 gene construct to HSCs would be highly desirable.

Acknowledgments

We thank Ms. Y. Tokuyama, Ms. K. Hayashi, and Ms. A. Kitajima for their expert technical assistance, and also Mr. Hilary Eastwick-Field and Ms. K. Ando for helping with the preparation of this manuscript. This study was supported by grants from the "21st Century COE Program" of the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Department of Transplantation for Regeneration Therapy (sponsored by Otsuka Pharmaceutical Co., Ltd.); the Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan); and Japan Immunoresearch Laboratories Co., Ltd. (Takasaki, Gunnma, Japan); a Grant-in-Aid for Scientific Research (C) 21590447; and an award from Dr. Toshiko Kitanishi.

Author Disclosure Statement

No competing financial interests exist.

References

1. Basilico C and D Moscatelli. (1992). The FGF family of growth factors and oncogenes. Adv Cancer Res 59:115–165.
2. Baird A. (1994). Fibroblast growth factors: activities and significance of non-neurotrophin neurotrophic growth factors. Curr Opin Neurobiol 4:78–86.
3. Krejci P, J Prochazkova, V Bryja, A Kozubik and WR Wilcox. (2009). Molecular pathology of the fibroblast growth factor family. Hum Mutat 30:1245–1255.
4. Fischer S, BW Draper and CJ Neumann. (2003). The zebrafish fgf24 mutant identifies an additional level of Fgf signaling involved in vertebrate forelimb initiation. Development 130: 3515–3524.
5. Böttcher RT and C Niehrs. (2005). Fibroblast growth factor signaling during early vertebrate development. Endocr Rev 26:63–77.
6. Braun S, U auf dem Keller, H Steiling and S Werner. (2004). Fibroblast growth factors in epithelial repair and cytoprotection. Philos Trans R Soc Lond B Biol Sci 359:753–757.
7. Ziche M, S Donnini and L Morbidelli. (2004). Development of new drugs in angiogenesis. Curr Drug Targets 5:485–493.
8. Powers CJ, SW McLeskey and A Wellstein. (2000). Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 7:165–197.
9. Wilson EL, DB Rifkin, F Kelly, MJ Hannocks and JL Gabrilove. (1991). Basic fibroblast growth factor stimulates myelopoiesis in long-term human bone marrow cultures. Blood 77:954–960.
10. Kashiwakura I and TA Takahashi. (2005). Fibroblast growth factor and ex vivo expansion of hematopoietic progenitor cells. Leuk Lymphoma 46:329–333.
11. Allouche M and A Bikfalvi. (1995). The role of fibroblast growth factor-2 (FGF-2) in hematopoiesis. Prog Growth Factor Res 6:35–48.
12. Dumont DJ, TP Yamaguchi, RA Conlon, J Rossant and ML Breitman. (1992). tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. Oncogene 7:1471–1480.
13. Sato TN, Y Qin, CA Kozak and KL Audus. (1993). Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc Natl Acad Sci U S A 90:9355–9358.
14. Arai F, A Hirao, M Ohmura, H Sato, S Matsuoka, K Takubo, K Ito, GY Koh and T Suda. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118:149–161.
15. Arai F and T Suda. (2007). Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche. Ann N Y Acad Sci 1106:41–53.
16. Matsunaga S, M Okigaki, M Takeda, A Matsui, S Honsho, A Katsume, E Kishita, C Jishan, T Kurihara, Y Adachi, A Mansukhani, M Kobara, Y Matoba, T Tatsumi and H Matsubara. (2009). Endothelium-targeted overexpression of constitutively active FGF receptor induces cardioprotection in mice myocardial infarction. J Mol Cell Cardiol 46:663–673.
17. Adachi Y, S Taketani, H Oyaizu, K Ikebukuro, R Tokunaga and S Ikehara. (1999). Apoptosis of colorectal adenocarcinoma induced by 5-FU and/or IFN-gamma through caspase 3 and caspase 8. Int J Oncol 15:1191–1196.
18. Koike-Kiriyama N, Y Adachi, K Minamino, M Iwasaki, K Nakano, K Koike, H Yamada, H Mukaide, A Shigematsu, T Mizokami, M Matsumura and S Ikehara. (2007). Human cord blood cells can differentiate into retinal nerve cells. Acta Neurobiol Exp 67:359–365.
19. Sugihara A, Y Adachi, M Inaba, H Hisha, K Sugiura, S Miyashima, Y Amoh, S Taketani, H Oyaizu, K Ikebukuro, M Kawamura, H Genba, T Horio and S Ikehara. (1999). Age-dependent abnormalities of hematopoietic stem cells in (NZW x BXSB)F1 mice. Stem Cells 17:357–365.
20. Kushida T, M Inaba, H Hisha, N Ichioka, T Esumi, R Ogawa, H Iida and S Ikehara. (2001). Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for

treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 97:3292–3299.
21. Dexter TM, E Spooncer, P Simmons and TD Allen. (1984). Long-term marrow culture: an overview of techniques and experience. Kroc Found Ser 18:57–96.
22. Okada S, H Nakauchi, K Nagayoshi, S Nishikawa, Y Miura and T Suda. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood 80:3044–3050.
23. Yamamoto O, T Hamada, N Tokui and Y Sasaguri. (2001). Comparison of three in vitro assay systems used for assessing cytotoxic effect of heavy metals on cultured human keratinocytes. J UOEH 23:35–44.
24. Garcia RL, MD Coltrera and AM Gown. (1989). Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues. Comparison with flow cytometric analysis. Am J Pathol 134:733–739.
25. van Engeland M, LJ Nieland, FC Ramaekers, B Schutte and CP Reutelingsperger. (1998). Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 31:1–9.
26. Ikehara S. (2003). New strategies for BMT, organ transplantation, and regeneration therapy. Hematology 8:77–81.
27. Ikebukuro K, Y Adachi, Y Suzuki, M Iwasaki, K Nakano, Y Koike, H Mukaide, Y Yamada, S Fujimoto, Y Seino, H Oyaizu, A Shigematsu, N Kiriyama, Y Hamada, Y Kamiyama and S Ikehara. (2006). Synergistic effects of injection of bone marrow cells into both portal vein and bone marrow on tolerance induction in transplantation of allogeneic pancreatic islets. Bone Marrow Transplant 38:657–664.
28. Suzuki Y, Y Adachi, K Minamino, Y Zhang, M Iwasaki, K Nakano, Y Koike and S Ikehara. (2005). A new strategy for treatment of malignant tumor: intra-bone marrow-bone marrow transplantation plus CD4- donor lymphocyte infusion. Stem Cells 23:365–370.
29. Frassoni F, F Gualandi, M Podestà, AM Raiola, A Ibatici, G Piaggio, M Sessarego, N Sessarego, M Gobbi, N Sacchi, M Labopin and A Bacigalupo. (2008). Direct intrabone transplant of unrelated cord-blood cells in acute leukaemia: a phase I/II study. Lancet Oncol 9:831–839.
30. Mauch P, JS Greenberger, L Botnick, E Hannon and S Hellman. (1980). Evidence for structured variation in self-renewal capacity within long-term bone marrow cultures. Proc Natl Acad Sci U S A 77:2927–2930.
31. Kiel MJ and SJ Morrison. (2008). Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol 8:290–301.
32. LaVallee TM, IA Prudovsky, GA McMahon, X Hu and T Maciag. (1998). Activation of the MAP kinase pathway by FGF-1 correlates with cell proliferation induction while activation of the Src pathway correlates with migration. J Cell Biol 141:1647–1658.
33. Lao DH, P Yusoff, S Chandramouli, RJ Philp, CW Fong, RA Jackson, TY Saw, CY Yu and GR Guy. (2007). Direct binding of PP2A to Sprouty2 and phosphorylation changes are a prerequisite for ERK inhibition downstream of fibroblast growth factor receptor stimulation. J Biol Chem 282:9117–9126.
34. Allouche M and A Bikfalvi. (1995). The role of fibroblast growth factor-2 (FGF-2) in hematopoiesis. Prog Growth Factor Res 6:35–48.
35. Allouche M, F Bayard, S Clamens, G Fillola, P Sié and F Amalric. (1995). Expression of basic fibroblast growth factor (bFGF) and FGF-receptors in human leukemic cells. Leukemia 9:77–86.
36. Gabrilove JL, K White, Z Rahman and EL Wilson. (1994). Stem cell factor and basic fibroblast growth factor are synergistic in augmenting committed myeloid progenitor cell growth. Blood 83:907–910.
37. Chen QS, ZY Wang and ZC Han. (1999). Enhanced growth of megakaryocyte colonies in culture in the presence of heparin and fibroblast growth factor. Int J Hematol 70: 155–162.
38. Koritschoner NP, P Bartůněk, S Knespel, G Blendinger and M Zenke. (1999). The fibroblast growth factor receptor FGFR-4 acts as a ligand dependent modulator of erythroid cell proliferation. Oncogene 18:5904–5914.

Address correspondence to:
Dr. Susumu Ikehara
First Department of Pathology
Kansai Medical University
10-15 Fumizono-cho
Moriguchi City 570-8506
Osaka
Japan

E-mail: ikehara@takii.kmu.ac.jp

Received for publication September 22, 2009
Accepted after revision March 25, 2010
Prepublished on Liebert Instant Online March 26, 2010

Cancer Immunol Immunother (2010) 59:1121–1130
DOI 10.1007/s00262-010-0840-2

ORIGINAL ARTICLE

Prolonged survival in mice with advanced tumors treated with syngeneic or allogeneic intra-bone marrow–bone marrow transplantation plus fetal thymus transplantation

Naoki Hosaka · Wenhao Cui · Yuming Zhang · Takashi Takaki · Muneo Inaba · Susumu Ikehara

Received: 23 August 2009 / Accepted: 23 February 2010 / Published online: 13 March 2010

Abstract Thymic function decreases in line with tumor progression in patients with cancer, resulting in immunodeficiency and a poor prognosis. In the present study, we attempted to restore thymic function by BALB/c (H-2^d) syngeneic (Syn), or B6 (H-2^b) allogeneic (Allo) bone marrow transplantation (BMT) using intra-bone marrow–bone marrow transplantation (IBM–BMT) plus Syn-, Allo- or C3H (H-2^k) 3rd-party fetal thymus transplantation (TT). Although the BALB/c mice with advanced tumors (Meth-A sarcoma; H-2^d, >4 cm^2) treated with either Syn- or Allo-BMT alone showed a slight improvement in survival compared with non-treated controls, the mice treated with BMT + TT showed a longer survival. The mice treated with Allo-BMT + Allo-TT or 3rd-party TT showed the longest survival. Interestingly, although there was no difference in main tumor size among the BMT groups, lung metastasis was significantly inhibited by Allo-BMT + Allo-TT or 3rd-party TT. Numbers of $CD4^+$ and $CD8^+$ T cells, Con A response, and IFN-γ production increased significantly, whereas number of Gr-1^+/$CD11b^+$ myeloid suppressor cells and the percentage of $FoxP3^+$ cells in $CD4^+$ T cells significantly decreased in these mice. Furthermore, there was a positive correlation between survival days and the number of T cells or T cell function, while there was a negative correlation between survival days and lung metastasis, the number of Gr-1^+/$CD11b^+$ cells, or the percentage of $FoxP3^+$ cells. These results suggest that BMT + TT, particularly Allo-BMT + Allo-TT or 3rd-party TT, is most effective in prolonging survival as a result of the restoration of T cell function in hosts with advanced tumors.

Keywords Advanced cancer · Bone marrow transplantation · Thymus transplantation · Metastasis · Regulatory T cell · Myeloid suppressor cell

Abbreviations

BMT	Bone marrow transplantation
IBM–BMT	Intra-bone marrow–bone marrow transplantation
TT	Thymus transplantation
BMC	Bone marrow cell
MHC	Major histocompatibility complex

Introduction

Patients with malignant tumors show reduced immune function, which strongly influences prognosis [1–3]. A number of mechanisms for the reduction of immune function have been postulated. One is that the thymus is, as a result of the tumor progression, involved in the maturation block of thymocytes, which leads to a reduction in the number and function of T cells [4–7]. The other is that the number of Gr-1^+ $CD11b^+$ myeloid suppressor cells increases [8], which results in inhibited T cell signaling with tumor growth factor (TGF)-β and IL-13 [9, 10]. In addition, recent studies have shown that regulatory T cells, which express $CD4^+FoxP3^+$ and inhibit T cell function, play a crucial role in the development of autoimmune disease and graft-versus-host

N. Hosaka and W. Cui contributed equally to this study.

N. Hosaka · W. Cui · Y. Zhang · T. Takaki · M. Inaba · S. Ikehara (✉)
First Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan
e-mail: ikehara@takii.kmu.ac.jp

Y. Zhang
Department of Pediatrics, Nanfang Hospital, Guangzhou 510515, China