TABLE 2. Effects of IBM-BMT With OT on serum estrogen and TRACP

Group	Serum estrogen (pg/mL)	TRACP (U/L)
Normal control	$23.71\pm7.80^{a,b}$	0.62 ± 0.19^{a}
OVX	$11.69\pm4.41^{c,d}$	$1.28\pm0.33^{c,d}$
BMT/OT	$21.47\pm13.44^{a,b}$	0.71 ± 0.39^{a}
BMT	$13.77\pm4.09^{c,d}$	1.08 ± 0.74

Serum estrogen and TRACP were measured using an ELISA kit. There were no significant differences between the normal control group and the BMT/OT group in the serum estrogen assay. The hormonal rise indicated the acceptance of allografts with function. In the TRACP assay, the TRACP level in the BMT/OT group decreased in comparison with the BMT group, indicating that bone resorption had decreased.

Data are expressed as mean±SD, n=8.

[a] $P<0.01$ vs. OvX group.

[b] $P<0.01$ vs. BMT group.

[c] $P<0.01$ vs. normal control group.

[d] $P<0.01$ vs. BMT/OT group.

IBM, intrabone marrow; BMT, bone marrow transplantation; TRACP, tartrate-resistant acid phosphatase; bone marrow transplantation; OT, ovarian transplantation.

duces not only ovarian failure but also uterine dysfunction. However, in this study, after BMT/OT, the weight of the uteri increased in the BMT/OT group. Moreover, the endometrial morphology, including the endometrial glands, was almost normal, although the uterine volume was in the normal range.

It is a doctrine that the mammalian neonatal ovary contains a finite stockpile of nongrowing primordial follicles, each of which encloses an oocyte arrested at the diplotene step in the meiotic prophase. Recent studies of mouse ovaries, however, propose that intra- and extraovarian germline stem cells replenish oocytes and form new primordial follicles after chemotherapy but not after ionizing radiation (*7–10*). Moreover, the notion of oocyte and follicular renewal in the postnatal mouse ovary from within the ovary or external to it has been supported by other authors (*34–38*).

The ovary is radiosensitive tissue, and half of the follicles are lost by 0.1 to 0.3 Gy in mice (*5*). Moreover, low-dose irradiation (0.5 Gy) can sterilize female mice (*19*). In this report, we used a lethal dose of 6 Gy ×2 as a conditioning regimen for IBM-BMT, and there were no oocytes in the host ovaries in the BMT group after 3 months. In the BMT/OT group, however, the host ovaries demonstrated oocytes within primordial and growing immature follicles. However, we have no evidence to support the hypothesis that renewing oocytes are derived from donor-derived bone marrow cells or germline stem cells. Our observations provide qualified support for an as-yet unknown mechanism for follicle renewal in the postnatal and adult mouse ovary, even after ionizing radiation. This is an important area for future study. Interestingly, all of the new oocytes in the host ovaries were observed in immature follicles up to the preantral stage of development, but never in maturing antral or Graafian follicles. Also, 2 weeks after the allogeneic BMT/OT described above, the mice were mated with male mice, but none were fertile.

Others have hypothesized that BMT functions primarily by reactivating host oogenesis (*7*), which becomes impaired in a BMT-reversible manner after chemotherapy but not after ionizing radiation (*4*). Our results demonstrate that BMT therapy after irradiation cannot, on its own, reverse the damage to the ovary. The gonadal environmental factors are essential for the oocyte's self-renewal, and our data indicate that, if a gonadal microenvironment is supplied after irradiation (such as by timely BMT/OT), new follicles may be produced in the host ovary. However, other laboratories have postulated that the frequencies of micronuclei, anaphase-telophase alterations and chromosomal aberrations increase after low-dose irradiation (*39*), and that DNA damage results in genetic instability. We suggest that it is possible that BMT/OT is insufficient to repair the damage induced by irradiation on DNA (*39*), blood vessels (*40*), and granulosa cells (*41*) or fertility genes (*42*) in the host ovaries, which can then no longer support the development of new immature oocytes, such as in the case of primordial germ cells lacking Nanog, which fail to mature on reaching the genital ridge (*43*).

There are direct and indirect toxic effects of irradiation on the bone. Irradiation can directly damage the osteogenic activity of marrow by suppressing osteoblasts, leading to postirradiation osteoporosis (*44*) and postradiation atrophy of mature bone (*45,46*); after irradiation, ovaries cannot secrete estrogen to regulate bone homeostasis (*47*). Estrogen deficiency becomes the main reason for bone loss in young females. The osteoprotective action of estrogen blocks the formation of new osteoclasts, shortens the lifespan of old osteoclasts, and promotes osteoblast proliferation. The data presented in this study report that the bone mass in the BMT/OT group achieved normal levels after 3 months. This finding implies that estrogen secreted by transplanted allogeneic ovaries can efficiently prevent bone loss after heavy irradiation. Hence, we propose that IBM-BMT with ovarian allografts can be advantageous for young women with POF and osteopenia or osteoporosis (due to chemotherapy and radiotherapy for malignant diseases). This is clearly an important area for future study.

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Research Report

Intra-bone marrow-bone marrow transplantation slows disease progression and prolongs survival in G93A mutant SOD1 transgenic mice, an animal model mouse for amyotrophic lateral sclerosis

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ABSTRACT

It has been reported that bone marrow transplantation (BMT) has clinical effects on not only hematopoietic diseases and autoimmune diseases but also solid malignant tumors and metabolic diseases. We have found that intra-bone marrow-bone marrow transplantation (IBM-BMT) is superior to conventional intravenous BMT, since IBM-BMT enables rapid recovery of donor hematopoiesis and reduces the extent of graft-versus-host disease (GVHD). In this experiment, we examined the effects of IBM-BMT on symptomatic G93A mutant SOD1 transgenic mice (mSOD1 Tg mice), a model mouse line for amyotrophic lateral sclerosis (ALS). Symptomatic mSOD1 Tg mice (12 weeks old) were irradiated with 6 Gy×2 at a 4-hour interval, one day before IBM-BMT. The mice were transplanted with bone marrow cells (BMCs) from 12-wk-old eGFP-transgenic C57BL/6 mice (eGFP Tg mice) or BMCs from 12-wk-old mSOD1 Tg mice. The ALS model mice transplanted with BMCs from eGFP Tg mice showed longer survival and slower disease progression than those transplanted with BMCs from mSOD1 Tg mice or untreated mSOD1 Tg mice. There was a significantly high number of eGFP$^+$ cells in the anterior horn of the spinal cord of the mSOD1 Tg mice transplanted with BMCs of eGFP Tg mice, some of which expressed Iba-1, a marker of microglia, although they did not differentiate into neural cells. These results suggest that the replacement with normal hematopoietic cells improved the neural cell environment, thereby slowing the progression of the disease.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal and progressive neurodegenerative disease, which selectively affects motor neurons in the spinal cord, lower brainstem and cerebral cortex (Strong and Rosenfeld, 2003). The disease clinically shows progressive skeletal muscle atrophy and paralysis, and finally induces individual death, mainly due to respiratory failure,

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Abbreviations: ALS, amyotrophic lateral sclerosis; SOD1, superoxide dimustase 1; mSOD1 Tg mouse, G93A mutant SOD1 transgenic mouse; GVHD, graft-versus-host disease; BMCs, bone marrow cells; BMT, bone marrow transplantation; IBM-BMT, intra-bone marrow-BMT; eGFP, enhanced green fluorescent; eGFP Tg mouse, eGFP-transgenic mouse

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within a few years after onset. Although most cases are sporadic, approximately 10% of cases are familial type, and the disease in approximately 20% of familial patients is caused by dominantly inherited mutations in the gene encoding the antioxidant enzyme copper- and zinc-dependent superoxide dismutase 1 (SOD1) (Rosen et al., 1993). The G93A mutant SOD1 transgenic mouse (mSOD1 Tg mouse) is a model mouse line of familial ALS, and exhibits progressive degeneration of lower motor neurons coincident with the onset of limb tremors and muscle wasting. The mouse eventually dies due to its inability to feed itself (Gurney et al., 1994). Therefore, the mSOD1 Tg mouse is now widely used as a convenient tool not only for understanding the pathogenesis of ALS but also for the development of new therapeutic strategies for this disease.

Until now, investigations of ALS patients and the mSOD1 Tg mouse models have suggested the existence of several pathogenic mechanisms underlying the degenerative processes of ALS, including oxidative stress, excitotoxicity, chronic inflammation, mitochondrial and neurofilamental dysfunction, and ultimately activation of a programmed cell death pathway (Bruijn et al., 2004; Shaw, 2005). Based on these results, several therapeutic approaches have been attempted with ALS model animals, such as anti-oxidants, anti-excitotoxics, anti-inflammatories, immunomodulators, and neurotrophics, and some amelioration of the symptoms in these models has been reported (Benatar, 2007). Nevertheless, clinical trials using these therapies with human patients have been extremely limited. There has been no effective therapy for ALS except for riluzole, which is the only FDA-approved therapeutic agent for ALS.

The transplantation of bone marrow (BM) stem cells has been found to be effective for the treatment of neurological disorders, including stroke (Li et al., 2002), spinal cord injury (Chopp et al., 2000), multiple sclerosis (Saccardi et al., 2005; Van Wijmeersch et al., 2007), Parkinson's disease (Rodríguez-Gómez et al., 2007), Alzheimer's disease (Yamasaki et al., 2007), and Huntington's disease (Keene et al., 2007; Kim et al., 2008). For the treatment of ALS, recent reports have demonstrated that the intra-peritoneal or intravenous injections of murine BM cells (Corti et al., 2004) or human umbilical cord blood cells (Ende et al., 2000; Garbuzova-Davis et al., 2003) into the mSOD1 Tg mice ameliorates the symptoms and prolongs survival.

In the bone marrow transplantation study from normal mice to the mSOD1 Tg mice, Corti et al. used 4-wk-old asymptomatic mSOD1 Tg mice as recipients, and they induced mixed chimerism of hematopoietic cells by transplanting donor bone marrow cells (BMCs) into the peritoneal cavity. They were able to show that the BMT helped retard the onset of the disease and slow its progression.

We have shown that complete chimerism is superior to mixed chimerism, since mixed chimerism is not sufficiently stable to maintain donor hematopoietic cells in the recipients (Hayashi et al., 1997). Recently, we established a new method of bone marrow transplantation (BMT); intra-bone marrow-BMT (IBM-BMT), which enables a reduction in the pretreatment for BMT, rapid recovery of donor hematopoietic cells, and also reduces the extent of graft-versus-host disease (GVHD) (Ikehara, 2002, 2003; Kushida et al., 2001).

In this study, we prepared complete chimerism of the hematopoietic cells from enhanced green fluorescence protein (eGFP)-transgenic C57BL/6 mice (eGFP Tg mice) to symptomatic ALS model mice at an earlier stage of ALS by the combination of split irradiation of 6 Gy×2 and IBM-BMT, and examined the effects of the complete chimerism of hematopoietic cells on the symptomatic mSOD1 Tg mice.

2. Results

2.1. *IBM-BMT from eGFP Tg mice to symptomatic mSOD1 Tg mice slows disease progression and prolongs survival of the mice*

First, we examined whether the IBM-BMT could ameliorate the symptoms of the ALS-like disease in the mSOD1 Tg mice. The recipient mSOD1 Tg mice (12-wk-old female) at an early stage of the disease were irradiated with 6 Gy×2 (4-hour interval) and were then transplanted with BMCs from 12-wk-old eGFP Tg mice or 12-wk-old male mSOD1 Tg mice the following day. In this experiment, we prepared 3 groups: 1) non-treated female mSOD1 Tg mice (non-transplanted group), 2) female mSOD1 Tg mice transplanted with BMCs of male mSOD1 Tg mice using IBM-BMT (mSOD1-BM-transplanted group), and 3) female mSOD1 Tg mice transplanted with BMCs of eGFP Tg mice using IBM-BMT (eGFP-BM-transplanted group). None of the recipient mice (n=25) suffered from adverse effects, and our transplantation protocol was successfully carried out. When the recipient and donor mSOD1 Tg mice showed the symptoms, we carried out the IBM-BMT. That is, on the day we identified the onset of the disease in each mouse, we irradiated that mouse with 6.0 Gy×2. The mean ages of onset in the eGFP-BM-transplanted group, the mSOD1-BM-transplanted group, and the non-transplanted group were 89.0±1.1, 88.0±0.5, and 87.7±0.8 days, respectively. There were no significant differences between these three groups. Flow cytometric analyses revealed that all of the recipient mice transplanted with BMCs from eGFP Tg mice showed more than 90% donor-derived cells in the peripheral blood (95.2±1.2%) at 2 weeks after IBM-BMT.

The survival (time period from birth to end-stage sacrifice) and survival interval (time period from onset to end-stage sacrifice) of the eGFP-BM-transplanted group were 145.7±2.8 days and 56.7±3.1 days, being significantly longer than those of the mSOD1-BM-transplanted group (survival 135.9±2.4 days; $p<0.01$, survival interval 47.9±2.1 days; $p<0.05$) and non-transplanted group (survival 132.4±1.8 days; $p<0.01$, survival interval 44.7±1.8 days; $p<0.01$) (Fig. 1A). On the other hand, there were no significant differences in either the survival or survival interval between the mSOD1-BM-transplanted group and the non-transplanted group. In addition, the mean time period from onset to the day when the mice reached a 50% decrease in their grip strength in the eGFP-BM-transplanted, mSOD1-BM-transplanted, and non-transplanted groups was 51.6±3.9, 40.6±2.3, and 41.0±2.3 days, respectively. Similarly, the mean time period from onset to the day of a 10% decrease in body weight in each group was 46.4±3.9, 46.6±2.1, and 43.9±2.4 days, respectively (Figs. 1B,C). The results of log-rank tests demonstrated significant differences in the grip strength between the eGFP-BM-transplanted and the mSOD1-BM-transplanted groups (p=0.0351) or eGFP-BM-transplanted and the non-transplanted groups (p=0.0081). On the other hand, there

were no significant differences between the mSOD1-BM-transplanted and non-transplanted groups ($p=0.5277$). In contrast, statistical analyses demonstrated no significant differences in loss of body weight between the 3 groups.

2.2. *Histological examination of spinal cord in recipient mice*

To clarify the mechanisms underlying the effects induced by the IBM-BMT, we examined the spinal cord histologically. We found donor-derived eGFP$^+$ cells in the lumbar spinal cord parenchyma of the recipient mice, mostly in the ventral grey matter (Fig. 2A). All of the eGFP$^+$ cells expressed CD45, a marker for hematopoietic cells (data not shown). H&E staining after immunofluorescent investigation using the same specimens revealed that some of the migrated eGFP$^+$ cells showed the morphological feature of ramified and ameboid microglia accompanied by several apophyses (Figs. 2B and C).

The double immunofluorescence technique for eGFP and for Iba-1, GFAP, O4, or NeuN revealed the colocalization of the expression of eGFP and the expression of Iba-1 in the migrated cells (Figs. 2D–F). We could not identify any eGFP$^+$ cells labeled with the anti-GFAP, anti-NeuN, or anti-O4 antibodies (data not shown). H&E staining after the immunofluorescent study revealed that the Iba-1$^+$ eGFP$^+$ cells showed the morphological feature of ramified and ameboid microglia (Fig. 2G).

2.3. *Numbers of axons, motor neurons and microglia in spinal cord*

It has been reported that the number of axons and motor neurons decrease in mSOD1 mice with the progression of the disease, and that the number of microglia increases (Corti et al., 2004; Kang and Rivest, 2007; Gowing et al., 2008). Therefore, we counted the number of axons, motor neurons and microglia in the specimens obtained at the end point of the observation: namely, when the mice were sacrificed at the end point of the experiment, the spinal cords were removed and fixed with 4% paraformaldehyde. We detected 3.4±1.9 motor neurons per sciatic motor pool area in the mSOD1-BM-transplanted groups and 3.3±2.5 in the eGFP-BM-transplanted group, and 11.0±2.7 in wild type (Wt). There was no significant difference in the number of motor neurons between the mSOD1-BM-transplanted group and the eGFP-BM-transplanted group. We also calculated the mean number of axons in three randomly selected areas of 100 μm×100 μm in the horizontal cross-section of the L5 ventral root. There was no significant difference in the numbers of axons between the mSOD1-BM-transplanted group (32.0±8.3) and the eGFP-BM-transplanted group (33.3±7.5). However, they were much lower than in the Wt (70±3.4) ($p<0.05$). The numbers of Iba-1 positive microglia were 52.6±5.8 in the mSOD1-BM-transplanted group and 54.5±6.8 in the eGFP-BM-transplanted group, and there were no significant

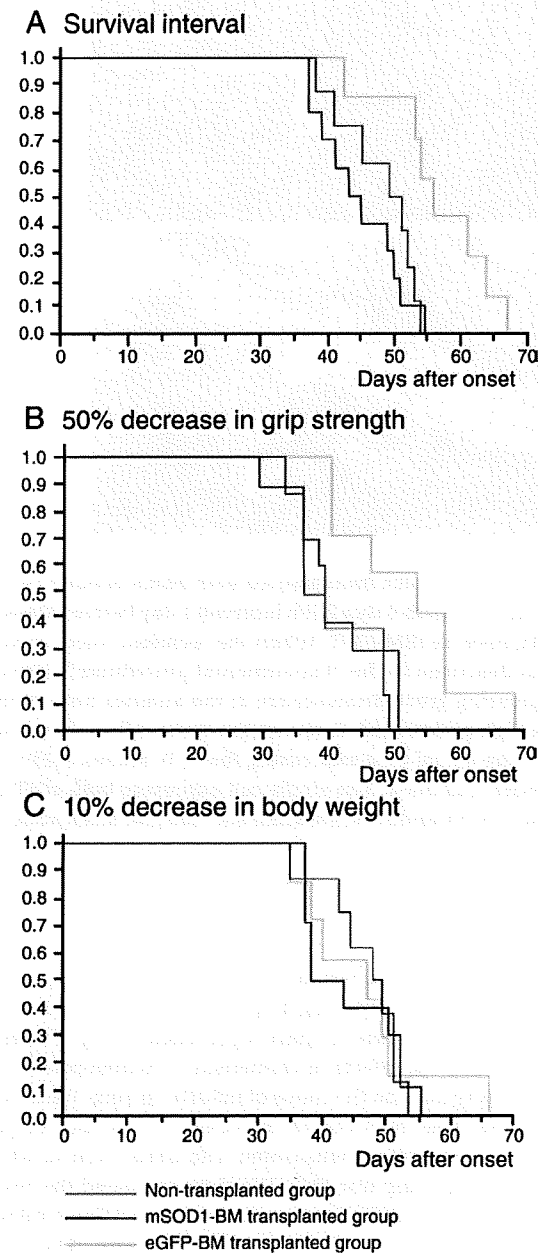

Fig. 1 – IBM-BMT can prolong the survival interval and slow the development of ALS in mSOD1 Tg mice. The mSOD1 Tg mice were randomly assigned into the following experimental groups: 1) non-treated female mSOD1 Tg mice (non-transplanted group) ($n=10$), 2) female mSOD1 Tg mice transplanted with BMCs of male mSOD1 Tg mice using IBM-BMT (mSOD1-BM-transplanted mice group) ($n=8$), and 3) female mSOD1 Tg mice transplanted with BMCs of male eGFP Tg mice using IBM-BMT (eGFP-BM-transplanted mice group) ($n=7$). All the donor mice were 12-wk-old symptomatic males. Each night, examiners who were blind to the information, weighed each mouse and measured the grip strength of their forelimbs using a Digital Grip Strength Meter. Each measurement was repeated 4 times and the maximum value was adopted. When each mouse lost the ability to autofeed or to right itself within 30 s. after having been placed on its side, it was sacrificed under deep anesthesia. "Survival interval" means the term from onset of the disease to the day that the mouse was sacrificed.

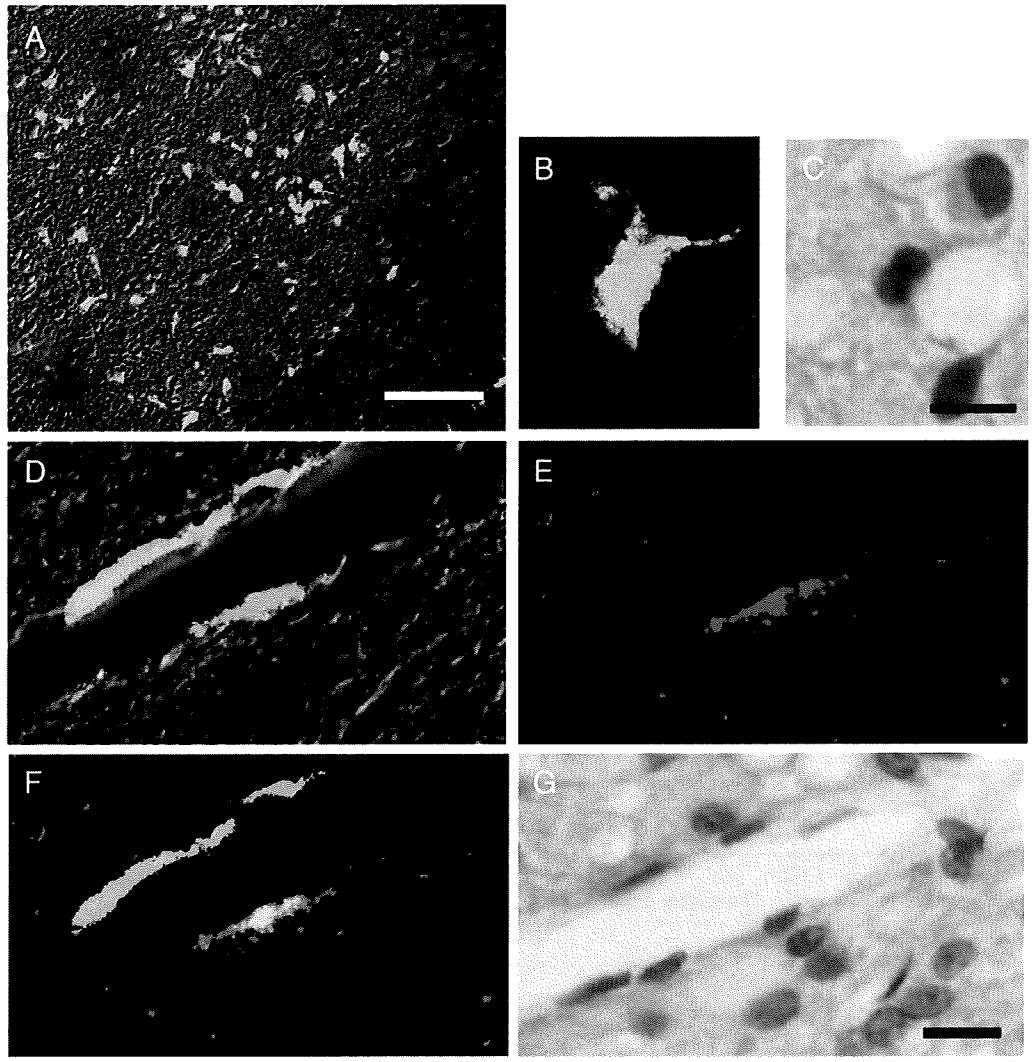

Fig. 2 – eGFP$^+$ cells infiltrated the anterior horn of the spinal cord in the recipient mice transplanted with BMCs of eGFP Tg mice by IBM-BMT. Symptomatic female mSOD1 Tg mice (12-wk-old) were irradiated with 6 Gy×2 (4h interval) 1 day before IBM-BMT. The recipient mice were transplanted with BMCs of 12-wk-old eGFP Tg mice by IBM-BMT. When the recipient mice could no longer autofeed, they were sacrificed, and specimens were prepared as described in the "Experimental procedures", followed by observation using a confocal microscope. A. shows eGFP$^+$ cells expressing green fluorescence in the anterior horn of the spinal cord in a low-power field, while B. shows a photograph of it in a high-power field. C. shows the same cell as shown in B. stained with H&E. D, E and F show an eGFP$^+$ cell in the anterior horn of the spinal cord expressing Iba-1. D. shows eGFP$^+$ cells (green), E. shows Iba-1$^+$ cells (red), while F. shows a merged figure of eGFP and Iba-1. The single cell expresses both eGFP and Iba-1 (yellow). G. shows the same cell stained with H&E as the cell shown in D,E and F. White scale bar: 100 μm. Black scale bar: 10 μm.

differences between them. However, these numbers were significantly more than that of Wt (13.5±3.7).

2.4. *Effects of chimerism of donor hematopoietic cells on number of infiltrating donor cells in spinal cord, and on survival*

Corti et al. reported that BMT from wild-type to asymptomatic mSDO1 Tg mice ameliorated the symptoms and prolonged the survival of the Tg mice (Corti et al., 2004). Our results are thus consistent with their results. However, Corti et al. prepared mixed chimera by combining 8 Gy-irradiation with the injection of donor BMCs into the recipient's peritoneal cavity. Therefore, we examined the effects of chimerism of hematopoietic cells from eGFP Tg mice on the status of mSOD1 Tg mice. We used an irradiation dose of 5.5 Gy×2 or 6.0 Gy×2 and the recipient mSOD1 Tg mice were transplanted with BMCs from 12-wk-old eGFP Tg mice using IBM-BMT. We then compared the results with those from mSOD1 Tg mice irradiated at 6.0 Gy×2 followed by IBM-BMT with BMCs from eGFP Tg mice. The mean

percentage of hematopoietic engraftment of the mSOD1 Tg mice irradiated with 5.5 Gy×2 was 87.5%, which was lower than the 95.2% in the mSOD1 Tg mice irradiated with 6.0 Gy×2.

On the other hand, the mean survival and survival interval in the 5.5 Gy×2 irradiated mice were 140 days and 52 days, being shorter than the 6 Gy×2 irradiated Group (145.7 days and 56.7 days). The mean number of migrated eGFP$^+$ cells in one recipient lumbar anterior horn of the spinal cord in one slice was 32.1 cells in 5.5 Gy×2 mSOD1 Tg mice and 58.8 cells in 6.0 Gy×2 mSOD1 Tg mice (Figs. 3A and B). We also examined the number of Iba-1$^+$/eGFP$^+$ cells in one recipient anterior horn of the spinal cord in the 5.5 Gy×2 irradiated mSOD1 Tg mice and 6.0 Gy×2 irradiated mSOD1 Tg mice. The mean number of Iba-1$^+$/eGFP$^+$ cells in the recipient anterior horn in one slice was 2.5 cells in the 5.5 Gy×2 irradiated mice and 4.1 cells in 6.0 Gy×2 irradiated mice.

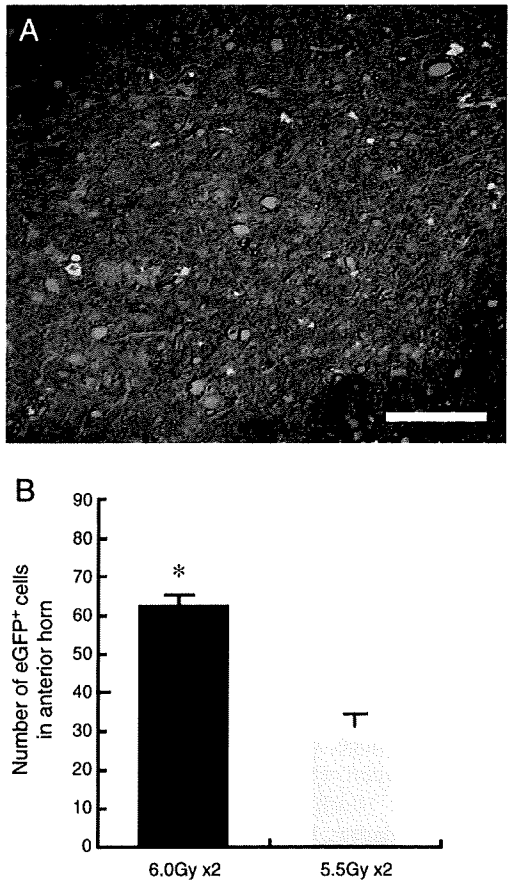

Fig. 3 – Reduced reconstitution rate of hematopoietic cells reduces the effect of BMT. Female 12-wk-old symptomatic mSOD1 Tg mice were irradiated with 5.5 Gy×2 one day before IBM-BMT. The mSOD1 Tg mice were transplanted with BMCs of eGFP Tg mice using IBM-BMT. When the mice could no longer autofeed, they were sacrificed and eGFP$^+$ cells in the spinal cord were histologically examined (A). eGFP$^+$ cells in the anterior horn in one slice were counted and compared with the 6 Gy×2 irradiated mSOD1 Tg mice. Representative data are shown in panel B. *: significant difference to 5.5 Gy×2 irradiated mSOD1 Tg mice.

3. Discussion

In this paper, we have examined the effects of allogeneic IBM-BMT on the symptomatic mSOD1 Tg mice. IBM-BMT slowed the disease progression and prolonged the survival in these mice. In the recipient mice, some microglia in the spinal cord expressed eGFP, which is a marker of donor cells, suggesting that the replacement with normal hematopoietic cells, including microglia, slowed the progression of the disease. As far as we can ascertain, this is the first report of the effects of BMT on symptomatic mSOD1 Tg mice.

Corti et al. transplanted BMCs from normal mice into mSOD1 Tg to induce mixed chimerism of hematopoietic cells, and retarded the onset of the disease and prolonged survival (Corti et al., 2004). In their experiment, they used 4-wk-old mice that still did not show any symptoms. However, it is very difficult to predict which human patients will develop ALS and it is also probably unethical to carry out BMT on asymptomatic patients even if they are carrying an abnormal gene. In this experiment, we used symptomatic 12-wk-old mSOD1 Tg mice, and induced significantly donor-predominant chimerism of the hematopoietic cells of normal mice, thereby slowing disease progression and prolonging survival.

It has been reported that IBM-BMT is superior to conventional BMT in the suppression of GVHD and tolerance induction (Kushida et al., 2001). Therefore, we carried out IBM-BMT, with the result that more than 90% of the peripheral blood nuclear cells were reconstituted into donor type without GVHD. For the pretreatment of recipient mice, we irradiated mSOD1Tg mice at "6.0 Gy×2" with a 4-hour interval, since we have found that fractionated irradiation provides better results than single irradiation (Cui et al., 2002; Kushida et al., 2001; Takeuchi et al., 1998), and that 4h is the optimal interval for fractionated irradiation in mice (Cui et al., 2002).

It has been reported that autoimmunity is related to the development of ALS (Appel et al., 1996). We have treated several model mouse strains for autoimmune diseases by BMT (Ikehara et al., 1994; Ikehara, 1998, 2002; Kushida et al., 2001). In our experiments, we showed that BMT had significant effects on the treatment of the autoimmune diseases in model mice: the autoimmune diseases disappeared and the transplanted mice survived as long as normal mice. However, in this study, we showed that the effects of BMT on mSOD1Tg mice were limited. Therefore, even if the development of ALS is associated with an autoimmune mechanism, it is unlikely that the autoimmune mechanism is the main factor in its development.

To investigate the efficacy of stem cell therapy for ALS, a variety of cell sources have been applied for mouse models for ALS, including human umbilical cord blood stem (HuCB) cells (Chen and Ende, 2000; Ende et al., 2000; Garbuzova-Davis et al., 2003; Habisch et al., 2007), human neural stem cells (Klein et al., 2005; Xu et al., 2006), rodent BM stem cells (Corti et al., 2004; Solomon et al., 2006), human BM stem cells (Hermann et al., 2004), and human neuron-like teratoma cells (hNT cells) (Garbuzova-Davis et al., 2001, 2002, 2006; Willing et al., 2001). These cells have been administered to ALS model mice intravenously (Ende et al., 2000; Garbuzova-Davis et al., 2001; Hermann et al., 2004; Solomon et al., 2006;), intraperitoneally (Corti et al., 2004), lumbar subcutaneously (Corti et al., 2007;

Garbuzova-Davis et al., 2001, 2002, 2006), or intrathecally (Habisch et al., 2007). There are also some reports indicating that neural stem cells improved neurological symptoms in rat models of the same phenotype (Klein et al., 2005; Xu et al., 2006).

Recently, the effects of stem cell therapy on mSOD1 Tg mice have been reported to possibly be due not to neurogenesis but neuroprotection as a result of the secretion of neurotrophic factor(s) derived from donor-derived cells (Beers et al., 2006; Corti et al., 2004; Kang and Rivest, 2007). The mutant SOD1 expressed in neurons alone or astrocytes alone failed to induce a significant motor neuron loss in G37R mutant SOD1 Tg or G86R mutant SOD1 Tg mice (Lino et al., 2002; Pamatarova et al., 2001; Gong et al., 2000). Clement et al. also reported that nonneuronal cells not expressing mutant SOD1 delay degeneration and significantly extend the survival of motor neurons expressing mutant SOD1, suggesting that an improvement in the environment of neural cells is crucial for the treatment of ALS (Clement et al., 2003). In our study, we found no donor-derived neural cells but we did find donor-derived microglia in the recipient spinal cord. Our results are consistent with the results of Corti et al. (2004) Beers et al. (2006) and Boillée et al. (2006). They show that the delayed onset, delayed progression of the symptoms and prolonged survival of mSOD1 Tg mice and $PU^{-/-}$ mSOD1 Tg mice were induced by BMT from normal mice to the mSOD1 Tg mice and $PU^{-/-}$ mSOD1 Tg mice in the preclinical stage.

It has been reported that the numbers of motor neurons and axons decrease after onset in the mSOD1 Tg mice (Corti et al., 2004), and that the number of microglia increases (Gowing et al., 2008). It has also been reported that BMT from normal mice to mSOD1 Tg mice retards the decrease in the numbers of motor neurons and axons. Therefore, we calculated the numbers of motor neurons, axons and microglia. The numbers of motor neurons and axons decreased in comparison with Wt mice, and the number of microglia increased. However, there were no significant differences in the numbers of motor neurons, axons and microglia between the mSOD1-BM-transplanted group and the eGFP-BM-transplanted group. In our experiment, the mice were sacrificed at the end point of the disease, when the mice could no longer feed by themselves. Namely, we sacrificed the mice at different ages. Corti et al. sacrificed the mice at the same age, 100 days after birth, and showed significant differences in the numbers of the motor neurons and axons between eGFP-BM-transplanted mice and mSOD1-BM-transplanted mice. Therefore, the differences between their results and ours could be attributable to the timing of sacrifice. It has been reported that BMT from mSOD1 Tg mice into $PU^{-/-}$ mSOD1Tg mice failed to ameliorate the symptoms of ALS-like disease but that BMT from eGFP Tg mice into $PU^{-/-}$ mSOD1Tg mice had some effects (Beers et al., 2006). They found donor-derived $eGFP^+$ microglia in the recipients' spinal cord. We also found donor-derived $eGFP^+$ microglia in the recipients' spinal cord. However, the number of microglia was too small (4.1 cells per lumbar anterior horn) in comparison with $eGFP^+$ cells (58.8 cells). Recently, it has been reported that T cells are also important in retarding the disease progression (Beers et al., 2008). Therefore, not only microglia but also other types of hematopoietic cells are associated with the retardation of the disease progression. Somehow, these results suggest that the infiltration of normal hematopoietic cells, including microglia (not carrying mutant SOD1), into the recipients' spinal cord is crucial for the retardation of the ALS-like disease. Further examinations are warranted with other types of mSOD1 Tg mice and with appropriate model animals to clarify the mechanisms underlying the effects of BMT on the amelioration of ALS.

As we previously described, the greater the predominancy of the donor hematopoietic cells induced, the more stable was the tolerance to donor antigens (Hayashi et al., 1997). Therefore, we examined the effects of the reduction of the irradiation dose from 6.0 Gy×2 to 5.5 Gy×2, on the status of ALS-like disease. The reduction in the irradiation induced a reduction in the numbers of donor-derived $eGFP^+$ cells and $Iba\text{-}1^+/eGFP^+$ cells infiltrating the spinal cord and a shortening of survival. These results suggest that the replacement of recipient hematopoietic cells carrying mutant SOD1 with donor hematopoietic cells carrying the normal genotype is necessary for an improvement in the disease.

In the present study, we have shown that IBM-BMT can also slow the progression of the symptoms of mSOD1 Tg mice and prolong survival, even after onset, and that the migration of donor hematopoietic cells, including microglia, into the recipient spinal cord is the most plausible explanation for the improvement.

4. Conclusions

We have shown the effects of allogeneic IBM-BMT on mice expressing the mutant SOD1, the ALS model mice. The effects are possibly due to an improvement in the environment of the spinal cord resulting from the replacement of hematopoietic cells, including microglia carrying the mutant gene with normal hematopoietic cells. However, the effect was not as pronounced as with our previous BMT therapy using autoimmune model mice. Therefore, we believe there may be merit in attempting other approaches, including a combination of new medicine(s) combined with IBM-BMT.

5. Experimental procedures

5.1. *Animals*

Male mSOD1 Tg mice, which are heterozygous for the ALS-linked G93A mutation of the human gene for SOD1 (TgN [B6SJL-Tg (SOD1-G93A) 1Gur]), were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Wild-type female B6SJLF1/J mice were bred and paired with the heterozygous mSOD1 Tg male mice. The mSOD1 Tg mice were identified using an ASTEC research thermal cycler for polymerase chain reaction amplification of mouse DNA extracted from tail snips (Rosen et al., 1993). The detailed protocol was described previously (Wate et al., 2005).

Heterozygous female mSOD1 Tg mice were used as recipients, since there are some differences in disease progression between genders in the mice (Heiman-Patterson et al., 2005).

As donors of therapeutic BM stem cells, eGFP Tg mice (C57BL/6 background) were kindly donated by Dr. Okabe (Okabe et al., 1997). Procedures involving animals and their care were conducted in conformity with our institutional

guidelines, which are in compliance with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

5.2. *Intra-bone marrow-bone marrow transplantation (IBM-BMT)*

We carried out IBM-BMT on 12-wk-old mSOD1 Tg mice at an early stage of the disease. We determined the onset of the symptom in each mouse by the manifestation of a leg tremor on 2 consecutive days. The symptomatic recipient mice were exposed to a total radiation dose of 5.5 Gy×2 or 6.0 Gy×2 (1.0 Gy/min with a 4 h interval) from a ^{137}Cs source (Gamma cell 40 Exactor; MDS Nordion International Inc., Ottawa, Ontario, Canada). The day after the total body irradiation, bone marrow cells (BMCs) from the eGFP Tg mice (male, 12-wk-old) or symptomatic mSOD1 Tg mice (male, 12-wk-old) were transplanted into the recipient female mice using the IBM-BMT technique.

Donor BMCs were transplanted into the bone marrow cavity of both tibias of the recipients (3.0×10^7 cells in each bone, in total 6.0×10^7 cells per mouse) using the IBM-BMT method, as previously described (Kushida et al., 2001). The mice were observed until they could no longer feed by themselves.

5.3. *Flow cytometric analysis of hematopoietic engraftment*

For analysis of the percentages of hematopoietic engraftment, peripheral blood was obtained from the tail vein of the recipient mice 2 weeks after transplantation. Red blood cells were lysed with 0.15 M NH_3Cl and the samples were stained with a PE-conjugated anti-CD45 antibody (BD Bioscience Pharmingen, San Jose, CA). The percentage of donor cells in the peripheral blood in each transplanted mouse was calculated as (percentage of both eGFP$^+$ and CD45$^+$ cells)×100 / (percentage of CD45$^+$ cells). Flow cytometric analysis was performed using a FACScan (Becton Dickinson; Mountain View, CA, USA).

5.4. *Histological examination*

When the recipient mice became unable to feed by themselves, they were anaesthetized using diethyl ether and transcardially perfused with 4% paraformaldehyde in 0.1 M PBS as a fixative. The spinal cord at the level of L3 to L4 was sampled from each mouse, embedded in paraffin, and sectioned sequentially throughout into sections of 7-micrometer thickness for histological study.

In order to avoid counting the same cell twice in consecutive sections, every 5th section from each mouse was deparaffinized, washed in PBS, incubated in 3% H_2O_2 in methanol for 15 min to inhibit endogenous peroxidase activity, washed in PBS, and blocked in PBS containing 3% normal bovine serum albumin (PBS-BSA) for 1 h at room temperature. The sections were then incubated with the rabbit polyclonal antibody against Iba-1 (Wako Pure Chemical Industries, Osaka, Japan; diluted 1:100 with PBS-BSA, with the rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP: Millipore Corporation, Billerica, MA, USA; 1:200), with the mouse monoclonal antibody against oligodendrocyte marker O4 (Millipore Corporation, 1:150), or with the mouse monoclonal antibody against neuronal nuclei (NeuN: Millipore Corporation; 1:200) overnight at 4 °C. After washing in PBS, the sections were incubated with the secondary antibody (Alexa Fluor 647 goat anti-rabbit or anti-mouse IgG (H+L) high cross-adsorbed: Invitrogen Corporation, Carlsbad, CA, USA; 1:500) for 1 h at room temperature. Alternatively, the samples were stained with PE-labeled anti-CD45 antibody. More than 10 samples were prepared for each Ab.

Using a laser scanning confocal microscope (Olympus FluoView™ FV300 Version 4.3, Olympus Corporation, Tokyo, Japan), neuropathologists unaware of the identity of sections counted the numbers of eGFP$^+$, eGFP$^+$/Iba-1$^+$, eGFP$^+$/GFAP$^+$, eGFP$^+$/O4$^+$, or eGFP$^+$/NeuN$^+$ cells within the anterior horn region.

After examination using a confocal microscope, some sections were rinsed and then stained with hematoxylin and eosin (H&E) to identify the morphological features of the eGFP$^+$ cells.

The staining specificity was assessed by replacing the primary antibody with PBS-BSA. No deposits of reaction products were seen in the sections thus treated.

5.5. *Evaluation of the effects of IBM-BMT on grip strength and body weight*

Each night, examiners who were blind to the information weighed each mouse, and measured the grip strength of their forelimbs using a Digital Grip Strength Meter (Columbus Instruments, OH, USA). Each measurement was repeated 4 times and the maximum value was adopted.

When each mouse had lost the ability to autofeed or to right itself within 30 s. after having been placed on its side, it was sacrificed under deep anesthesia. This provided us with the "survival day" and "survival interval" for each mouse. The "survival interval" was defined as the time period from onset of the disease to the day when the mouse was sacrificed.

5.6. *Statistical analyses*

All of the data were expressed as the mean±standard error of the mean (SEM). Statistical analyses were performed using JMP statistical discovery software version 7 (SAS Institute, Cary, NC, USA). The data concerning the numbers of the GFP$^+$ cells in the anterior horn of the mSOD1 Tg mice irradiated with 6 Gy×2 and 5.5 Gy×2 were analyzed by the Wilcoxon rank sum.

The data of survival interval, days for reduction of the grip strength by more than 50% of that at onset, and days for decrease in body weight by more than 10% of that at onset were analyzed by the Kaplan–Meyer method with Wilcoxon rank sum test.

Differences between groups were analyzed using Wilcoxon rank sum test and $p<0.05$ was considered to be significant.

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The Future of Stem Cell Transplantation in Autoimmune Disease

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Abstract We have previously found that conventional allogeneic bone marrow transplantation (allo BMT) can be used to treat various spontaneously developed autoimmune diseases in mice. In addition, we have found that autoimmune diseases can be transferred into the normal mice by conventional BMT from autoimmune-prone mice. Based on these findings, we have proposed that autoimmune diseases are "stem cell disorders." To apply allo BMT to humans, we extensively carried out BMT to clarify which cells are essential for successful BMT, and finally found that three types of cells are essential for successful allogeneic BMT: (1) hemopoietic stem cells (HSCs), (2) natural suppressor cells, and (3) stromal cells (including mesenchymal stem cells, MSCs). We have very recently found that MSCs play a crucial role in preventing graft failure, since there is a major histocompatibility complex restriction between HSCs and MSCs. To recruit donor-derived MSCs, we have found that the injection of whole bone marrow cells into the bone marrow cavity (intra-bone marrow-BMT, IBM-BMT) is the best strategy for the treatment of various otherwise intractable diseases, including autoimmune diseases. In this review article, we provide evidence that IBM-BMT heralds a revolution in the field of transplantation and regeneration medicine.

Keywords Bone marrow transplantation (BMT) · Autoimmune disease · Intra-bone marrow (IBM)-BMT · Hemopoietic stem cell (HSC) · Mesenchymal stem cell (MSC)

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Humana Press

Introduction

In 1985, we found that allogeneic (but not syngeneic or autologous) bone marrow transplantation (BMT) could be used to treat autoimmune diseases in autoimmune-prone mice [1, 2]. Since then, we have confirmed our findings using a variety of autoimmune-prone mice [3–5].

Conversely, we have succeeded in inducing autoimmune diseases in normal mice by transplanting T-cell-depleted bone marrow cells (BMCs) or partially purified hemopoietic stem cells (HSCs) from autoimmune-prone mice to normal mice [6, 7]. Based on these findings, we proposed that autoimmune diseases were "stem cell disorders (SCDs)" [6–8].

Our findings have also been confirmed in humans: patients with autoimmune diseases were cured after allogeneic BMT, while autoimmune diseases were found to be transferred to recipients of BMT from donors who were suffering from autoimmune diseases [9].

In this article, we show that various otherwise intractable diseases (including autoimmune diseases) can be cured by our novel BMT method.

Strategies for recruitment of donor stromal cells

Using radiosensitive and chimeric-resistant MRL/lpr mice, we have found that the recruitment of donor stromal cells is essential for successful allogeneic BMT. We found that three methods are effective in replacing recipient stromal cells with donor-derived stromal cells: (1) conventional intravenous BMT (IV-BMT) plus bone grafts [10, 11]; (2) BMT from the portal vein (PV-BMT) [12], since tolerance can be easily induced when the antigen is portal venously (PV) injected into the liver; and finally (3) intra-bone

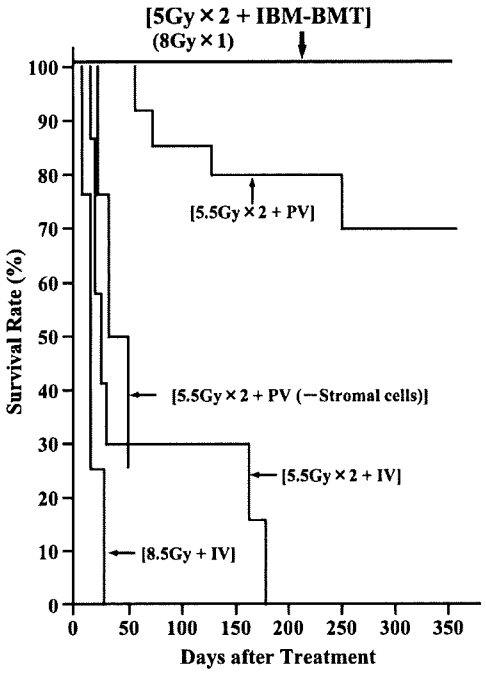

Fig. 1 Treatment of autoimmune diseases in MRL/lpr mice by IBM-BMT (5 Gy×2). IBM-BMT can be used to treat autoimmune diseases in MRL/lpr mice even when the radiation dose is reduced to 5 Gy×2

marrow (IBM)-BMT [13]. IBM-BMT was ultimately found to be best, since it allows us to use a mild conditioning regimen (5 Gy×2), as shown in Fig. 1. We therefore used IBM-BMT instead of the conventional IV-BMT in subsequent experiments.

IBM-BMT for organ transplantation

Since we have previously found that the combination of organ allografts and conventional IV-BMT from the same donors prevents the rejection of organ allografts [14], we attempted to apply IBM-BMT to organ allografts. IBM-BMT was the most effective strategy, since the radiation dose could be reduced to 4.0 Gy×2 in skin allografts [15]. In addition, we found that IBM-BMT is applicable to allografts of other organs and tissues in rats, such as pancreas islets [16], legs [17], lungs [18], and heart [19].

IBM-BMT for regeneration therapy

As it was apparent that donor stromal cells could be effectively recruited by "IBM-BMT", we next attempted to treat osteoporosis in SAMP6 mice; the SAMP6 mouse (a substrain of senescence-accelerated mice) spontaneously develops osteoporosis early in life and is therefore a useful model for examining the mechanisms underlying osteoporosis. After IBM-BMT, the hematolymphoid system was completely reconstituted with donor-type cells. Thus-treated SAMP6 mice (8 months after IBM-BMT) showed marked increases in trabecular bone even at 20 months of age (Fig. 2) and the bone mineral density remained similar to that of normal B6 mice. Bone marrow stromal cells in "IBM-BMT"-treated SAMP6 mice were replaced by donor stromal cells [20, 21]. Thus, we succeeded in curing osteoporosis in SAMP6 mice by IBM-BMT, which can recruit both donor- derived HSCs and MSCs.

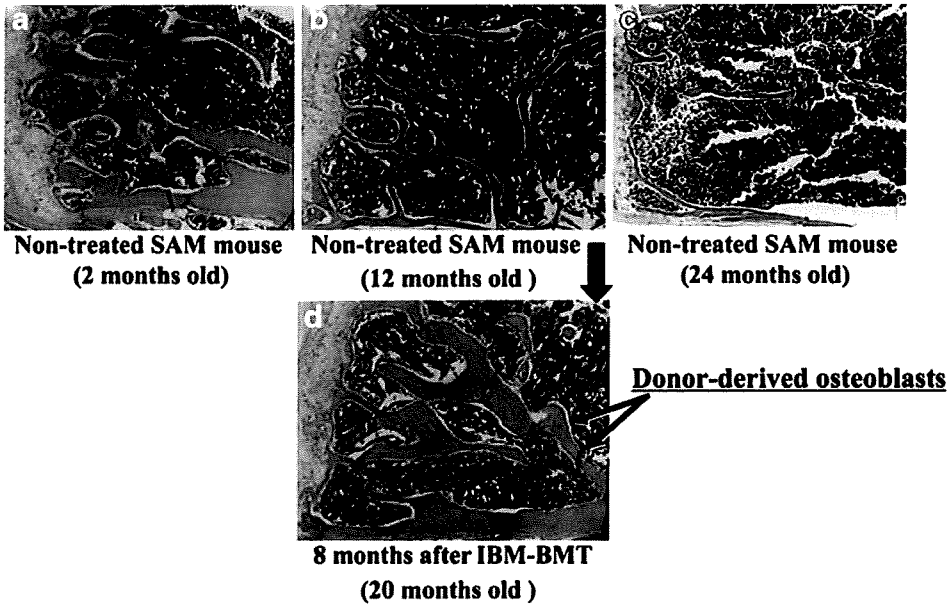

Fig. 2 Treatment of osteoporosis in SAMP6 mice by IBM-BMT from normal B6 mice

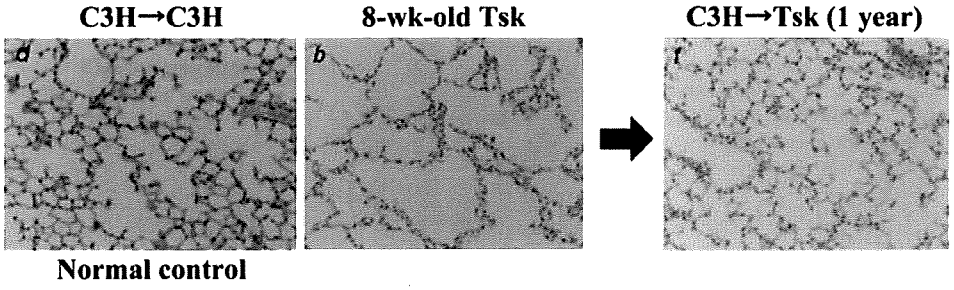

Fig. 3 Amelioration of emphysema in [C3H→Tsk] mice by IBM-BMT

Since IBM-BMT appeared to be a powerful strategy in regeneration therapy, we next used tight-skin (Tsk) mice (an animal model for emphysema) to examine whether emphysema could be cured by IBM-BMT.

IBM-BMT was carried out from C3H mice into Tsk mice (8–10 weeks old) that had already shown emphysema. Eight months after the transplantation, the lungs of all the Tsk mice treated with IBM-BMT [C3H→Tsk] showed structures similar to those of normal mice, whereas the [Tsk→Tsk] mice showed emphysema, as seen in age-matched Tsk mice (Fig. 3). Next, we attempted to transfer emphysema from Tsk mice to C3H mice by IBM-BMT. Six months after IBM-BMT, the [Tsk→C3H] mice showed emphysema [22]. These results strongly suggested that emphysema in Tsk mice originates from defects in the stem cells (probably MSCs and/or HSCs) in the bone marrow [22].

IBM-BMT + donor lymphocyte infusion for treatment of malignant tumors

It is well known that the graft-versus-leukemia reaction can cure patients of a variety of hematological malignancies [23, 24]. Recently, it has been reported that graft-versus-tumor (GvT) effects can induce partial (complete in some) remission of metastatic solid tumors such as breast cancer [14, 25, 26] and renal cell carcinoma [27–33]. Based on these findings, donor lymphocyte infusion (DLI) has recently been used for the treatment of malignant solid tumors even in humans. However, it is very difficult to completely eradicate the tumors, since extensive DLI induces graft-versus-host disease (GvHD). We therefore attempted to establish a new method for the treatment of malignant tumors, this method consisting of intra-bone marrow-IBM-BMT plus DLI, since we have recently found that IBM-BMT can allow a reduction in radiation doses as a conditioning regimen and prevent GvHD in mice [13, 34]. Using the Meth-A cell line (BALB/c-derived fibrosarcoma), we found that IBM-BMT plus the injection of $CD4^+$ T-cell-depleted (but not $CD8^+$ T cell depleted) spleen cells (as DLI) can prevent GvHD while suppressing tumor growth (Fig. 4). In addition, we have found that IBM-BMT plus extensive DLI (three times every 2 weeks) leads to the complete rejection of the tumor, although the success rate (three out of 50) is not high so far [34].

In addition, we have examined whether this strategy (IBM-BMT plus DLI) is applicable to other tumors in other animals. We have obtained similar results in another system (colon cancer, ACL-15 in rats) [35]. We are now establishing more efficient strategies to eradicate malignant tumors.

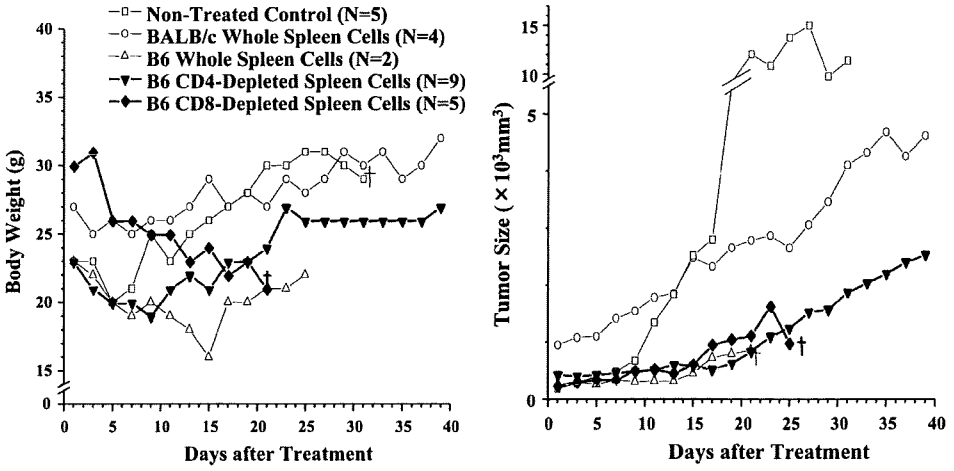

Fig. 4 Prevention of GvHD and suppression of tumor growth by IBM-BMT + DLI ($CD4^-$)

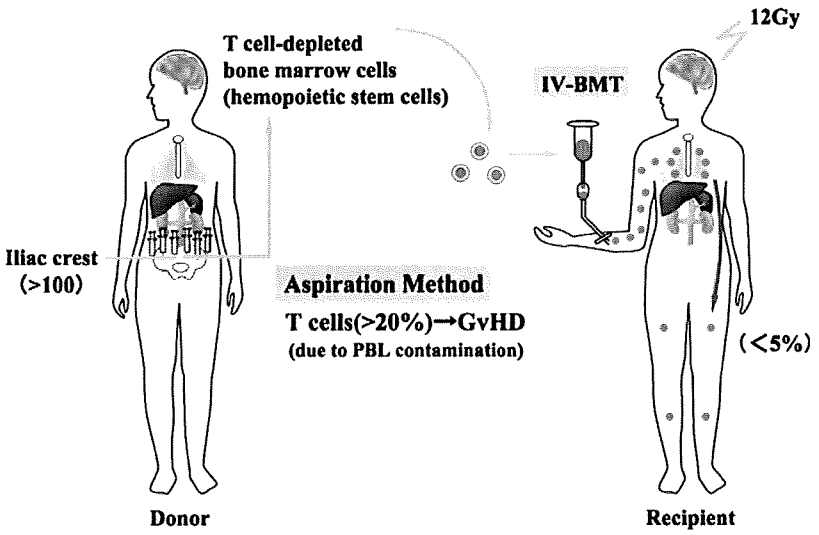

Fig. 5 Conventional BMT method for allogeneic BMT. Conventional BMT is carried out using an aspiration method (AM), followed by the intravenous injection of BMCs (IV-BMT)

Conventional BMT versus novel BMT

Conventional BMT is carried out as follows: bone marrow needles are inserted into the iliac bones more than 100 times, and the BMCs are collected by the aspiration method (AM; Fig. 5). Therefore, contamination with peripheral blood (particularly T cells) is inevitable. When thus-collected cells are intravenously injected, most cells become trapped in the lung and only a few cells migrate into the bone marrow (Fig. 5).

To apply our new BMT methods to humans, we established, using cynomolgus monkeys, a "perfusion method (PM)", which minimizes the contamination of BMCs with T cells. As shown in Fig. 6, two needles are inserted into a long bone such as the humerus, femur, or tibia. The end of the extension tube is connected to a needle. The other end is placed in a syringe containing 0.5 ml heparin. The second needle is connected to a syringe containing 30 ml of saline, and the saline is then pushed gently from the syringe into the medullary cavity to flush out the bone marrow. The saline containing the BM fluid is then collected (Fig. 6).

There is significantly less contamination with T cells when using the PM (<10%) than with the conventional AM (>20%). Therefore, T cell depletion is unnecessary with the PM, and whole BMCs can be used. However, in the case of the conventional AM, T cell depletion is necessary, and the loss of some important cells such as MSCs during the

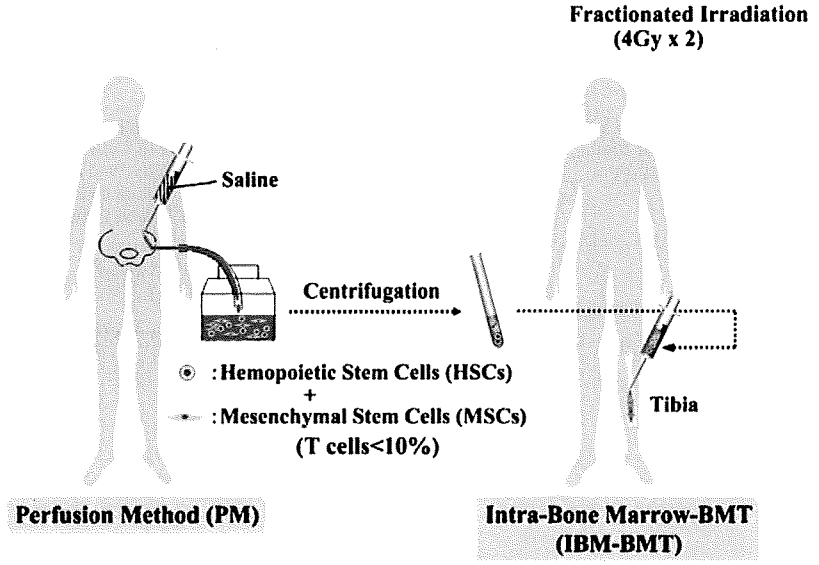

Fig. 6 New BMT method for allogeneic BMT. New BMT method is carried out using a perfusion method (*PM*), followed by IBM-BMT

process of T cell depletion is inevitable. Furthermore, the number and progenitor activities of the cells harvested using the PM are greater than when using the conventional AM [36, 37].

Future directions

We have also found that the PM is applicable to the iliac bones as well as the long bones not only in monkeys but also in humans.

As described here, the new BMT method (PM + IBM-BMT) can be used to treat various otherwise intractable diseases, including (1) autoimmune diseases, (2) age-associated diseases (osteoporosis, emphysema, etc.), (3) diseases curable by organ transplantation, and (4) malignant tumors (including solid tumors). The PM can efficiently be used to collect whole BMCs (including HSCs and MSCs) without them being contaminated with T cells, and no GvHD therefore develops. IBM-BMT can efficiently transfer donor whole BMCs (both HSCs and MSCs) into recipients and this method can therefore be used to quickly replace not only HSCs but also MSCs with donor-derived cells.

From the findings to date, it is conceivable that all the body's cells originate in the bone marrow, and that all diseases might therefore originate from defects in the bone marrow. One paper already suggests that gastric cancer originates from bone-marrow-derived cells [38].

We believe that the development of our BMT method heralds a revolution in the field of transplantation (BMT and organ transplantation) and regeneration therapy.

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LETTERS

edited by Jennifer Sills

China's Environmental Civilian Activism

IN THE POLICY FORUM "CHINA'S ROAD TO SUSTAINABILITY" (2 APRIL, P. 50), J. LIU OVERLOOKS AN important cultural force. China's worsening environmental conditions have catalyzed a spirit of environmental civilian activism.

For example, in 2003, a consortium proposed erecting 13 dams on the Nujiang River. China's environmental nongovernmental organizations and scholars launched a protest campaign through the Internet and newspapers. The critics argued that as reservoirs behind the dams filled up, flooding and landslides would imperil habitats. In response, Premier Wen Jiabao suspended the dam project pending an environmental review in 2004 (*1*).

The landmark of environmental civilian activism occurred in Xiamen City in 2007. The local government supported construction of a $1.4-billion paraxylene plant near the center of the city. Information about the environmental impact of this project was not made available to the local residents. The people of Xiamen City were outraged when—through cell phone messages and the Internet—they learned of the plant's environmental risks. A phone text message was circulated among Xiamen citizens in late May calling for a "collective walk" (demonstration). On 1 June 2007, more than 1000 citizens gathered in front of the municipal building to protest. The demonstration forced the local government to cancel the largest industrial project in the history of Xiamen (*2*).

Protests. Chinese citizens protest against the planned Guangzhou trash incinerator in 2009. Their banners read, "Oppose the trash incinerator."

The burgeoning middle class has become the driver of environmental civilian activism. For example, operation of the Likeng trash incinerator in Guangzhou City started in 2005 without any protest, although local farmers worried about health risk (*3*). In contrast, the proposed Panyu trash incinerator in Guangzhou City in 2009 triggered protests that were led by the middle class (*4*), who used science-based evidence to openly challenge prevailing notions formulated by the authorities. (In earlier years, standard practice was to obey Beijing-based experts in environmental protection.) In addition, the self-organized middle class forced the local government to open discussion by Internet. By seizing the opportunity for an open discussion, the newly empowered locals took to the streets to protect their environmental rights (*4*).

Recent years have witnessed an impressive growth in environmental protests in China. The number of petitions and mass public protests related to environmental issues has increased by 30% per year in the past few years, although the number of petitions lodged with the Chinese government has dropped (*5*).

The current environmental civilian activism movements have several common characteristics: (i) They are confined to one specific geographical space. (ii) Their goal is protecting the environment, rather than political rights or commercial interests. (iii) They focus on a specific pollutant, rather than general environmental degradation. The local nature of the movement enables the organization of a large number of citizens with little effort in a very short time. Given more open social and political conditions and the increasing size of the middle class in China, environmental civilian activism will certainly be a key driver in China's transition to sustainability.

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6. The views expressed in this paper are the author's own and not necessarily those of QIBEBT-CAS, GIG-CAS.

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Effects of China's Economic Growth

IN A RECENT POLICY FORUM, J. LIU REVIEWED "China's road to sustainability" (2 April, p. 50). Liu focused on population growth and an increase in the number of households, but he failed to adequately address the most important socioeconomic driver behind environmental degradation in China: rapid economic growth that is not offset by efficiency improvements (*1*, *2*).

In China, exports and capital investments contribute significantly more to gross domestic product (GDP) than household and government consumption combined (*3*), and this also holds true for emissions (*1*, *2*). From 2002 to 2005, the production of exports was responsible for 50% of the growth in carbon dioxide emissions and capital formation was responsible for 35%;

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Global warming and lizards

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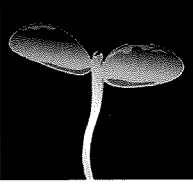

Mobile small RNA

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household and government consumption contributed 15% (*4*, *5*).

Liu discusses population control and household size, but a more dominant issue in terms of population dynamics is the migration from rural to urban areas (*6*). From 1990 to 2007, the urban population increased by 292 million, whereas the rural population decreased by 116 million (*3*). Urban dwellers, even if migrants from rural areas, have a higher income (*3*) and hence higher energy use and environmental impacts (*2*, *6*).

A key challenge for China is to continue strong economic growth while minimizing environmental impacts. Reductions in emissions per unit of GDP are unlikely to reduce total emissions if economic growth continues (*1*). China will need to combine aggressive domestic policies with international support to reverse the current growth in coal-dominated energy use and emissions.

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TECHNICAL COMMENT ABSTRACTS

COMMENT ON "Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome"

Cathie Sudlow, Malcolm Macleod, Rustam Al-Shahi Salman, Jon Stone

Lombardi *et al.* (Reports, 23 October 2009, p. 585) reported an association between the human gammaretrovirus XMRV and chronic fatigue syndrome. However, their results may be misleading because of various potential sources of bias and confounding. If real, the association may lack generalizability because of the specific characteristics of the cases studied and could be due to reverse causality.

Full text at www.sciencemag.org/cgi/content/full/328/5980/825-a

COMMENT ON "Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome"

Andrew Lloyd, Peter White, Simon Wessely, Michael Sharpe, Dedra Buchwald

Lombardi *et al.* (Reports, 23 October 2009, p. 585) reported a significant association between the human retrovirus XMRV and chronic fatigue syndrome (CFS). However, the cases with CFS and the control subjects in their study are poorly described and unlikely to be representative. Independent replication is a critical first step before accepting the validity of this finding.

Full text at www.sciencemag.org/cgi/content/full/328/5980/825-b

COMMENT ON "Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome"

Jos W. M. van der Meer, Mihai G. Netea, Jochem M. D. Galama, Frank J. M. van Kuppeveld

Lombardi *et al.* (Reports, 23 October 2009, p. 585) reported detection of the human gammaretrovirus XMRV in the blood cells of patients with chronic fatigue syndrome (CFS). However, the patient description provided was incomplete. The inclusion of patients from a "CFS outbreak" previously linked with a viral infection, without confirmation in sporadic CFS cases, casts doubt on the role of XMRV in the pathogenesis of CFS.

Full text at www.sciencemag.org/cgi/content/full/328/5980/825-c

RESPONSE TO COMMENTS ON "Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome"

Judy A. Mikovits and Francis W. Ruscetti

We reported the detection of the human gammaretrovirus XMRV in 67% of 101 patients with chronic fatigue syndrome (CFS) and in 3.7% of 218 healthy controls, but we did not claim that XMRV causes CFS. Here, we explain why the criticisms of Sudlow *et al.*, Lloyd *et al.*, and van der Meer *et al.* regarding the selection of patients and controls in our study are unwarranted.

Full text at www.sciencemag.org/cgi/content/full/328/5980/825-d

Risks of Immune System Treatments

WE WISH TO ADD SEVERAL POINTS TO THE News Focus story "Replacing an immune system gone haywire" (J. Couzin-Frankel, 12 February, p. 772).

First, a great deal of research had already been done before the 1996 Basel meeting mentioned in the story. Stem cell transplants had been studied in animal models of autoimmune disease (*1*–*5*). Patient stem cell transplant protocols had been written, and a few human patients had already been treated specifically for autoimmune disease (*6*–*10*).

Second, we would like to stress the varying levels of risk in the treatment strategies described in the story. The immune system originates from hematopoietic stem cells (HSCs). Before receiving a transplant, patients with autoimmune diseases receive "conditioning" chemotherapy or radiation that destroys lymphocytes, inducing an immediate immune cease-fire. Subsequently, HSCs are infused to regenerate a new self-tolerant immune system. Sullivan and Nash advocate conditioning regimens with high doses of radiation. These extreme regimens cause irreversible bone marrow failure, thus requiring mandatory HSC reinfusion. The rationale for this high-dose strategy is that maximal ablation of the immune system will translate into longer and more durable disease remission. In contrast, we advocate less extreme regimens of chemotherapy, which can halt inflammation without altering the bone marrow's ability to recover. The News Focus article also comments on the risk of infertility when patients are pretreated with chemotherapy. We emphasize that the risk of infertility is higher for the more extreme regimens.

Finally, although the News Focus story comments on problems obtaining insurance approval in the United States, medical funding is a worldwide issue, including in countries with government-funded health services. In addition to patient safety benefits, less toxic regimens also cost any health care system less money, because patients are less likely to suffer complications such as secondary cancers.

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CORRECTIONS AND CLARIFICATIONS

Letters: "Climate change and the integrity of science" by P. H. Gleick *et al.* (7 May, p. 689). Due to an editorial error, the original image was not a photograph but a collage. It was a mistake to have used it. The image (link available at www.sciencemag.org/cgi/content/full/328/5979/689/DC2) has been replaced in the HTML version and in the online PDF by an unaltered photograph from National Geographic (CREDIT: Paul Nicklen/National Geographic/Getty Images) of two polar bears on an ice floe.

News Focus: "Meeting briefs: The ins and outs of HIV" by J. Cohen (5 March, p. 1196). The earliest report of HIV predominantly entering cells through endocytosis appeared in C. D. Pauza, T. M. Price, *J. Cell Biol.* **107**, 959 (1988).

Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the previous 3 months or issues of general interest. They can be submitted through the Web (www.submit2science.org) or by regular mail (1200 New York Ave., NW, Washington, DC 20005, USA). Letters are not acknowledged upon receipt, nor are authors generally consulted before publication. Whether published in full or in part, letters are subject to editing for clarity and space.