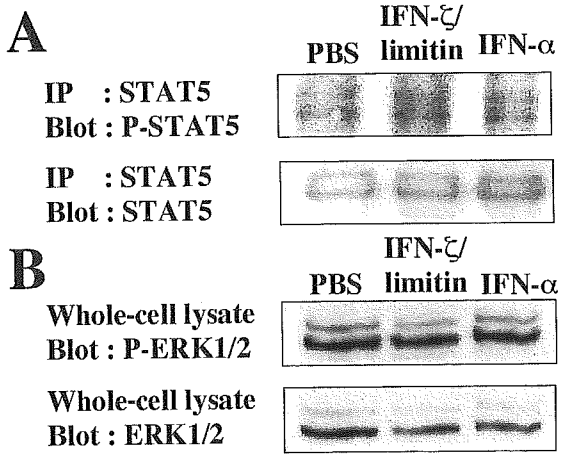

Figure 6. Effects of IFN-ζ/limitin and IFN-α on STAT5 and ERK1/2 in cultured megakaryocytes. Bone marrow mononuclear cells (1 X 10^6 cells/mL) were cultured in serum-free conditioned medium with 10 ng/mL TPO and 10 ng/mL IL-11 for 5 days. At 24 hours before the end of the culture, 1000 IU/mL IFN-ζ/limitin or IFN-α was added. Whole-cell lysates, which were obtained from the cultured megakaryocytes (1 x 10^7), were subjected to immunoprecipitation followed by Western blot for STAT5 (**A**) as well as Western blot for ERK1/2 (**B**). Similar results were obtained in two independent experiments.

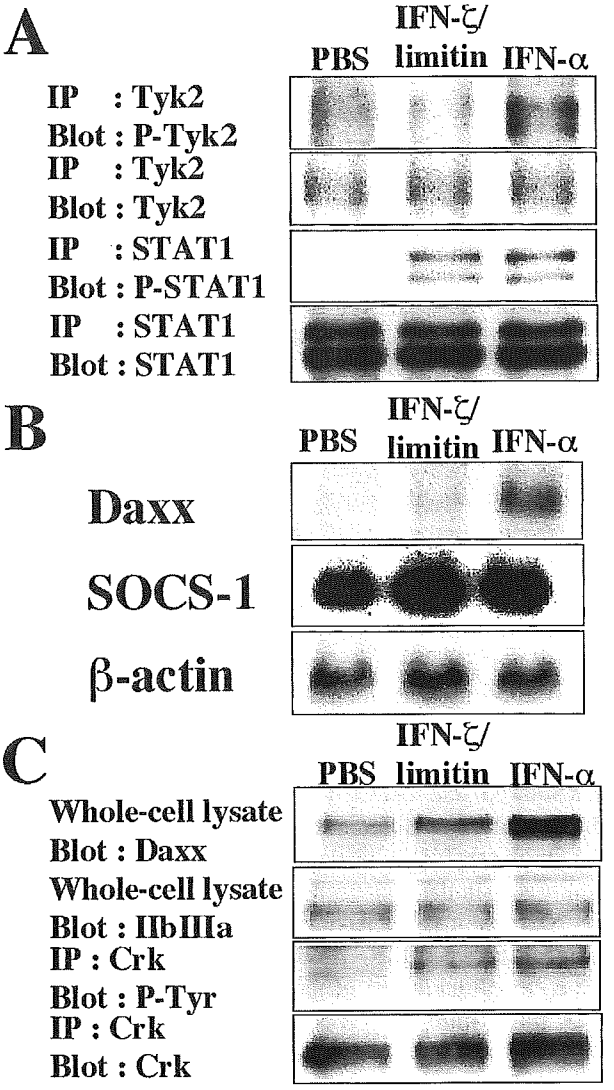

Figure 7. Comparison of signals between IFN-ζ/limitin and IFN-α in megakaryocytes. **(A):** Megakaryocytes in serum-free suspension cultures were starved for 8 hours, and then stimulated with 1000 IU/mL IFN-ζ/limitin for IFN-α for 5 minutes (Tyk2) or 30 minutes (STAT1). Whole cellular lysates, which were obtained from 10^7 cells, were immunoprecipitated and Western blotted with the indicated antibodies. Similar results were obtained in two independent experiments. **(B):** Megakaryocytes (1 X 10^7 cells) in serum-free suspension cultures were starved for 6 hours, and then stimulated with 250 IU/mL IFN-ζ/limitin or IFN-α. After the indicated period of stimuli (24 hours for Daxx and 8 hours for SOCS-1), total RNAs (15 μg/lane) were isolated and subjected to Northern blot analysis using the cDNAs of SOCS-1, Daxx, and β-actin as probes. Similar results were obtained in three independent experiments. **(C):** Megakaryocytes in serum-free suspension cultures were starved for 6 hours, and then stimulated with 1000 IU/mL IFN-ζ/limitin or IFN-α. After the stimuli (24 hours for Daxx and 15 minutes for Crk), whole cellular lysates, which were obtained from 10^7 cells, were immunoprecipitated and Western blotted with the indicated antibodies. Similar results were obtained in three independent experiments.

is also dependent on Tyk2, because these effects of IFN-α were not observed in Tyk2-deficient cells [20]. Our colony assays using knockout mice clearly revealed that both STAT1- and Tyk2-dependent signaling pathways are important for the IFN-α-induced growth inhibition of megakaryocyte progenitors. Among STAT1- dependent molecules, the expression of double-stranded RNA-dependent protein kinase, which inhibits translation initiation factor-2, and IL-1β-converting enzyme gene, which is a mammalian homologue of the caenorhabditis elegans cell death gene *ced3,* are IRF-1-dependent [32,33]. A tumor suppressor, p53, is also induced by IFN-α and IFN-β, depending on the binding of ISGF3 to ISRE in the promoter of the *p53* gene [34]. Recently, Wang et al. reported that SOCS-1, whose gene expression is induced by some STATs including STAT1, blunts TPO-induced signaling in IFN-α-treated megakaryocytes [11]. Among Tyk2-dependent molecules, Crk and Daxx are the most promising candidates. In myeloid and erythroid progenitors, the Crk antisense oligonucleotides canceled the IFN-α-induced growth inhibition [22]. The Daxx antisense oligonucleotides rescued the IFN-α-treated B lymphocytes from growth arrest and apoptosis in parallel with the reduction of Daxx [23]. We here clarified that both Crk and Daxx play a role in growth inhibitory effects of IFN-ζ/limitin and IFN-α on megakaryocyte progenitors. The antisense oligonucleotides against Crk and Daxx canceled the IFN-induced reduction of CFU-Meg colony numbers. Possible molecular mechanisms of growth suppression by Crk have been considered to be a result of the interaction between Crk and C3G, a guanine exchange factor for Rap-1 [35,36]. The activated Rap-1 then antagonizes the Ras pathway, which plays a role in cell proliferation [37]. Possible mechanisms of growth suppression by Daxx are complicated. Daxx

was first identified as a Fas-binding protein and has been known to enhance Fas-mediated apoptosis through Jun N-terminal kinase activation [38]. Daxx also acts as a corepressor of some transcription factors such as Ets-1, whose interaction causes the decrease of *bcl*-2 gene expression [39]. In addition, Daxx is a nuclear protein, which localizes to the sub-nuclear structures referred to as PML-oncogenic domains [40]. The interaction of Daxx with PML may regulate the PML–nuclear body formation, which controls the actylation of p53 and the Rb transcription [41]. Although precise mechanisms have not been clarified yet, our data indicate that Crk and Daxx, both of which are downstream molecules of Tyk2, participate in IFN-ζ/limitin- and IFN-α-induced growth inhibition of megakaryocyte progenitors besides the involvement of SOCS-1 as reported by Wang et al. [11].

The most important and strange issue of IFN-ζ/limitin is why it has the narrow range of biological activities as compared with IFN-α. There are some explanations for the different functions among type I IFNs. The β-R1 gene is induced by IFN-β, but not by IFN-α [42]. Association of a 95- to 100-kDa tyrosine-phosphorylated protein with the IFN-α/β receptor was found in an IFN-β- but not an IFN-α-treated myeloma cell line [43]. In the previous report, we also showed that IRF-1 dependency for the induction of antiviral state was different between IFN-ζ/limitin and IFN-α in fibroblasts [2], indicating that signals induced by IFN-ζ/limitin are similar but distinct as compared with those of IFN-α. Thus, it is interesting to investigate the differences of signals between IFN-ζ/limitin and IFN-α that are related to the growth inhibition of megakaryocytes. In megakaryocytes, IFN-ζ/limitin and IFN-α induced similar levels of the phosphorylation of STAT1 and the gene expression of SOCS-1. These facts indicated that signals for STAT1–SOCS-1 are similar between IFN-ζ/limitin and IFN-α. However, IFN-ζ/limitin could induce lower expression of Daxx and weaker phosphorylation of Tyk2 and Crk than IFN-α. Because Daxx induction and Crk phosphorylation are downstream events of Tyk2, weaker phosphorylation of Tyk2 by IFN-ζ/limitin than IFN-α is likely to lead to lower expression of Daxx and weaker phosphorylation of Crk, which participate in IFN-mediated inhibition of megakaryocytes. Thus, lower activation of Tyk2 by IFN-ζ/limitin may result in the restricted biological activities of IFN-ζ/limitin as compared with IFN-α.

The results reported here extend not only our understanding about thrombocytopenia in patients with IFN-α treatment but also the possibility for clinical application of human homologue of IFN-ζ/limitin or an engineered cytokine with useful features of the IFN-ζ/limitin structure. In addition, further analysis of molecular mechanisms of the functional differences will develop new strategies to reduce adverse effects of IFNs including a pegylated IFN and a consensus IFN.

Acknowledgments

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Cell Cycle Regulation in Hematopoietic Stem/progenitor Cells

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Abstract: Hematopoietic Stem Cells (HSCs) are characterized by two distinct abilities, that is, self-renewal ability and multipotency. To keep the homeostasis of hematopoiesis and protect the exhaustion of HSCs throughout the life, most of HSCs are kept quiescent and only a limited number of HSCs enter cell cycle to supply mature blood cells. Cell cycle state of HSCs is crucially regulated by external factors such as cytokines, Notch ligands and Wnt signals in the Bone Marrow (BM) microenvironment, so called hematopoietic niche. In addition, the intrinsic factors expressed in HSCs such as c-Myb, GATA-2, HOX family proteins and Bmi-1 also control their growth through the gene transcription. Cell cycle regulation in HSCs is not so unique but rather common to other cell types. However, the specific function of each cell cycle regulatory molecule in HSCs has been clarified during the last few years. Especially, $p21^{WAF1}$ and $p18^{INK4C}$ keep the quiescence of HSCs and $p27^{CIP1}$ keeps that of progenitor cells, respectively, thereby governing their pool sizes and/or preventing their exhaustion. On the other hand, the inactivation or deletion of $p16^{INK4A}$ and $p15^{INK4B}$ genes is supposed to contribute to malignant transformation of hematopoietic cells. These results imply that appropriate cell cycle control at the stage of stem/progenitor cells in the BM is required for maintaining normal hematopoiesis.

Key words: Hematopoietic stem/progenitor cell, cell cycle

INTRODUCTION

HSCs are characterized by two distinct abilities; self-renewal ability and multipotency. With these activities, HSCs are capable of maintaining a life-long supply of all lineages of hematopoietic cells according to systemic needs. The durability of the output potential of HSCs is believed to be dependent on their ability to execute self-renewal divisions; that is, an ability to proliferate without activation of a latent readiness to differentiate along restricted lineages. To maintain the homeostasis of hematopoiesis and protect the exhaustion of HSC population, most of HSCs are kept quiescent and only a limited number of cells enter cell cycle to supply mature blood cells. During this cell division, HSCs are obliged to undergo self-renewal, differentiation, or apoptosis. This step is controlled by external stimuli transmitted from the Bone Marrow (BM) microenvironment, including cytokines, Notch ligands, Wnt signals and sonic hedgehog (Shh) signals. Also, intrinsic factors expressed in HSCs, such as transcription regulators and cell cycle regulatory molecules, are crucially involved in this regulation (Fig. 1).

During the last decade, a number of cell cycle regulatory molecules such as cyclins, Cyclin-dependent Kinases (CDKs) and CDK inhibitors (CKIs) have been identified and their roles and regulation have been well characterized in various types of cells[1-3]. Cell cycle is positively regulated by CDKs associated with cyclins and their activities are negatively regulated by CKIs also included in these complexes at the same time. CKIs are classified into two families based on their structures and CDK targets. One class of inhibitors including $p21^{WAF1}$ (hereafter indicated as p21), $p27^{KIP1}$ (p27) and$p57^{KIP2}$ share a CDK2-binding motif in the N-terminus and inhibit the activities of cyclinD-, E- andA-dependent kinases. The other class of inhibitors also known as the INK4 family, including $p16^{INK4A}$ (p16), $p15^{INK4B}$(p15), $p18^{INK4C}$ (p18) and $p19^{INK4D}$, contain fourfold ankyrin repeats and specifically inhibit CDK4 and CDK6. Members of both families are important for executing cell cycle arrest in response to a variety of stimuli such as DNA damage, contact inhibition and transforming growth factor-β1 (TGF-β1) treatment.

Molecular mechanisms governing the stemness of HSCs from a viewpoint of cell cycle regulation are presented in this study.

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Characteristic of HSCs: The procedure for the purification of HSCs has made great progress along with the identification of molecular markers that characterize the cells having reconstitution activities in transplanted mice. The most primitive HSCs are considered to be with the $CD34^{low/-}c\text{-}Kit^{+}Sca\text{-}1^{+}Lin^{-}$ (CD34-KSL) phenotype, since a single cell with this phenotype could reconstitute whole hematopoiesis *in vivo* with high probability[4]. In addition to the specific surface phenotype, HSCs present in steady-state adult mouse BM are functionally characterized by their ability to efflux Rhodamine-123 and Hoechst 33342[5,6]. When adult mouse BM cells are stained with Hoechst 33342, exposed to the UV light and examined at 2 emission wavelengths simultaneously, HSCs are found in the rare Side Population (SP) with the dim fluorescence because of this ability[7]. The low fluorescence of HSCs after staining with Rhodamine-123 and Hoechst 33342 is attributed to their selective expression of different ABC transporters, P-glycoprotein and bcrp-1, respectively[8-10]. In addition, a more recent study proved that the cells having the strongest dye efflux capacity (Tip-SP cells) with the CD34-KSL phenotype are the most primitive HSCs, which can reconstitute long-term hematopoiesis with almost 100% probability after the single cell transplantation[11]. The cells in the SP fraction is considered to be in the G0 phase and this state is supposed to be restrictedly regulated by "hematopoietic niche" in the BM as described later.

Cytokines involved in cell cycle regulation in HSCs: A number of cytokines regulate growth, differentiation and survival of HSCs both positively and negatively. Among these, stem cell factor (SCF), Flt3 ligand (FL), thrombopoietin (TPO), interleukin-3 (IL-3) and IL-6 are known to promote the growth of HSCs *in vitro*[12-14]. In fact, Sl/Sl and W/W mice each having homozygous defect in the SCF gene and its receptor c-kit gene reveal severe anemia[15]. Also, total number of HSCs was reduced in the BM of c-mpl (TPO receptor)-null mice[16]. In addition, HSCs obtained from $c\text{-}mpl^{-/-}$ mice reveled severely decreased activities in reconstitution assays. These lines of evidence indicate that cytokine signals are required for the growth and survival of HSCs *in vivo* as well as *in vitro*[17].

During the last few years, the number of cases with hematologic malignancies receiving cord blood transplantation has increased more and more. However, the insufficient number of HSCs in each cord blood and the delayed recovery of hematopoiesis have limited their applicability to transplantation for adults. So, it has been of particular interest to expand hematopoietic cells *ex vivo*. Regarding the effects of cytokines in the *ex vivo* expansion of HSCs, a number of cytokine combinations were employed and their effects were evaluated by long-term reconstitution assays in transplanted mice. Among these, the combination of SCF, FL, TPO and IL-6/soluble IL-6 receptor seems to induce the *ex vivo* expansion of HSCs most efficiently with 4.2-fold increase of SRC (SCID-repopulating cells)[18].

TGF-β1 is a 25 kd protein produced by the stromal cells and by the hematopoietic progenitors, which induces the growth arrest in HSCs in autocrine and/or paracrine manners[19-23]. Using antisense oligonucleotides, it was demonstrated that the inhibition of TGF-β1 production could release HSCs in the umbilical cord blood or BM from quiescence[22,24,25]. Furthermore, the inhibition of the TGF-β1 signaling pathways in human HSCs using blocking antibodies against TGF-β1 or its receptor allowed quiescent cells to enter the cell cycle[26]. TGF-β1 has been supposed to induce cell-cycle arrest through p21 and p27 in various cell types including HSCs[27-33]. However, a recent paper provided evidence that TGF-β1 induces growth arrest independently of p21 or p27 by demonstrating that TGF-β1 can suppress the growth of HSCs and progenitor cells lacking both p21 and p27[34]. As for the other possible mechanisms of TGF-β1-induced growth arrest, TGF-β1 was reported to transcriptionally induce the expression of p15[35,36] and to down regulate the expression of c-Kit, FLT3 andIL-6 receptor on HSCs, thereby disrupting cytokine-dependent growth signals[37,38].

In contrast, another TGF-β super family protein, bone morphogenetic protein-4 (BMP-4) was recently reported to induce self-renewal of HSCs[39].

Effects of the BM microenvironment "hematopoietic niche" on cell cycle regulation in HSCs: HSCs receive critical signals for proliferation and differentiation from the BM microenvironment consisting of stromal cells and the extracellular matrix (ECM)[40-42]. ECM is composed of a variety of molecules such as fibronectin (FN), collagens, laminin and proteoglycans. ECM in the BM is not merely an inert framework but mediates specialized functions[43-45]. Some components of ECM have been shown to bind to growth factors produced by stromal cells and to immobilize them around cells, resulting in giving spaces where hematopoietic cells and growth factors colocalize. In addition, ECM can bind to glycoproteins expressed on HSCs. FN, collagens and laminin are ligands for integrins that not only control anchorage, spreading and migration of HSCs but also activate signal transduction pathways in these cells[43,44,46,47].

As were the cases with the niches for gut and certain skin stem cells[48-50], it has been supposed that HSCs also receive critical signals for proliferation and differentiation from the BM microenvironment "hematopoietic niche". However, it has been unknown where the hematopoietic niche is located in the BM or what types of cells

contribute to it. Recently, two groups individually generated mice lacking the BMP receptor type A (BMPRIA) and those engineered to produce osteoblast-specific, activated Parathyroid Hormone (PTH) and PTH-related protein (PTHrP) receptors (PPRs). In these mice, the osteoblast population was found to increase in the specific regions of bone, 'trabecular bone-like areas'. Also, the increase of the osteoblast population caused the parallel increase of the HSC population, particularly long-term repopulating HSCs[51,52]. As for this mechanism, Zhang *et al.*[51] demonstrated that the long-term HSCs were attached to spindle-shaped N-cadherin$^+$CD45$^-$ osteoblastic (SNO) cells. Two adherent junction molecules, N-cadherin and β-catenin, were asymmetrically localized between the SNO cells and the long-term HSCs, suggesting that SNO cells function as a key component of the niche to support HSCs and that BMP signaling through BMPRIA controls the number of HSCs by regulating niche size. Meanwhile, in the latter study, Calvi *et al.*[52] demonstrated that PPR-stimulated osteoblasts produced high levels of the Notch1 ligand, Jagged1 and supported the activity of HSCs through the Notch signaling. Together, these papers indicate that the interaction with osteoblasts contributes to the maintenance of the HSCs.

HSCs expressing the receptor tyrosine kinase Ties were quiescent and Ang-1, the ligand for Tie2 expressed on endothelial cells and HSCs, enhanced the quiescence of HSCs and their adhesion to fibronectin and collagen[53,54]. Therefore, it has been assumed that the Ang-1/Tie2 signaling pathway plays some role in the quiescence of HSCs. In accord with this hypothesis, a recent paper proved that Tie2$^+$ HSCs were in close contact with sub-endosteal osteoblasts expressing Ang-1 and that these Tie2$^+$ cells were included in SP and in the G0 phase of the cell cycle in the pyronin Y staining[55]. These results suggest that HSCs attaching to the specific osteoblasts in the hematopoietic niche are kept quiescent and protected from the myelosuppressive stress such as the treatment with 5-Fluorouracil (5-FU), a cell cycle-specific myelotoxic agent that kills cycling cells. However, it remains unknown which fraction of osteoblasts expresses Ang-1 and how it is regulated. Furthermore, the molecular mechanisms how Tie2/Ang-1 signaling prevents cell cycle progression also remain elusive.

Effects of the signals from the Notch ligand, Wnt and sonic hedgehog (Shh) on self-renewal of HSCs: In addition to the cytokines and molecules consisting of the extracellular matrix, various stimuli such as the Notch ligand, Wnt and Shh are transmitted to HSCs in the BM microenvironment. The activation of Notch transmembrane receptors expressed on HSCs by their ligand (Jagged 1 or Jagged 2) expressed on stromal cells promotes self-renewal of HSCs[56-60]. As for the critical target molecule of Notch signals that mediates self-renewal of HSCs, we recently found that c-Myc was transcriptionally induced by Notch[61]. In addition, the ectopic expression of c-Myc induced the growth of HSCs without disrupting their biologic properties in terms of surface phenotypes, colony-forming activities and reconstituting activities. Thus, c-Myc was supposed to play a major role in self-renewal of HSCs as an effector molecule of Notch signals.

Like Jagged1/Notch, a number of Wnt proteins are expressed in the BM and their receptor frizzled was detectable on BM-derived HSCs and progenitor cells[62,63]. Reya *et al.*[64] recently demonstrated that the Wnt signaling is important for the *in vitro* and *in vivo* self-renewal of normal HSCs. Moreover, they demonstrated that the activation of Wnt signaling in HSCs induces the increased expression of HOXB4 and Notch1, thereby inducing proliferation of HSCs. Besides Wnt3a that activates the canonical pathway through Frizzled/β-catenin/TCF/LEF, non-canonical Wnt, Wnt-5a, was also reported to expanded HSCs *in vitro*[65]. However, its mechanisms remain to be clarified.

Shh is a family member of human homologs of *Drosophila* Hedgehog (Hh) and expressed on the cell surface as transmembrane proteins. Hh signals can be mediated through cell-to-cell contact between adjacent cells expressing the Patched (Ptc) receptor. Alternatively, NH2-terminal cleavage of Hh can generate a soluble Hh ligand that can interact with distal cells expressing Ptc[66,67]. In the BM, Shh and their receptors Ptc and Smoothened (Smo) are expressed in highly purified HSCs. Cytokine-induced proliferation of HSCs could be inhibited by the anti-Hh Ab, implying that endogenously produced Hh proteins play a role in the expansion of HSCs. Addition of soluble forms of Shh resulted in an increase in the number of HSCs with pluripotent repopulating capacities. In addition, Noggin, a potent BMP-4 inhibitor, was found to inhibit the mitogenic effects of Shh, indicating that Shh signaling acts upstream of BMP-4 signaling to induce proliferation of HSCs[68].

Intrinsic factors that regulate the growth of HSCs: In addition to extrinsic factors, accumulated evidence indicates that cell cycle state of HSCs is regulated by intrinsic transcription regulatory factors, such c-Myb, GATA-2, HOX proteins and Bmi-1 (Fig. 2).

A transcriptional factor, c-Myb promotes the growth of HSCs, probably through the induction of c-myc and upregulated expression of c-kit and Flt3[69,70] and c-Myb-deficient mice die at embryonic day 15.5 (E15.5) due to the defect of definitive hematopoiesis[71]. Similarly, GATA-2$^{-/-}$

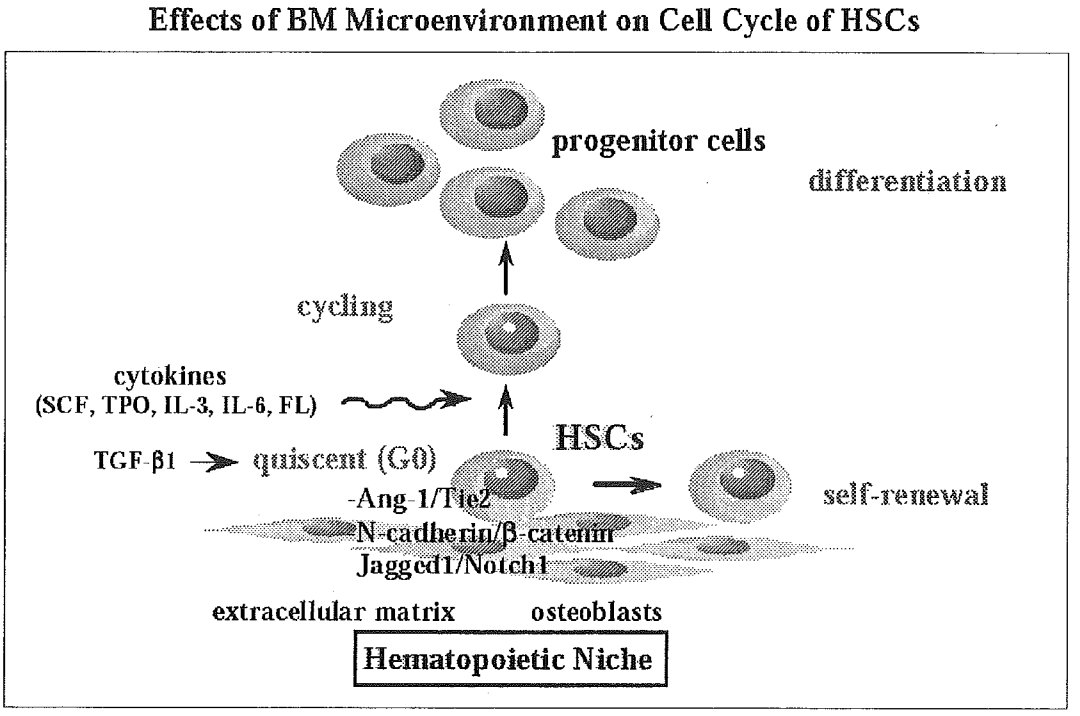

Fig. 1: Effects of BM microenvironment on cell cycle of HSCs

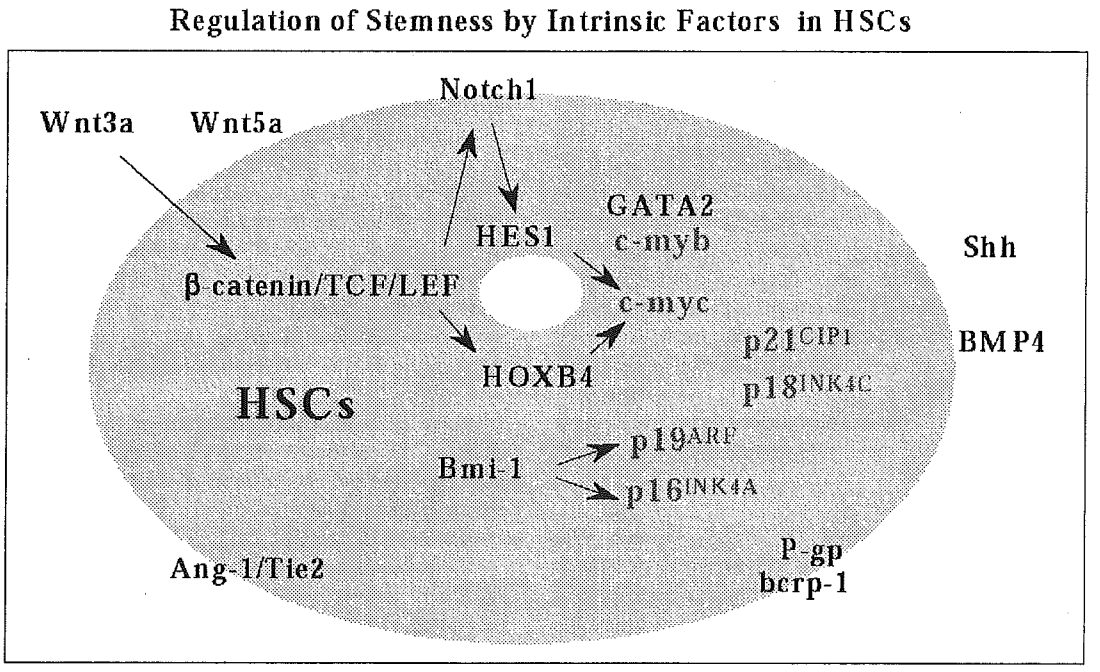

Fig. 2: Regulation of stemness by intrinsic factors in HSCs

mice are embryonic lethal around E11.5 because of the defect in the development and/or maintenance of HSCs[72]. However, functional roles of GATA-2 in the growth of HSCs are still controversial[73-76]. So, it remains unknown whether GATA-2 enhances or suppresses the growth of HSCs.

Among HOX family of transcription factors, HOXB4 is of particular remark as it promotes the growth of HSCs without the induction of leukemias[77-79]. As a result of the HOXB4 gene transfer or the protein delivery, HSCs could be expanded retaining their normal *in vivo* potential of differentiation and long-term repopulation[78,80]. Moreover, a recent study using HOXB4$^{-/-}$HOXB3$^{-/-}$ mice demonstrated that both HOXB4 and HOXB3 are required for the maximal growth potential of HSCs[81].

Bmi-1, a member of the Polycomb Group family of transcriptional repressors[82], was recently shown to be essential for maintenance of adult self-renewing HSCs[83]. Although the number of HSCs in the fetal liver of Bmi-1$^{-/-}$ mice was normal, the number of HSCs was markedly reduced in postnatal Bmi-1$^{-/-}$ mice. Furthermore, transplanted fetal liver and bone marrow cells obtained from Bmi-1$^{-/-}$ mice were able to contribute to hematopoiesis only transiently. Regarding this mechanism, in accord with the previous data obtained from embryonic fibroblasts[84], the micro array analysis on the BM mononuclear cells isolated from wild-type and Bmi-1$^{-/-}$ mice showed that the expression of p16 and p19^{ARF}, which is generated from the same INK4A locus by alternative splicing and inhibits MDM-2-mediated p53 degradation, was upregulated in Bmi-1$^{-/-}$ BM cells.

During neural development in mouse embryos, the cell-cycle regulator geminin controls replication by binding to the licensing factor Cdt1[85,86]. Recently, Luo *et al.*[87] reported that murine geminin transiently associated with members of the HOX-repressing polycomb complex, with the chromatin of HOX regulatory DNA elements and with HOX proteins[87]. Through these interactions, geminin displaces HOX proteins from their target genes and/or can interact with polycomb proteins to influence HOX activities. Therefore, the activities of HOX and polycomb protein families might be similarly regulated in HSCs.

Roles of p21 and p27 in quiescence of HSCs and progenitor cells: Embryonic fibroblasts obtained from p21$^{-/-}$ mice had a defect in their ability to achieve cell cycle arrest after irradiation[88,89] and antisense oligonucleotides against p21 was shown to release human mesenchymal cells from G0[90]. Therefore, p21 is required for the cell cycle arrest in G0 or G1 in some cell types. As for the roles for p21 in hematopoiesis, the expression level of p21 was reported be low in CD34$^+$ cells[91,92] and p21$^{-/-}$ mice did not exhibit an apparent hematologic defect[88,89]. However, in a subsequent analysis, Cheng *et al.*[93] found that p21 was highly expressed in the quiescent stem cell-like fraction of BM cells[93]. They also found that, under normal homeostatic conditions, the proportion of quiescent HSCs in the G0 phase was reduced and that total number of HSCs increased in p21$^{-/-}$ mice. In accord with these findings, when p21$^{-/-}$ mice were treated with 5-FU, the survival percentage was much lower in p21$^{-/-}$ mice than in littermate controls. They also directly assessed stem cell self-renewal capability using a serial transplantation approach. As a result, no mice transplanted with p21$^{-/-}$ BM cells survived after the fifth transplant due to the exhaustion of HSC population, whereas those transplanted with p21$^{+/+}$ BM cells had a 50% survival. Together, these results indicate that p21 is a key molecule that restricts cell cycle entry of HSCs, thereby keeping their pool size and preventing their exhaustion under certain stress.

p27 is molecularly distinct from p21 in its carboxyl terminus; it interacts with similar, though not identical, cyclin-CDK complex and lacks p53-regulated expression. In hematopoietic system, the expression of p27 is observed in more mature progenitors than p21[91,92]. The p27$^{-/-}$ mice have a larger body and hyperplasia of most organs including hematopoietic organs[94-96]. In striking contrast to p21$^{-/-}$ mice, the number, cell cycling and self-renewal of HSCs were normal in p27$^{-/-}$ mice, while these mice had an increase in hematopoietic progenitor cells[97]. In addition, these progenitor cells in p27$^{-/-}$ mice were more proliferative than p27$^{+/+}$ progenitor cells. Furthermore, progenitor cells from p27$^{-/-}$ mice were able to expand and regenerate hematopoiesis after serial transplantation, while p27$^{+/+}$ progenitors were markedly depleted. Thus, p21 and p27 govern the divergent stem and progenitor cell populations, respectively.

Roles for the INK4 family in self-renewing division of HSCs and as tumor suppressor genes: Several of INK4 proteins have been supposed to be implicated in the regulation of HSCs numbers and self-renewal. Yuen *et al.*[98] recently clarified a function of p18 in HSCs and the early progenitor cells[98]. Mice deficient for p18 had an increased number of HSCs in the bone marrow. Also, competitive repopulation assays showed that p18$^{-/-}$ HSCs are far more competitive than normal HSCs with 14-fold activities. In contrast to p21$^{-/-}$ HSCs, the exhaustion of p18$^{-/-}$ HSCs was not observed during serial bone marrow transplants, indicating that p18 is a strong inhibitor limiting the potential of stem cell self-renewal *in vivo*.

On the other hands, p16 is highly expressed in CD34$^+$ cells and its expression is down regulated during differentiation process towards all lineages[99]. So, p16 was assumed to play some role in cell cycle arrest in HSCs. However, since p16$^{-/-}$ mice did not show an apparent

abnormality in hematopoiesis, p16 was supposed to be dispensable for the quiescence of HSCs[100,101]. In contrast to the expression pattern of p16, the expression of p15 was not detected in $CD34^+$ cells, but increased specifically during myeloid differentiation[99,102]. However, the functional role of p15 in HSCs remained to be clarified. Both p16 and p15 inhibit the function of cyclin D-CDK4/6 complex and suppress the phosphorylation of pRb, thereby inducing cell cycle arrest at G0/G1 phase. Especially, under tumorgenetic stress such as the presence of oncogenic ras gene, p16 and p15 are induced to express and suppress tumor progression through the induction of premature senescence[103,104]. With these activities, both p16 and p15 are supposed to act as tumor suppressor genes. In fact, inactivation and/or deletion of p16 and p15 genes are observed in various human cancers very frequently[105,106]. As for hematologic malignancies, their defects caused by the homozygotic deletion or methylation were observed in a substantial proportion of AML, ALL, ATL, malignant lymphoma and MDS cases[107-111]. These results indicate that appropriate cell cycle control, particularly at the stage of stem/progenitor cells, is required for maintaining normal hematopoiesis.

CONCLUSIONS

Although a great advance has been made in stem cell biology, particularly in terms of purifying and evaluating the function of HSCs, precise mechanisms of cell cycle regulation that assign self-renewal or differentiation to HSCs remain unknown. So, further studies are required to disclose the whole feature of cell cycle regulation in HSCs. These studies would undoubtfully bring about useful information to establish therapeutic strategies for *ex vivo* stem cell expansion.

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Myelodysplastic Syndromes in Atomic Bomb Survivors in Nagasaki: A Preliminary Analysis

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Myelodysplastic syndromes (MDS) are a heterogenous hematological group characterized by an ineffective hematopoiesis resulting in a variety of cytopenias, morphological abnormalities of blood cells, chromosomal aberrations, and an increases risk of transformation into acute myeloid leukemia. Despite of its nature of close relation to leukemia, MDS has been not well investigated in atomic bomb (A-bomb) survivors. We conducted a retrospective cohort study with over 80,000 A-bomb survivors in Nagasaki to assess the incidence of MDS and its relation with A-bomb exposure status. In a preliminary analysis, we confirmed 162 MDS cases during 1980 to 2004. The median age at diagnosis was 71 years old. The incidence rate was higher in men than women, and an inverse relationship was observed between incidence of MDS and the distance from the hypocenter. We suggest that A-bomb radiation may affect the occurrence of MDS in A-bomb survivors even more than 50 years passed after the explosion. Further detail analyses are necessary to confirm these results.

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Keywords: Atomic bomb survivors; Myelodysplastic syndromes; Radiation exposure; Epidemiology; Retrospective cohort study

Introduction

Myelodysplastic syndromes (MDS) are characterized by an ineffective hematopoiesis resulting in a variety of cytopenias, morphological abnormalities of blood cells, chromosomal aberrations, and an increases risk of transformation into acute myeloid leukemia (AML).[1] Prevalence of MDS is high in elderly people and more common in developed countries.[2] The etiology of MDS has been still unclear. Chemotherapeutic agents, benzene, herbicides, pesticides, and ionizing radiation have been reported as possible etiological factors for MDS, but the actual causal-relationship between such factors and MDS has been not established.[3]

A wealth of information has been reported concerning an association between the atomic bomb (A-bomb) radiation and the leukemia risk since physicians in Nagasaki and Hiroshima began to notice an increased number of leukemia in the late 1940s.[4-7] Because of a close relationship between MDS and AML, the occurrence of MDS is supposed to be affected by A-bomb radiation in a similar fashion with radiation-induced leukemia. However, MDS has been not well investigated in A-bomb survivors so far. Only a few studies with small sample size reported with respect to the relationship between A-bomb irradiation and the incidence of MDS.[8,9] Therefore, a comprehensive large-scaled study has been needed for a long time.[10,11]

There has been some challenges to conduct a study for MDS: (1) MDS is a relatively new concept of disease that was first defined by French-American-British (FAB) Cooperative group in 1982,[12] (2) MDS has been not registered actively into any tumor-registries even in Nagasaki or Hiroshima because of non-malignant nature, and (3) a heterogeneous and vaguely condition of MDS makes its diagno-

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sis difficult. To overcome these difficulties of research situation, we established the Nagasaki-City MDS Project, in January 2004, consisting of the Nagasaki University Hospital, 5 affiliated hospitals in Nagasaki city, and the Nagasaki cancer registry. Our aim was to assess the incidence of MDS in A-bomb survivors during 1980 to 2004 and the effects of age, sex, and the exposure status including age at the time of the bombing (ATB) and exposure distance from the hypocenter on the risk of MDS. Here we report a preliminary analysis of this study.

Methods

A study population was 87,506 Nagasaki A-bomb survivors (35,529 men and 51,967 women) who were alive as of January 1, 1980 and were registered in the database of the Scientific Data Center at the Atomic Bomb Disease Institute of the Nagasaki University Graduate School of Biomedical Science. Since the operation of the database started in 1977, it has kept the data of approximately 120,000 A-bomb survivors in Nagasaki.[13] The median of attained age as of 1980 was 54 years with the range of 34 to 100 years. The distribution of survivors as of January 1980 by distance of exposure and age at the time of the bombing is shown in Figure 1.

After we obtained approvals by the institutional ethical committees of all institutes, MDS cases diagnosed from January 1980 to December 2004 were retrospectively accumulated into the study list irrespective of whether they were atomic bomb survivors or not. During the study period, 647 MDS cases were collected and we identified A-bomb survivors among them using the database of the Scientific Data Center at the Atomic Bomb Disease Institute of the Nagasaki University Graduate School of Biomedical Sciences. Diagnosis of MDS was made according to FAB criteria[12] by conducting hematological review of bone marrow specimen and clinical information. Cases diagnosed before 1980, secondary MDS, and cases not confirmed as MDS were excluded from the analysis.

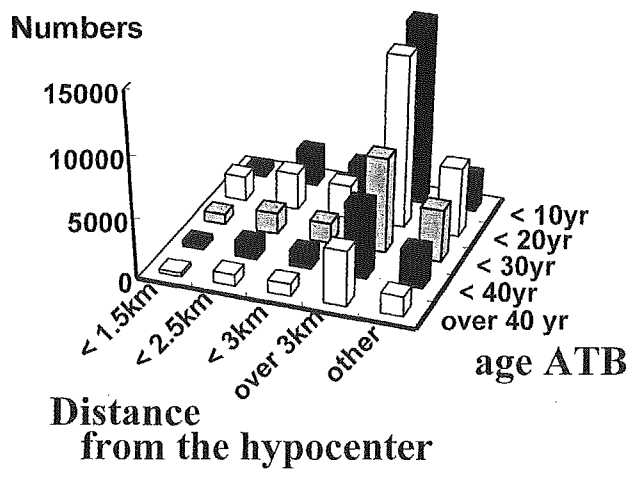

Figure 1. Distribution of study population by distance from the A-bombing hypocenter (km) and age at the time of the bombing (age ATB) (years).

The A-bomb survivors were followed up from the latest of January 1, 1980 and the time of the first registration to the database through the earliest of the following: time of death, time of migration out of Nagasaki city, time of diagnosis of MDS and the end of December 2004. We calculated the cumulative incidence per 100,000 persons during 24 years and the incidence rates per 100,000 person-years with stratification by sex, age ATB in years (0-9, 10-19, 20-29, 30-39, $\geq$ 40) and the distance from the hypocenter in km (0-1.49, 1.5-2.49, 2.5-2.99, $\geq$3.0). We used the distance from the hypocenter as a substitute for the radiation dose. A comparison of incidence among groups was performed by the rate ratio (RR), the chi-square statistic, nonparametric statistic, and when necessary, the Cochran-Armitage test for trend. All statistical analyses were performed with SAS 8.2 software (SAS Japan Institute, Tokyo, Japan). All tests were two tailed, and a statistically significance level was set at 0.05.

Results

A total of 162 MDS cases were confirmed in A-bomb survivors by the end of 2004. Among them, 119 were directly exposed to the A-bombing and the rest were early entrants to the city who were engaged in rescue service and fetuses. One hundred twenty-four cases were classified into FAB subtypes so far. In a preliminary analysis, the cumulative incidence of MDS during 24 years was 185 (the crude incidence rate, 10.7) per 100,000 A-bomb survivors in total. Almost all patients were diagnosed at the age over 60 years, and the median age at diagnosis was 70.7 with the range of 42.0 to 97.4 years (Figure 2). There was no significant difference in age at diagnosis between men and women (p = 0.12). The incidence of MDS was higher in men than women (RR = 1.7). An inverse relationship was observed between the incidence of MDS and the distance from the hypocenter; the more proximally exposed A-bomb survivors, the higher incidence of MDS (p < 0.0001).

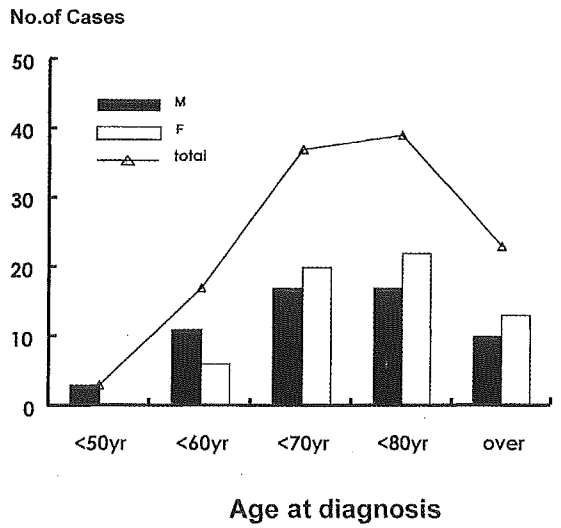

Figure 2. Distribution of MDS patients by sex and age at diagnosis (years).

Discussion

Several European studies reported that the crude annual incidence of MDS in general population was between 2 to 8 cases per 100,000 people and that the rate increased by age.[14-17] One study on the skewed elderly population in the United Kingdom reported 12.6 cases per 100,000 people of the crude annual incidence.[18] In our preliminary analysis, the crude incidence rate of MDS in A-bomb survivors in Nagasaki showed 10.7 cases per 100,000 person-years, which is slightly higher than that of general European population. Such a relatively high incidence in A-bomb survivors may due to that they are more aged than the general European population or even the general Japanese population; the youngest A-bomb survivors were aged 34 years in 1980. To ascertain whether the incidence rate of MDS in A-bomb survivors is truly higher or not compared to the general population, further age-adjustment analyses will be required. The incidence rate of MDS was significantly higher in men than women in A-bomb survivors, which is consistent with all of the previous reports of general population.[14-18] The median age at diagnosis was 70.7 years in A-bomb survivors, which was almost same as the general German patients, 72 years old.[19]

One of the important concerns in A-bomb survivors is whether the incidence of MDS is increasing yearly or not. Although our preliminary analysis showed a slightly upward trend of the incidence (data not shown), it remains unclear whether the trend is actually true or not. Even in general population, the trend of the incidence of MDS is controversial. Several investigators have reported increasing trends of MDS,[20-22] while others found no evidence for an increase in the incidence of MDS in general population over time based on their well-defined epidemiological studies.[14,17,23] Studies including cases diagnosed early 1980s tend to report a significant increase in the incidence of MDS, which might be affected by physicians' awareness of MDS and the improvement of case ascertainment through the publication of the FAB criteria for classifying MDS in 1982. Our results also might be affected by the misclassification bias due to the improvement of case ascertainment and a selection bias because of a retrospective study. To ascertain whether the incidence rate of MDS in the atomic bomb survivors is actually increasing yearly or not, further well-designed epidemiological analyses will be required.

There were some reports supporting that high-dose radiation exposure may induce MDS.[24-26] The link between radiation therapy and secondary MDS with abnormalities of chromosome no.5 and 7 were also well known.[27] However, MDS risk in occupational and environmental exposure to radiation was argued.[28,29] In the population of the atomic bomb survivors, 12 MDS cases were reported out of the LSS cohort of Radiation Effects Research Foundation (RERF) during 1950 to 1990,[8] and 26 cases were detected at Hiroshima University Hospital from 1985 to 1999.[9] Both studies showed a dose-dependent increase in the risk of MDS, but they were too small in the number of cases for the statistical evaluation to show a precise relationship between radiation and MDS. Too few cases of MDS were also reported in the Chernobyl accident cleanup workers.[30,31] Such previous studies might be failed to case detection of MDS in view of the fact that MDS is actually as common as AML. In our preliminary analysis based on a large-scaled study of A-bomb survivors in Nagasaki, we found the incidence of MDS was higher in A-bomb survivors who were proximally exposed than in those distally exposed. This result suggests that high-dose atomic bomb irradiation affected the occurrence of MDS in a long latency, around 40 to 60 years passed after the bombing. Further detailed analyses are necessary to confirm this result. Also, a systematic case detection of A-bomb survivors from both Hiroshima and Nagasaki cities is left for further challenges.

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Difference in clinical features between Japanese and German patients with refractory anemia in myelodysplastic syndromes

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Several reports indicate that there might be differences in clinical features between Asian and Western myelodysplastic syndrome (MDS) cases. We analyzed refractory anemia (RA) in French-American-British (FAB) classification cases diagnosed in Japan and Germany to perform a more exact comparison between Asian and Western MDS types. In the first step, we analyzed agreement of morphologic diagnosis between Japanese and German hematologists. Blood and bone marrow slides of 129 patients diagnosed with FAB-RA, FAB-RA with ringed sideroblasts (RARS), or aplastic anemia were selected randomly and evaluated separately by each group. The agreements of diagnoses according to FAB and World Health Organization (WHO) classifications were 98.4% and 83.8%, respectively. Second, we compared clinical features between 131 Japanese and 597 German patients with FAB-RA. Japanese patients were significantly younger than German patients. Japanese patients had more severe cytopenias. However, prognosis of Japanese patients was significantly more favorable than that of German patients. Japanese patients had a significantly lower cumulative risk of acute leukemia evolution than did German patients. Frequency of WHO-RA in Japanese patients with FAB-RA was significantly higher than that in German patients. In conclusion, our results indicate that the clinical features of Japanese patients with FAB-RA differ from those of German patients. (Blood. 2005;106:2633-2640)

Introduction

Myelodysplastic syndromes (MDSs) are acquired clonal stem cell disorders characterized by ineffective hematopoiesis with myelodysplasia[1] and are associated with a high risk of progression to acute leukemias.[2] MDSs are very heterogeneous in terms of their morphology, clinical features, and survival.[3] Refractory anemia (RA) according to the French-American-British (FAB) classification is generally classified as a low-risk group in MDS,[4] comprising 30% to 40% of all MDS cases. It was reported that the International Prognostic Scoring System (IPSS) was useful for assessing the prognosis in the whole group of patients with MDS according to the FAB classification.[5] According to the World Health Organization (WHO) classification,[6] most patients with FAB-RA are classified as refractory cytopenia with multilineage dysplasia (RCMD) or, less frequently, as WHO-RA. It was reported that patients with WHO-RA had more favorable prognoses than did patients with RCMD.[7-9]

There are several reports indicating possible differences in clinical features between Western MDS types and Eastern MDS types. It has been reported that the median age of Japanese patients with MDS is 60 years.[10] The median age of patients with MDS in Korea and Thailand and the mean age of those in Central Africa were reported to be 57,[11] 56,[12] and 57 years,[13] respectively. However, large MDS studies from Western countries showed a median or mean age of 68 to 73 years.[5,14-16] We have reported that the clinical features of RA with excess of blasts (RAEB) or RAEB in transformation according to FAB classification seemed to be similar between Japanese and Western patients.[17] However, previous reports indicate that Japanese patients with MDS have a lower frequency of RA with ringed sideroblasts (RARS) according to FAB classification and a higher frequency of FAB-RA than the Western IPSS study[17,18] and that there are different prognostic factors between Japanese and Western patients with MDS.[10,17] From cytogenetic analysis, it was indicated that the frequency of Japanese MDS with isolated del(5q) was lower than that in the IPSS study.[18] We additionally reported that patients with FAB-RA demonstrated favorable outcomes compared with those of the IPSS study.[17] We consider that there are different clinical features between Asian and Western patients with low-risk MDS. In the present study, after conducting an interobserver morphologic variation study for diagnosis of MDS subgroups between the Japanese and German hematologists, we compared

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in detail the clinical features of Japanese and German patients in FAB-RA.

Patients and methods

Patients

A total of 728 consecutive patients (Japan, 131 cases; Germany, 597 cases) with a diagnosis of primary RA according to FAB classification (FAB-RA) were included in this retrospective analysis. Japanese patients were diagnosed at the Saitama Medical School Hospital, Nagasaki University Hospital or affiliated hospitals in Japan between April 1976 and January 1997. German patients were diagnosed at the Department of Hematology, Oncology, and Clinical Immunology of the Heinrich-Heine University in Germany between January 1973 and December 2002. Patients who had previously been treated with antineoplastic drugs or ionizing radiation were excluded from the study. Informed consent was provided according to the Declaration of Helsinki. This study was performed according to the guideline of the institutional review board of the Saitama Medical School.

Interobserver variation study

Hematologic examinations were performed using standard methods (peripheral blood [PB] and bone marrow [BM] Wright-Giemsa or May-Giemsa stained films). PB and BM differential counts were performed on 100 and 500 cells, respectively. Evaluations of bone marrow cellularity were performed using the specimens of BM trephine biopsy and/or clot section.

In the first step, we reviewed all the training slides of FAB-RA (25 Japanese and 20 German cases) by Japanese and German hematologists separately. After this training review, the first joint review meeting for morphologic consensus was performed by 4 Japanese and 4 Germany hematologists for 4 days in February 2002 at Heinrich-Heine-University. At the first joint review, we mainly discussed evaluation of dysplasia and diagnosis using the training slides.

In the second step, the slides of 129 patients (110 FAB-RA, 7 FAB-RARS, 12 aplastic anemia [AA] diagnosed by Japanese or German groups) were selected randomly and were evaluated for morphologic diagnosis according to FAB and WHO classifications by the Japanese and German groups separately in each country. Patients with FAB-RA were reclassified to WHO subgroups according to the criteria of a previous German report.[7] After this separate review, the second joint review meeting for morphologic consensus was performed by 4 Japanese and 4 German hematologists for 4 days in October 2004 at Heinrich-Heine-University. The observers were blinded to the clinical and laboratory data, including cytogenetics, until finishing this separate review. Diagnoses of FAB classification or AA were performed using only morphologic findings. Concerning diagnoses of WHO classification, morphologic and cytogenetic findings were used. In the second joint review meeting, the concordance rate of morphologic diagnosis according to the FAB and WHO classifications between Japanese and German hematologists was analyzed, and we discussed cases whose diagnoses did not agree between Japanese and German hematologists in this separate review.

Cytogenetic analysis

Cytogenetic analyses were performed with a trypsin-Giemsa banding technique on BM cells from aspirates. Ordinarily 20 to 30 metaphases were examined. Cytogenetic aberrations were grouped according to the IPSS publication.[5]

Clinical studies

Comparisons of the clinical features and the prognostic factors between 131 Japanese and 597 German patients with FAB-RA were analyzed. Patients were followed for overall survival (OS) and leukemic progression through June 2004 in Japanese cases and July 2003 in German cases. OS was measured from the date of diagnosis until death from any cause, the date of stem cell transplantation, or until the last patient contact. Leukemic progression was measured from the date of diagnosis until the date of diagnosis of acute leukemia. We classified causes of death into 3 subtypes: MDS related (MDS death), MDS unrelated (non-MDS death), and unclear cause (unclear death). MDS death was defined as acute leukemia, infection, bleeding, or heart failure resulting from anemia or iron overload. Non-MDS death was defined as causes of certain independence from MDS. Unclear death was defined as causes of death without any obvious MDS-related sign and without causes of certain independence from MDS. We also measured survival period censored non-MDS death or unclear death (modified survival). Measurements of modified survival were censored at the date of death in patients with non-MDS death or unclear death.

Statistical methods

The chi-square test and the nonparametric Mann-Whitney test were used to compare the proportions of patients and continuous data, respectively. The Kaplan-Meier method was used to generate the estimate of cumulative probabilities of OS, modified survival, and cumulative risk of acute leukemia evolution. The difference in the cumulative probabilities within subcategories of patients was compared using a 2-sided log-rank test. Age- and sex-adjusted effects of clinical parameters on outcomes were performed with use of 2 different models of multivariate Cox proportional hazards regression. Model A included age category, sex, dichotomized peripheral blood counts, and chromosome category of IPSS. Model B included age category, sex, and IPSS score. An examination for interaction between parameters was performed with the inclusion of interaction terms into each model. The effects of clinical parameters were evaluated as hazard ratios and their 95% confidence intervals. The interobserver concordance was evaluated using the simple κ coefficient. A 2-sided P value of less than .05 was considered to be statistically significant. All statistical analyses were performed with the use of SAS software (version 8.2; SAS Institute, Cary, NC), and all graphic presentations were performed with the use of StatView (version 5.0; SAS Institute).

Results

Morphologic consensus

Of the 129 cases reviewed, the agreement of morphologic diagnosis according to FAB classification between Japanese and German hematologists was 98.4%. A significant concordance was achieved while using FAB classification (κ, 0.94; $P < .001$). There were 2 cases whose diagnoses did not agree between Japanese and German hematologists by separate review. One case was diagnosed as AA by the Japanese group, but the diagnosis by the German group was FAB-RA. The final diagnosis of this case as AA was reached by consensus among the Japanese and German groups by joint review. Another case was diagnosed as RAEB by the Japanese group, but the diagnosis by the German group was FAB-RA. The Japanese hematologists judged that percentage of BM blasts of this patient was slightly higher than 5%. Each group performed morphologic examination again. As a result, the blasts percentage was judged to be less than 5%. The final diagnosis of this case as FAB-RA was reached by consensus among the Japanese and German groups by joint review. Of the 110 FAB-RA and 7 FAB-RARS cases reviewed for WHO classification, the agreement of morphologic diagnosis according to WHO classification between Japanese and German hematologists was 83.8%. A significant concordance was achieved while using WHO classification (κ, 0.73; $P < .001$).

Comparison of clinical features and prognostic factors between Japanese and German patients with FAB-RA

Clinical and laboratory features at the time of diagnosis. The age of Japanese patients with FAB-RA was significantly younger