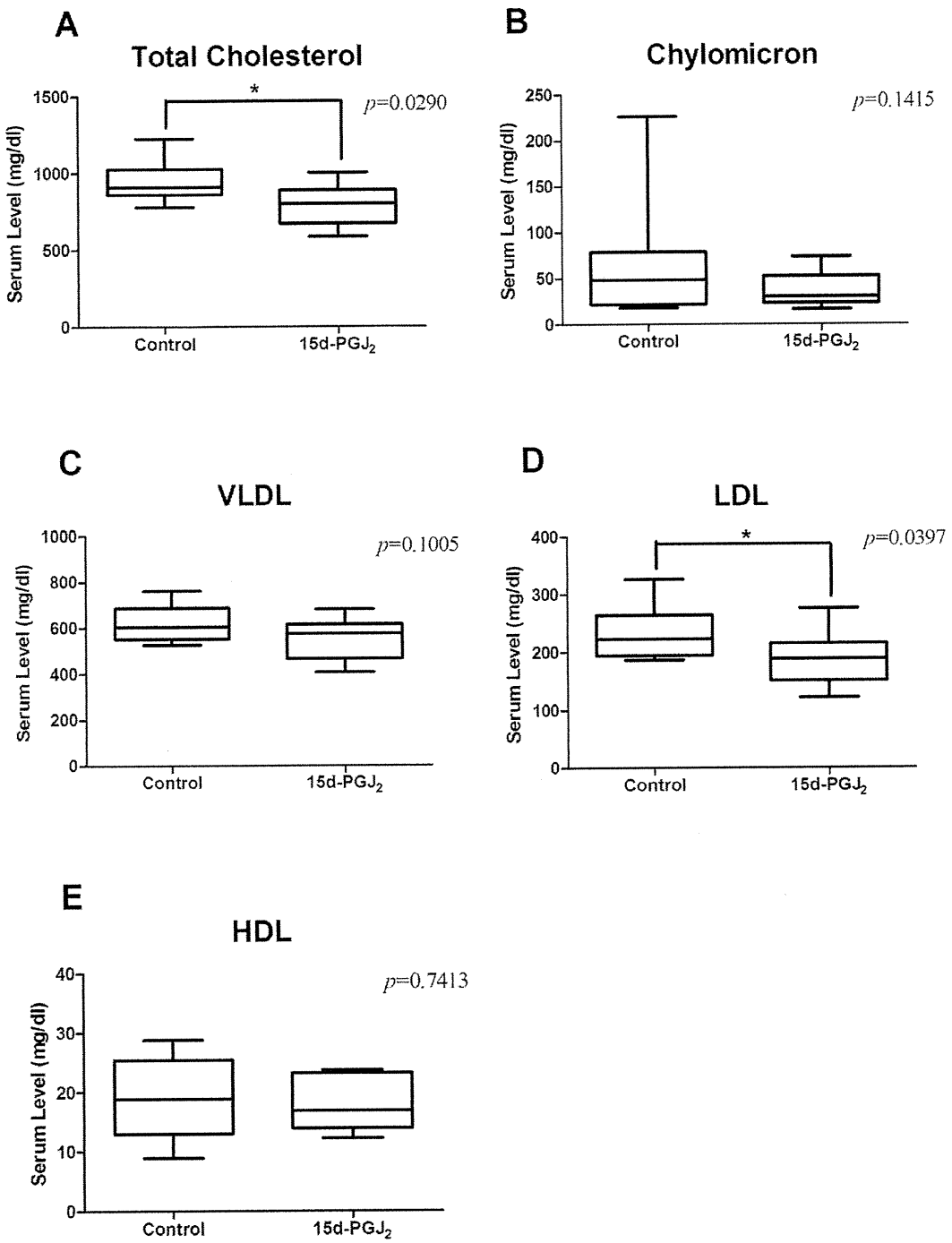

Figure 6. Serum lipid levels in the controls and the 15d-PGJ_2 group. Blood was collected from the cardiac cavity of mice aged 16 wk and analyzed for the lipid profile. The plasma chylomicron (CM) (B), very low density lipoprotein (VLDL) (C), low density lipoprotein (LDL) (D), and high density lipoprotein (HDL) (E) levels were determined by use of a high-sensitivity lipoprotein-profiling system by high-performance liquid chromatography. The total serum cholesterol level (A) was significantly lower in the15d-PGJ_2 group than in the control group (795.5±39.31 mg/dl vs 944.1±49.04 mg/dl, $p=0.029$). Especially LDL was significantly reduced in the 15d-PGJ_2 group (186.9±13.49 mg/dl vs 234.3±16.60 mg/dl, $p=0.0397$). CM, VLDL and HDL were not different between the control and 15d-PGJ_2 groups, 36.96±4.999 mg/dl vs 68.13±23.98 mg/dl, 553.5±26.67 mg/dl vs 622.7±28.02 mg/dl, 18.14±1.264 mg/dl vs 19.01±2.562 mg/dl, respectively. *$p<0.05$, with Student's t test.
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RelA (p65), c-Rel, and RelB, as well as p105 and p100 and their processed forms, p50 and p52, respectively. NF-κB primarily exists as a p50/p65 heterodimer [27]. In our data, PPARγ expressed in atherosclerotic lesions in both controls and 15d-PGJ_2 groups. In addition, the expression of RelA was decreased in 15d-PGJ_2 groups. It is generally known that high dose of ligands lead to downregulation of its receptor expressions. Although it has not been reported about PPARγ agonist, a previous study revealed that treatment with GW1929, a selective PPARγ antagonist, enhanced PPARγ mRNA expressions in kidneys from hypertension model rats [28]. This study shows the possibility that high dose PPARγ also downregulate its receptor expression. But PPARγ expression was not changed in our results. It indicated that 15d-PGJ_2 did not induce the negative-feedback in our study. On the other hand, the reduction of RelA was owing to NF-κB inhibition. 15d-PGJ_2 induces some PPARγ-independent biological actions, such as inhibition of NF-κB signaling through covalent modifications of critical cysteine residues in IκB kinase and the DNA-binding domains of NF-κB subunits [29]. We presumed that 15d-PGJ_2 inhibited NF-κB not only as a PPARγ agonist but also as PPARγ-independent actions.

High plasma concentrations of cholesterol, in particular those of LDL cholesterol, are one of the principal risk factors for atherosclerosis [24]. In this study, 15d-PGJ_2 decreased serum total cholesterol level and LDL cholesterol level. This shows that 15d-PGJ_2 reduces the principal risk factor of atherosclerosis. But the detail mechanisms remain an open question. Further studies are needed to elucidate this matter.

In conclusion, this is the first study demonstrating an anti-atherosclerotic effect of 15d-PGJ_2 in vivo, using a rodent model. The mechanism of its effect remains to be elucidated in detail. However, our data indicate that 15d-PGJ_2 exhibits ability as to an anti-atherosclerotic effect. These findings suggest that 15d-PGJ_2 is a beneficial therapeutic reagent for both atherosclerosis.

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Author Contributions

Conceived and designed the experiments: TS MH MK YK. Performed the experiments: TS MH AY MK KN. Analyzed the data: TS MH. Contributed reagents/materials/analysis tools: TS MH EA HI AY MK KM. Wrote the paper: TS MK SM YK. Assisted editing paper: EA MK SM TM YK.

References

1. Libby P, Ridker PM (2006) Inflammation and Atherothrombosis From Population Biology and Bench Research to Clinical Practice. Journal of the American College of Cardiology 48: A33–A46. Available: http://linkinghub.elsevier.com/retrieve/pii/S0735109706019966. Accessed 23 Jul 2010.
2. Ricote M, Huang J, Fajas L, Li A, Welch J, et al. (1998) Expression of the peroxisome proliferator-activated receptor gamma (PPARgamma) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. Proceedings of the National Academy of Sciences of the United States of America 95: 7614–9. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid = 22700&tool = pmcentrez&rendertype = abstract. Accessed 31 Aug 2010.
3. Chen Z, Ishibashi S, Perrey S, Osuga Ji, Gotoda T, et al. (2001) Troglitazone inhibits atherosclerosis in apolipoprotein E-knockout mice: pleiotropic effects on CD36 expression and HDL. Arteriosclerosis, thrombosis, and vascular biology 21: 372–7. Available: http://www.ncbi.nlm.nih.gov/pubmed/11231916.
4. Shinohara E, Kihara S, Ouchi N, Funahashi T, Nakamura T, et al. (1998) Troglitazone suppresses intimal formation following balloon injury in insulin-resistant Zucker fatty rats. Atherosclerosis 136: 275–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/9543098. Accessed 14 Oct 2010.
5. Jiang C, Ting AT, Seed B (1998) PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 391: 82–6. Available: http://www.ncbi.nlm.nih.gov/pubmed/9422509. Accessed 31 Aug 2010.
6. Toriumi Y, Hiraoka M, Watanabe M, Yoshida M (2003) Pioglitazone reduces monocyte adhesion to vascular endothelium under flow by modulating RhoA GTPase and focal adhesion kinase. FEBS letters 553: 419–22. Available: http://www.ncbi.nlm.nih.gov/pubmed/14572662. Accessed 21 Jul 2010.
7. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature 391: 79–82. Available: http://www.ncbi.nlm.nih.gov/pubmed/9422508.
8. Bonfield TL, Thomassen MJ, Farver CF, Abraham S, Koloze MT, et al. (2008) Peroxisome proliferator-activated receptor-gamma regulates the expression of alveolar macrophage macrophage colony-stimulating factor. Journal of immunology (Baltimore, Md. : 1950) 181: 235–42. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid = 2819287&tool = pmcentrez&rendertype = abstract. Accessed 22 Oct 2010.
9. Zhang W-Y, Schwartz EA, Permana PA, Reaven PD (2008) Pioglitazone inhibits the expression of inflammatory cytokines from both monocytes and lymphocytes in patients with impaired glucose tolerance. Arteriosclerosis, thrombosis, and vascular biology 28: 2312–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/18818415. Accessed 22 Oct 2010.
10. Jackson SM, Parhami F, Xi XP, Berliner JA, Hsueh WA, et al. (1999) Peroxisome proliferator-activated receptor activators target human endothelial cells to inhibit leukocyte-endothelial cell interaction. Arteriosclerosis, thrombosis, and vascular biology 19: 2094–104. Available: http://www.ncbi.nlm.nih.gov/pubmed/10479650.
11. Pasceri V, Wu HD, Willerson JT, Yeh ET (2000) Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-gamma activators. Circulation 101: 235–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/10645917.
12. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP (2002) Prostaglandins as modulators of immunity. Trends in immunology 23: 144–50. Available: http://www.ncbi.nlm.nih.gov/pubmed/11864843.
13. Ide T, Egan K, Bell-Parikh LC, FitzGerald GA (2003) Activation of nuclear receptors by prostaglandins. Thrombosis research 110: 311–5. Available: http://www.ncbi.nlm.nih.gov/pubmed/14592554. Accessed 14 Oct 2010.
14. Bishop-Bailey D, Hla T (1999) Endothelial cell apoptosis induced by the peroxisome proliferator-activated receptor (PPAR) ligand 15-deoxy-Delta12, 14-prostaglandin J2. The Journal of biological chemistry 274: 17042–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/10358055.
15. Xin X, Yang S, Kowalski J, Gerritsen ME (1999) Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis in vitro and in vivo. The Journal of biological chemistry 274: 9116–21. Available: http://www.ncbi.nlm.nih.gov/pubmed/10085162.
16. Dong Y-G, Chen D-D, He J-G, Guan Y-Y (2004) Effects of 15-deoxy-delta12,14-prostaglandin J2 on cell proliferation and apoptosis in ECV304 endothelial cells. Acta pharmacologica Sinica 25: 47–53. Available: http://www.ncbi.nlm.nih.gov/pubmed/14704122.
17. Blanco M, Moro MA, Dávalos A, Leira R, Castellanos M, et al. (2005) Increased plasma levels of 15-deoxyDelta prostaglandin J2 are associated with good outcome in acute atherothrombotic ischemic stroke. Stroke; a journal of cerebral circulation 36: 1189–94. Available: http://www.ncbi.nlm.nih.gov/pubmed/15879329. Accessed 22 Oct 2010.
18. Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM, Maeda N (1992) Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America 89: 4471–5. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid = 49104&tool = pmcentrez&rendertype = abstract.
19. Kawahito Y, Kondo M, Tsubouchi Y, Hashiramoto A, Bishop-Bailey D, et al. (2000) 15-deoxy-delta(12,14)-PGJ(2) induces synoviocyte apoptosis and suppresses adjuvant-induced arthritis in rats. The Journal of clinical investigation 106: 189–97. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid = 314310&tool = pmcentrez&rendertype = abstract. Accessed 13 Jul 2010.
20. Hamaguchi M, Seno T, Yamamoto A, Kohno M, Kadoya M, et al. (2010) Loxoprofen Sodium, a Non-Selective NSAID, Reduces Atherosclerosis in Mice by Reducing Inflammation. Journal of Clinical Biochemistry and Nutrition 47: 138–147. Available: http://joi.jlc.jst.go.jp/JST.JSTAGE/jcbn/10-33?from = CrossRef.
21. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA (1987) Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 68: 231–240. Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd = Retrieve&db = PubMed&dopt = Citation&list_uids = 3426656.
22. Usui S, Hara Y, Hosaki S, Okazaki M (2002) A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. Journal of lipid research 43: 805–14. Available: http://www.ncbi.nlm.nih.gov/pubmed/11971952.
23. Okazaki M, Usui S, Ishigami M, Sakai N, Nakamura T, et al. (2005) Identification of unique lipoprotein subclasses for visceral obesity by component

analysis of cholesterol profile in high-performance liquid chromatography. Arteriosclerosis, thrombosis, and vascular biology 25: 578–84. Available: http://www.ncbi.nlm.nih.gov/pubmed/15637308. Accessed 16 Oct 2010.

24. Ross R (1999) Atherosclerosis–an inflammatory disease. The New England journal of medicine 340: 115–26. Available: http://www.ncbi.nlm.nih.gov/pubmed/9887164.
25. Pan J-H, Sukhova GK, Yang J-T, Wang B, Xie T, et al. (2004) Macrophage migration inhibitory factor deficiency impairs atherosclerosis in low-density lipoprotein receptor-deficient mice. Circulation 109: 3149–53. Available: http://www.ncbi.nlm.nih.gov/pubmed/15197138. Accessed 6 Jul 2011.
26. Tanaka T, Fukunaga Y, Itoh H, Doi K, Yamashita J, et al. (2005) Therapeutic potential of thiazolidinediones in activation of peroxisome proliferator-activated receptor gamma for monocyte recruitment and endothelial regeneration. European journal of pharmacology 508: 255–65. Available: http://www.ncbi.nlm.nih.gov/pubmed/15680279. Accessed 10 Sep 2010.
27. Ghosh S, Karin M (2002) Missing pieces in the NF-kappaB puzzle. Cell 109 Suppl: S81–96. Available: http://www.ncbi.nlm.nih.gov/pubmed/11983155. Accessed 6 Sep 2010.
28. Yousefipour Z, Oyekan A, Newaz M (2009) Role of G protein-coupled receptor kinase-2 in peroxisome proliferator-activated receptor gamma-mediated modulation of blood pressure and renal vascular reactivity in SHR. American journal of nephrology 30: 201–8. doi:10.1159/000218061.
29. Straus DS, Pascual G, Li M, Welch JS, Ricote M, et al. (2000) 15-deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. Proceedings of the National Academy of Sciences of the United States of America 97: 4844–9. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid = 18320&tool = pmcentrez&rendertype = abstract. Accessed 7 Aug 2011.

Galectin-3 (Gal-3) induced by leukemia microenvironment promotes drug resistance and bone marrow lodgment in chronic myelogenous leukemia

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Bone marrow (BM) microenvironment (BMME) constitutes the sanctuary for leukemic cells. In this study, we investigated the molecular mechanisms for BMME-mediated drug resistance and BM lodgment in chronic myelogenous leukemia (CML). Gene-expression profile as well as signal pathway and protein analyses revealed that galectin-3 (Gal-3), a member of the β-gal–binding galectin family of proteins, was specifically induced by coculture with HS-5 cells, a BM stroma cell-derived cell line, in all five CML cell lines examined. It was also found that primary CML cells expressed high levels of Gal-3 in BM. Enforced expression of Gal-3 activated Akt and Erk, induced accumulation of Mcl-1, and promoted in vitro cell proliferation, multidrug resistance to tyrosine kinase inhibitors for Bcr-Abl and genotoxic agents as a result of impaired apoptosis induction, and chemotactic cell migration to HS-5–derived soluble factors in CML cell lines independently of Bcr-Abl tyrosine kinase. The conditioned medium from Gal-3–overexpressing CML cells promoted in vitro cell proliferation of CML cells and HS-5 cells more than did the conditioned medium from parental cells. Moreover, the in vivo study in a mice transplantation model showed that Gal-3 overexpression promoted the long-term BM lodgment of CML cells. These results demonstrate that leukemia microenvironment-specific Gal-3 expression supports molecular signaling pathways for disease maintenance in BM and resistance to therapy in CML. They also suggest that Gal-3 may be a candidate therapeutic target to help overcome BMME-mediated therapeutic resistance.

Philadelphia-positive leukemia | bone marrow niche | chemoresistance | minimal residual disease

Chronic myelogenous leukemia (CML) is characterized by Bcr-Abl fusion tyrosine kinase (TK) as a result of the Philadelphia chromosome (Ph). Major advances in the treatment of CML have resulted from molecularly targeted therapeutic agents, such as imatinib mesylate (IM), which is the first-in-class Bcr-Abl TK inhibitor (TKI) and the more potent second-generation TKIs, such as nilotinib and dasatinib (Das) (1, 2). However, the complete elimination of CML clones has rarely been achieved by TKIs because of a variety of cell-intrinsic and cell-extrinsic protective mechanisms. The former include Bcr-Abl–related mechanisms, such as point mutations in the Abl kinase domain, and a variety of molecular abnormalities unrelated to Bcr-Abl (3–9). The latter include support of the bone marrow (BM) microenvironment (BMME), the so-called leukemia niche, which consists of soluble factors and supporting tissues, such as BM stromal cells (BMSCs), extracellular matrix (ECM), or hypoxia (10–19). Various new agents have been proposed for overcoming cell-intrinsic mechanisms for drug resistance (20, 21), and the precise molecular mechanisms for CML cell protection and maintenance by BMME sanctuary are not yet fully understood.

With the aim of developing new therapeutic strategies to overcome BMME-mediated protection of CML cells, we investigated the molecular mechanisms regulated by BMME, e.g., BMSCs and ECM, which enable leukemic cells to reside in the BM niche. Our study identified the involvement of galectin-3 (Gal-3), a member of the β-gal–binding galectin family of proteins, in BMME-mediated cell proliferation, protection, and BM lodgment. Gal-3 associates with cell proliferation, migration, adhesion, and apoptosis (22–26), and moreover is associated with disease progression, metastasis, and drug resistance in various cancers (27–31), but the role of Gal-3 in leukemia has remained largely unknown.

Results

Identification of Gal-3 as Candidate Mediator of Leukemia Proliferation and Drug Resistance Caused by BMME. Leukemic cells are supported not only by BMSCs (and their secretion) but also by ECM (13, 32). We first used coculture with HS-5, a BMSC-derived cell line, to examine whether the acquisition of resistance to cell death by TKIs or by genotoxic agents is induced in Ph-positive (Ph^+) CML cell lines (MYL, K562, BV173, and KCL22). HS-5, an immortalized human BMSC-derived cell line, potently secretes various hematopoietic growth factors (33). The coculture rendered both MYL cells and K562 cells partly resistant to TKIs, doxorubicin (DOX), cytarabine (CA), etoposide (i.e., VP16), and vincristine (VCR). BV173 cells, which are primarily resistant to induction of cell death by TKIs, did not become more resistant to IM and Das as a result of coculture with HS-5, but they acquired resistance to cell death induced by DOX, CA, VP16, and VCR. KCL22 also acquired resistance to cell death by DOX (Fig. S1*A*). Ph-negative (Ph^-) cell lines and Jurkat T (i.e., Jurkat) and HL60 cells also acquired resistance to cell death by DOX by the coculture with HS-5 (Fig. S1*B*). We next used microarray-based assays to investigate the changes in gene expression profiles in MYL cells as a result of coculture with HS-5 and adhesion to fibronectin (FN). In MYL with HS-5 or MYL with FN, 902 and 910 genes were up-regulated more than 2.0-fold, respectively, whereas 563 and 550 genes were down-regulated by less than half of their expression levels, respectively, in comparison with levels of control (Fig. S1*C* and Table S1).

Author contributions: J.K. and M.T. designed research; M.Y.-S., J.K., E.A., H.N., T.K., Y.M., N.S., Y.S., M.K., R.N., K.A., T.T., S.H., and T.M. performed research; K.A., N.U., Y.K., and T.M. contributed new reagents/analytic tools; M.Y.-S., J.K., E.A., H.N., T.K., Y.M., N.S., Y.S., M.K., R.N., T.T., N.U., Y.K., S.H., and T.M. analyzed data; and M.Y.-S., J.K., and M.T. wrote the paper.

The authors declare no conflict of interest.

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Among the 284 genes commonly up-regulated in MYL with HS-5 and MYL with FN, we focused on Gal-3 as one of the candidate mediators of BMME-mediated leukemia proliferation/protection because of its pleiotropic cellular function, the interaction with cell signaling molecules downstream of Bcr-Abl TK (Fig. S1*D*), and the association with progression of various cancers (27–31). Because the levels of *galectin-3* mRNA increased 3.84-fold as a result of coculture with HS-5, and 2.83-fold as a result of adhesion to FN in MYL cells, it was likely that Gal-3 was induced by cell adhesion and was further increased by HS-5–derived soluble factors. The induction of Gal-3 by the coculture with HS-5 was also confirmed at the protein level not only in MYL cells, but also in all leukemic cell lines examined regardless of their Ph status, whereas Gal-3 protein expression was absent or extremely low in normal liquid culture (Fig. 1*A*), suggesting that Gal-3 is especially inducible in the presence of BMME components in leukemic cells.

Gal-3 Is Highly Expressed in Primary Treatment-Naive CML Cells in BM. We next investigated the expression of Gal-3 in BM-derived primary leukemic cells from 25 Ph^+ leukemias (20 CMLs and five acute leukemias) and six Ph^- patients with acute leukemia. Of the leukemic cells of 20 patients with CML, those of all but one Ph^+ patient with blast crisis phase leukemia were positive for Gal-3. Ph^+ cells from CML in chronic phase (CP) were especially highly positive for Gal-3 expression. In contrast, the frequency of Gal-3–positive cells from most patients with acute leukemia was as low as that of BM hematopoietic cells from healthy volunteers, regardless of Ph status (Table S2 and Fig. 1*B*). In normal BM, cells of myeloid/monocytic series, but not of lymphoid or erythroid series, were positive for Gal-3. These results suggest that Gal-3 expression in the BM milieu is more predominant in CML, especially in CML-CP.

Gal-3 Overexpression Promotes Cell Proliferation and Chemotactic Cell Migration and Confers Drug Resistance to Leukemic Cells in Vitro. To characterize the role of Gal-3 in CML, we generated Gal-3 stably overexpressing MYL (MYL/G3) and K562 (K562/G3) subcell lines (Fig. S2). Gal-3 overexpression conferred moderately higher in vitro proliferation potency to both cell lines in medium containing 10% FCS as well as in low-nutrient 1% FCS-containing medium (Fig. 2*A*), whereas the growth of cells transfected with the mock plasmid was not significantly different from that of their respective parental cells. Both MYL/G3 and K562/G3 were less sensitive than their respective parental cells to cell death induced by TKIs as well as by conventional anticancer agents (Fig. 2*B* and Fig. S3*A*). Gal-3–overexpressing Ph^- Jurkat/G3 cells were also less sensitive to conventional anticancer agents (Fig. S3*B*). This diminished sensitivity to cell death caused by chemotherapeutic agents was caused by a reduction in apoptosis (Fig. 2*C*). In contrast, the drug sensitivity of cells transfected with the mock plasmid was not significantly impaired. To confirm that MYL/G3 acquired a drug-resistant phenotype as result of Gal-3 overexpression, we examined the effect of an inhibitor for Gal-3, fractionated citrus pectin powder (FPP) (34), on MYL cells and MYL/G3 cells. MYL and MYL/G3 showed similar sensitivity to cell death induced by FPP, whereas the addition of FPP overcame resistance to IM-induced cell death in MYL/G3 cells (Fig. 2*D*). Furthermore, the addition of FPP overcame HS-5–induced resistance against IM in MYL cells (Fig. S3*C*). We also investigated the role of Gal-3 in cell migration of leukemic cells by using conditioned medium (CM) from HS-5 cells (CM/HS-5) as the source of BMSC-derived chemotactic stimuli. CM/HS-5–stimulated cell migration of MYL cells and Gal-3 overexpression further promoted chemotactic and nonchemotactic cell migration in MYL cells (Fig. 2*E*). These findings revealed that Gal-3 overexpression promotes cell proliferation, multidrug resistance, and cell migration in CML cells.

We also examined the involvement of extracellular Gal-3 in the resistance to cell death by chemotherapeutic agents and in the cell migration ability of leukemic cells. Gal-3 concentrations in CM from MYL (CM/MYL), CM from MYL/G3 (CM/MYL/G3), CM from K562 (CM/K562), and CM from K562/G3 (CM/K562/G3) were 0.25 ng/mL, 0.77ng/mL, 0.19 ng/mL, and 9.49 ng/mL, respectively. The addition of recombinant human Gal-3 protein (rhGal-3; ProSci) up to 10.0 ng/mL did not confer CML cell lines more resistance to IM or DOX (Fig. S4*A*), and did not promote cell migration of leukemic cell (Fig. S4*B*), indicating that intracellular Gal-3 expression is essential for the higher resistance to apoptosis and the higher cell migration ability of leukemic cells.

CM from Gal-3–Overexpressing CML Cells Contains More Proliferative Factors for Leukemic Cells and BMSCs. Leukemic cells excrete growth factors, which stimulate the growth of adjacent leukemic cells as well as BM supporting cells via autocrine and paracrine loops, and thereby create a malignant niche (35). To investigate the involvement of Gal-3 in this scenario, MYL cells or HS-5 cells were cultured with media containing various ratios of CM/MYL and CM/MYL/G3 individually. MYL cells and HS-5 cells proliferated more at higher concentrations of CM/MYL/G3 (Fig. 3), indicating that MYL/G3 cells excrete more growth factors for both MYL cells themselves and BMSCs. These findings were the same for K562 and K562/G3 (Fig. S5). In contrast, the addition of rhGal-3 did not enhance the cell proliferation of MYL or HS-5 cells (Fig. 3 *C* and *D*), suggesting that an undefined soluble factor other than Gal-3 promotes the growth of leukemic cells and HS-5.

Molecular Sequelae Following Gal-3 Overexpression in Leukemic Cells. Coculture with HS-5 and enforced Gal-3 overexpression led to the activation of Akt and Erk in MYL and K562 (Fig. 4*A*). Moreover, coculture with HS-5 and Gal-3 overexpression resulted in the accumulation of Mcl-1, a member of the antiapoptotic Bcl-2 family of proteins, in MYL and K562, as well as a slight increase of Bim_{EL}, a proapoptotic BH3-only protein (Fig. 4*B*). This accumulation of Bim_{EL} may be the result of the accumulation of Mcl-1, which binds to Bim_{EL} in cytoplasm (36, 37). Although recent studies have identified Gal-3 as the substrate of c-Abl in solid cancers (38, 39), Gal-3 expression was not reduced by TKI in MYL and MYL/G3 (Fig. 4*C*), indicating that Gal-3 expression does not depend on Bcr-Abl TK activity in CML cells.

In Vivo Role of Gal-3 in Leukemia. Finally, we examined the in vivo role of Gal-3 in leukemia by studying a mouse CML model transplanted with MYL/mock cells (group A) or MYL/G3 cells

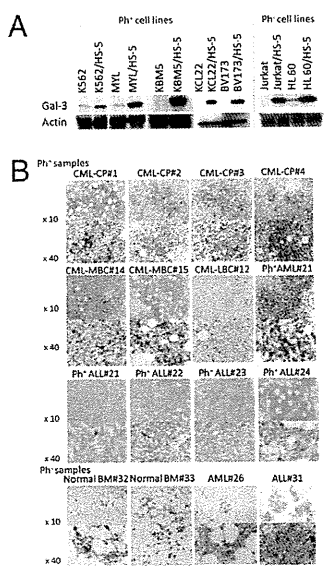

Fig. 1. Gal-3 expression in CML cell lines and primary CML cells. (*A*) Induction by coculture with HS-5 in CML cell lines. (*B*) Immunohistochemical staining of Gal-3 in patient-derived BM samples. Data are representative of the results for all patients examined (Table S2). LBC, lymphoid blast crisis; MBC, myeloid blast crisis.

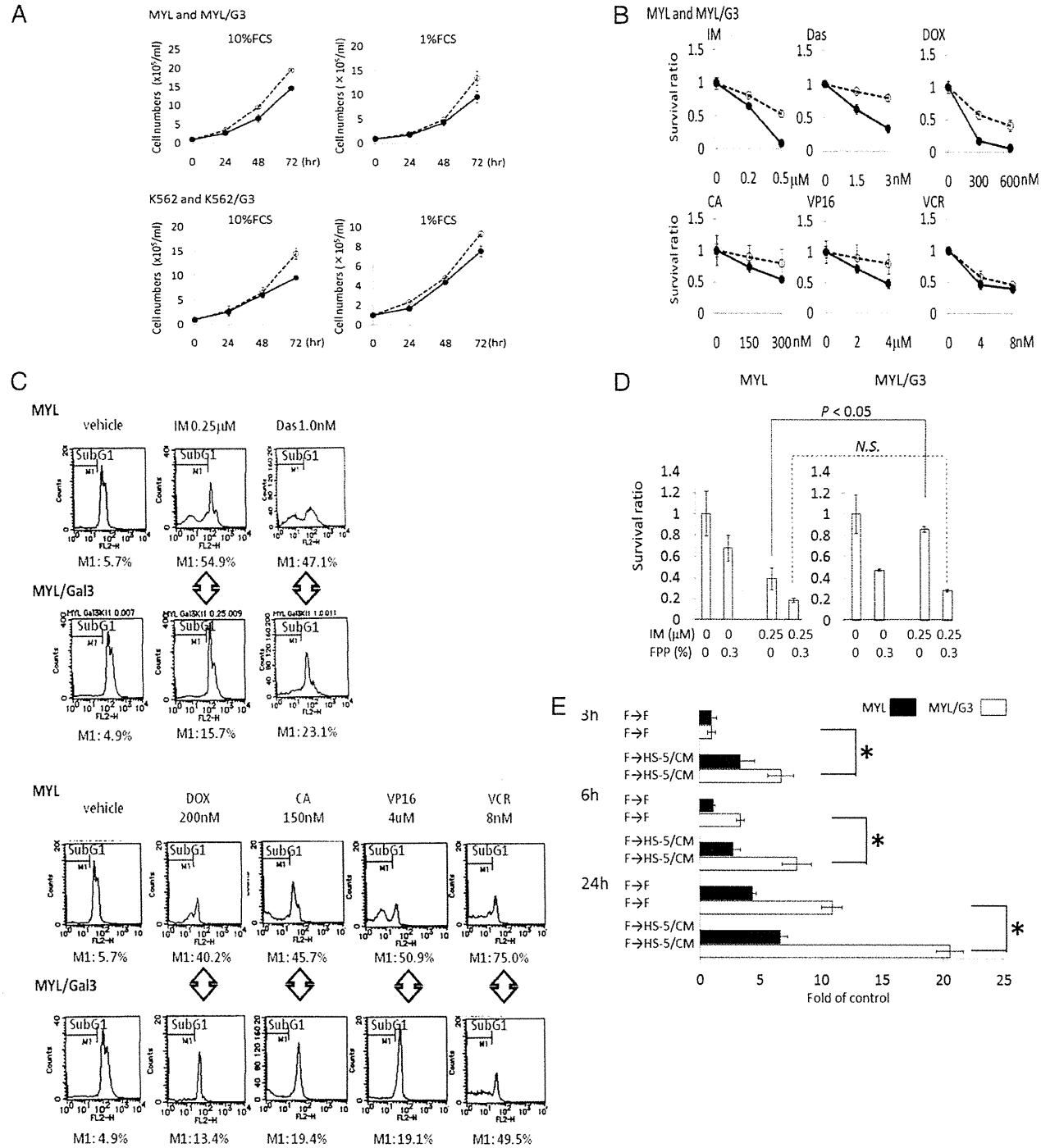

Fig. 2. Effects of Gal-3 overexpression on leukemic cell lines. (*A*) Cell proliferation potency. Solid lines represent parental cells and dotted lines represent Gal-3–overexpressing cells. (*B*) Cell-killing effects of TKIs and genotoxic agents. The *x* axis shows the drug concentration and the *y* axis shows survival cell ratio relative to untreated cells after 48 h treatment. Solid lines represent parental cells and dotted lines represent Gal-3–overexpressing cells. (*C*) Apoptosis induction determined by DNA content analyses. MYL and MYL/G3 cells were treated for 48 h with the agents indicated. The proportions of subG1 fractions (M1) were assumed to be cells undergoing apoptosis. (*D*) Gal-3 inhibitor overcomes Gal-3–induced resistance to cell death by IM in MYL cells. Cells were treated with IM and/or FPP for 48 h. *N.S.*, not statistically significant. (*E*) The effect of Gal-3 on chemotactic cell migration. The number of migrated cells in MYL after 3 h incubation was assumed to be 1.0. Asterisks indicate statistically significant differences ($P < 0.05$). F, serum-free medium; HS-5/CM, CM of HS-5 cells; F→HS-5/CM, upper chamber supplemented with serum-free medium and lower chamber filled with HS-5/CM. Bars indicate SD.

(group B). Although transplanted leukemic cells increased in a similar manner in the peripheral blood (PB) of both groups during the first 3 wk, the number of PB leukemic cells of group A mice then gradually decreased, whereas those of group B mice were preserved until death (Fig. 5*A*). The survival period of group A was significantly shorter than that of group B ($P = 0.025$; Fig.

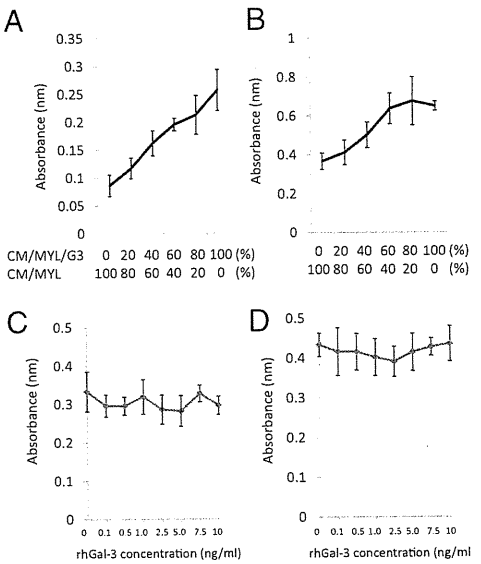

Fig. 3. CM of Gal-3–overexpressing cells contains more growth-promoting soluble factors. MYL cells (*A*) or HS-5 cells (*B*) were grown with mixtures of various concentrations of CM/MYL and CM/MYL/G3. MYL cells (*C*) or HS-5 cells (*D*) were grown in complete medium containing various concentrations of rhGal-3 for 96 h. Cell proliferation was determined by means of methylthiazol-diphenyl-tetrazolium (MTT) assay. An increasing in concentration of CM/MYL/G3 promoted the cell proliferation of MYL cells and HS-5 cells, whereas the addition of rhGal-3 up to 10 ng/mL did not.

5*B*); namely, all mice of group A died by day 48, whereas only one of seven mice in group B died during the observation period. Surprisingly, the sites of disease involvement at the mice's death showed major differences between the two groups. Most mice from group A showed extensive extramedullary involvement, such as intraabdominal, mediastinal, and/or s.c. tumors isolated from

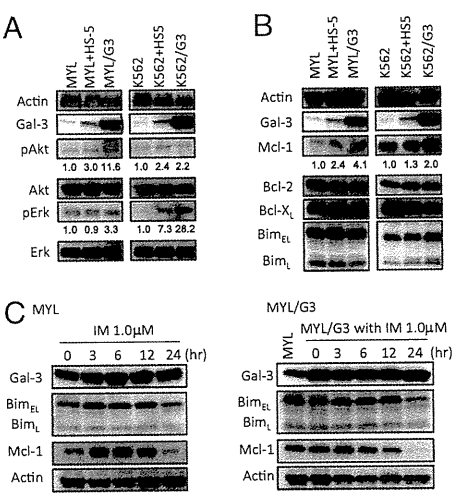

Fig. 4. Western blot analyses. Coculture with HS-5 and enforced Gal-3 overexpression induce phosphorylation of Akt and Erk (*A*), and causes Mcl-1 (*B*) to accumulate in MYL and K562. The expression levels of pAkt, Akt, pErk, Erk, Mcl-1, and Actin were calculated by using ImageJ software. The relative ratios of expression levels of pAkt/Akt, pErk/Erk, and Mcl-1/Actin of parental cells in normal cell culture were considered to be 1.0. (*C*) IM treatment (1.0 μM) for the indicated periods did not reduce Gal-3 in MYL and MYL/G3, whereas it caused accumulation of dephosphorylated Bim$_{EL}$ (faster migrated bands) and Bim$_L$. Mcl-1 accumulation was observed only when parental MYL cells were treated with IM.

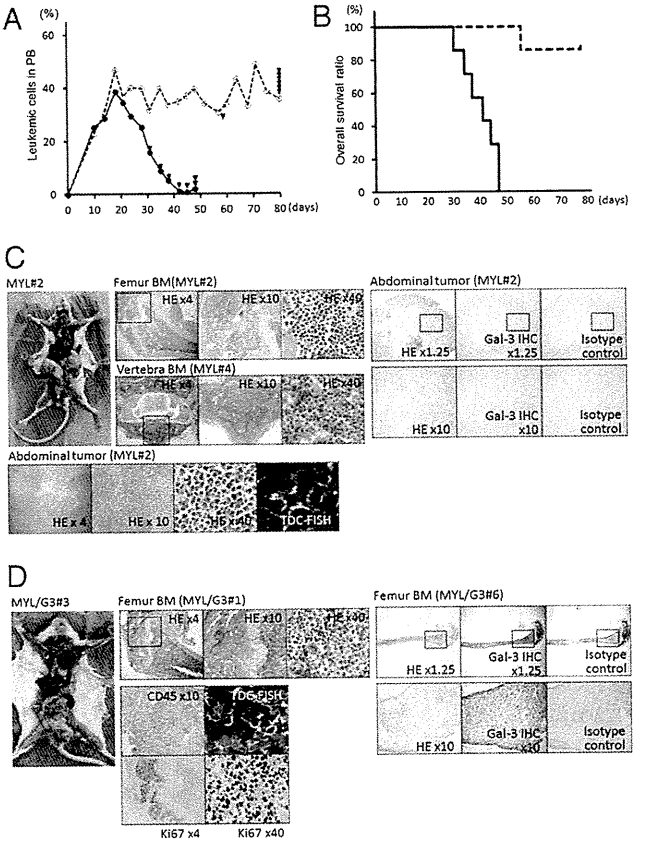

Fig. 5. In vivo role of Gal-3. (*A*) Percentages of transplanted leukemic cells in peripheral leukocytes of mice transplanted with MYL/mock cells (group A; solid line) and mice transplanted with MYL/G3 cells (group B; dotted line). The *x* axis shows days after transplantation and the *y* axis shows means ± SD of percentages of peripheral leukemic cells. Triangles indicate points of mouse deaths. (*B*) Overall survival periods of group A (solid line) and group B (dotted line). (*C* and *D*) Macroscopic and microscopic findings of mice transplanted with MYL/mock cells (*C*) or MYL/G3 cells (*D*). Data shown are representative of all mice examined (Table 1). In mice transplanted with MYL/mock cells, extensive extramedullary tumors (arrows) with Ph$^+$ Gal-3–negative leukemic cells [detected by tissue double color (TDC)-FISH and immunohistochemistry (IHC)] were identified in all mice (*C*). In contrast, none of the mice transplanted with MYL/G3 showed extramedullary involvement, whereas BM was at least partly replaced by Ph$^+$ leukemic cells expressing high level of Gal-3, which were also positive for human CD45 and Ki67 antigens (*D*). Direct invasion of leukemic cells outside BM was sometimes observed. HE, H&E staining.

BM, whereas only one of seven mice showed BM involvement at their time of death. In contrast, all mice in group B showed BM involvement, and sometimes outgrew BM, but none exhibited tumors isolated from BM (Fig. 5 *C* and *D*, Table 1, and Fig. S6). These findings suggest that Gal-3 overexpression facilitates BM homing and lodgment of CML cells. We also speculate that the reason for the shorter survival of group A mice is that the tumors expanded much faster when leukemic cells had advanced outside the BM, and that this may have had a significantly more deleterious effect on mice in group A than on mice in group B.

Discussion

The present study demonstrates that Gal-3 was specifically induced when leukemic cells were cultured with BMSCs in vitro, and that Gal-3 is predominantly expressed in CML cells, but not in acute leukemias. These findings prompted us to further investigate BMME-specific roles of Gal-3 in CML. As the results, enforced Gal-3 overexpression caused at least partial resistance to apoptotic induction by TKIs and genotoxic agents. As the

Table 1. Date of mice used in the present study

				Organ involvement at death				
		Max. leukemic cells in PB		BM invasion		Extramedullary tumor		
Mouse no.	Survival, d	%	Day	Femoral	Vertebral	Expansion from BM	Isolated from BM	Cause of death
MYL 1	31	36.8	14	—	—	—	Mediastinum	D
MYL 2	35	40.0	18	—	—	—	Abdominal cavity	D
MYL 3	38	53.6	18	—	—	Lower jaw	s.c. Tissue abdominal cavity	D
MYL 4	42	52.9	21	—	—	—	Abdominal cavity axillary LN	D
MYL 5	45	38.0	28	—	—	—	Abdominal cavity s.c. tissue	D
MYL 6	48	42.6	18	+	+	—	s.c. Tissue abdominal cavity	D
MYL 7	48	42.0	21	—	—	—	s.c. Tissue abdominal cavity, soft tissue around the testis	D
MYL/G3 1	57	73.3	14	+	+	Sacrum	—	B
MYL/G3 2	80	57.7	71	+	+	Sacrum	—	E
MYL/G3 3	80	50.0	48	+	+	—	—	E
MYL/G3 4	80	54.0	71	+	+	Sacrum	—	E
MYL/G3 5	80	48.0	18	+	+	Sacrum, right femur	—	E
MYL/G3 6	80	48.0	68	+	+	—	—	E
MYL/G3 7	80	57.14	24	+	+	—	—	E

B, Loss of >10% body wight; D, deterioration caused by tumor; E, euthanasia; LN, lymph node; Max.: maximum.

levels of drug resistance in Gal-3 gene transferred leukemic cells were similar to those in parental leukemic cell lines cocultured with HS-5, the inducibility of Gal-3 may at least partly explain the underlying molecular mechanisms of BMME-mediated drug resistance. As the molecular sequelae of Gal-3 overexpression, Erk and Akt, which are the essential downstream signaling molecules of Bcr-Abl (40), are activated in CML cells in a Bcr-Abl–independent manner. Simultaneously, Mcl-1 increased as the result of Gal-3 overexpression in CML cells. These results were consistent with those of previous studies showing that BMSC support activates Erk and Akt and increases Mcl-1 (41, 42), and the present study suggested Gal-3 as one of the positive mediators for these processes. Moreover, it has been reported that Gal-3 has an NWGR motif seen in the BH1 domain of Bcl-2 and may promote cell survival by interacting with Bcl-2 (27, 43). Bcl-2 family proteins have been shown to directly regulate cellular fate in the context of Bcr-Abl TK signaling, and Bim is essential for apoptosis by means of the blockade of Bcr-Abl TK signaling (36, 44–46). Because Mcl-1 protects mitochondrial integrity by binding to and keeping Bim_{EL} in check, and also inactivates other BH3-only proteins essential for genotoxic damage-induced apoptosis (47), Mcl-1 overexpression induced by Gal-3 may constitute one of the mechanisms for drug resistance of CML cells in BMME. Because Gal-3 expression induced by the BM milieu was not influenced by Bcr-Abl TK activity, Gal-3 induced by BM milieu stimuli may further augment the signaling for leukemia progression in combination with Bcr-Abl TK signaling, and also may maintain downstream pathways active even during treatment with TKIs.

In addition, the present study suggested the model that Gal-3 overexpression in CML cells exerts cell-extrinsic growth-promoting effects on CML cells as well as BMSCs, thereby accelerating the positive feedback mechanisms for leukemia proliferation and maintenance in the BM milieu in CML (Fig. S7), and promotes BM lodgment of CML cells in vivo. Although a number of studies have aimed to establish CML animal models by using xenograft models with human leukemic cells or transgene of *bcr-abl* into murine hematopoietic cells, most models have failed to recapitulate human CML-CP, which is clinically silent with persistent leukemic cell proliferation in BM and PB. Like the mice in group A in the present experiments, the survival periods of most previous CML models are frequently short as a result of progressive extramedullary involvements with or without BM leukemic lesion (48–51). The underlying molecular mechanism for this difference has remained unverified so far, but BMME-specific induction of Gal-3 expression in leukemic cells may be a clue to help solve this uncovered question. Also, soluble factors excreted by Gal-3–overexpressing CML cells, which promote this positive feedback machinery, are currently under investigation.

With respect to therapeutic applications, Gal-3 overexpression is expected to contribute to the generation of minimal residual disease as a result of the simultaneous promotion of BM lodgment and drug resistance, which makes the association between the expression levels of Gal-3 and the degree of response to TKIs a matter of considerable interest. However, because most patients with CML-CP with high levels of Gal-3 showed optimal response to TKIs (52), we underwrite the hypothesis that Gal-3 is a possible universal target in most patients with CML-CP, but is not a specific target in poor responders to TKIs. On the contrary, Gal-3 expression in leukemic cells in the advanced phase of CML and Ph^+ acute lymphoblastic leukemia is less than that of CML-CP; in addition, its expression does not differ significantly at onset and at relapse (Table S3). It is therefore important to verify that the loss of Gal-3 expression is mechanistically involved in disease stage progression and systemic organ dissemination in CML.

In conclusion, the present study disclosed that BMME-induced Gal-3 in CML cells may play an important role in drug resistance and leukemia lodgment in the BM milieu. Molecular-targeted agents against Gal-3, such as GCS-100, actually cause a decrease in Mcl-1 (53). The combined use of such compounds and TKIs is expected to be valuable for overcoming BMME-mediated protection of CML cells.

Materials and Methods

Cell Lines and Generation of Gal-3–Overexpressing Leukemic Cell Sublines. K562, BV173, KBM5 (American Type Culture Collection), MYL (54), and KCL22 (55) cell liens were established from Ph^+ patients with CML. Jurkat T is a human T-cell lymphoblast-like cell line, and HL60 (American Type Culture Collection) was established from cases of acute myelogenous leukemia; both are Ph^-. Gal-3–overexpressing subcell lines of MYL, K562, and Jurkat cells were generated by means of transfection of pEF1Galec3.neo plasmid (gift from Fu-Tong Liu, University of California, Davis, CA) (56). MYL and K562 cells were also transfected with a mock pEF1 plasmid empty vector as control, and were designated as MYL/mock and K562/mock, respectively. Following coculture assays, leukemic cells were positively isolated from HS-5 cells by using CD45 Microbeads and MiniMacs Separator (Miltenyi Biotec).

Microarray Analysis and Signal Pathway Analysis. MYL cells were cultured in normal medium on a noncoated plate as control, on a FN-coated plate, or on a plate preseeded with HS-5 for 48 h. Total RNA was isolated, and gene expression was analyzed with Affymetrix Gene Chip arrays and GeneChip Scanner 3000 (Affymetrix). Array data analysis was carried out with Affymetrix GeneChip operating software, version 1.0., and genes showing at least

a 2.0-fold difference in expression levels from control were considered to be positive. For signal pathway analysis, data were also analyzed with the Ingenuity pathway analysis software (Ingenuity Systems).

Mouse Xenografte Model for CML. Approval was obtained from the institutional review board at Kyoto University Hospital for a study using mice. Fourteen male NOD/SCID mice at 6 wk of age were sublethally irradiated (2 Gy), and 1.0×10^6 MYL/mock cells (group A) or 1.0×10^6 MYL/G3 cells (group B) were transplanted i.v. via their tail veins into seven mice each. Body weight and the percentage of leukemic cells in PB were monitored at least twice per week until day 80. For survival analysis, death was determined by spontaneous death or elective killing as a result of pain, the loss of more than 10% of maximum body weight of the individual mouse, or suffering or dying according to established criteria. All survived mice were subjected to euthanasia on day 80. We performed a macroscopic as well as microscopic analysis of BM of femoral bone and vertebra, and also of the tumors in each mouse at death. Tissue dual-color FISH was performed as previously described (57). The data shown are representative of three independent experiments.

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1. Jabbour E, Fava C, Kantarjian H (2009) Advances in the biology and therapy of patients with chronic myeloid leukaemia. *Best Pract Res Clin Haematol* 22:395–407.
2. Santos FP, Ravandi F (2009) Advances in treatment of chronic myelogenous leukemia —new treatment options with tyrosine kinase inhibitors. *Leuk Lymphoma* 50(suppl 2): 16–26.
3. Donato NJ, et al. (2004) Imatinib mesylate resistance through BCR-ABL independence in chronic myelogenous leukemia. *Cancer Res* 64:672–677.
4. Gorre ME, et al. (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 293:876–880.
5. Hu Y, et al. (2004) Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. *Nat Genet* 36:453–461.
6. La Rosée P, Deininger MW (2010) Resistance to imatinib: Mutations and beyond. *Semin Hematol* 47:335–343.
7. Milojkovic D, Apperley J (2009) Mechanisms of resistance to imatinib and second-generation tyrosine inhibitors in chronic myeloid leukemia. *Clin Cancer Res* 15:7519–7527.
8. Perrotti D, Jamieson C, Goldman J, Skorski T (2010) Chronic myeloid leukemia: Mechanisms of blastic transformation. *J Clin Invest* 120:2254–2264.
9. Soverini S, et al. (2005) ABL mutations in late chronic phase chronic myeloid leukemia patients with up-front cytogenetic resistance to imatinib are associated with a greater likelihood of progression to blast crisis and shorter survival: A study by the GIMEMA Working Party on Chronic Myeloid Leukemia. *J Clin Oncol* 23:4100–4109.
10. Damiano JS, Hazlehurst LA, Dalton WS (2001) Cell adhesion-mediated drug resistance (CAM-DR) protects the K562 chronic myelogenous leukemia cell line from apoptosis induced by BCR/ABL inhibition, cytotoxic drugs, and gamma-irradiation. *Leukemia* 15: 1232–1239.
11. Gregory MA, et al. (2010) Wnt/Ca2+/NFAT signaling maintains survival of Ph+ leukemia cells upon inhibition of Bcr-Abl. *Cancer Cell* 18:74–87.
12. Krämer A, et al. (1999) Adhesion to fibronectin stimulates proliferation of wild-type and bcr/abl-transfected murine hematopoietic cells. *Proc Natl Acad Sci USA* 96: 2087–2092.
13. Meads MB, Gatenby RA, Dalton WS (2009) Environment-mediated drug resistance: A major contributor to minimal residual disease. *Nat Rev Cancer* 9:665–674.
14. Takeuchi M, et al. (2010) Glyoxalase-I is a novel target against Bcr-Abl+ leukemic cells acquiring stem-like characteristics in a hypoxic environment. *Cell Death Differ* 17: 1211–1220.
15. Vianello F, et al. (2010) Bone marrow mesenchymal stromal cells non-selectively protect chronic myeloid leukemia cells from imatinib-induced apoptosis via the CXCR4/CXCL12 axis. *Haematologica* 95:1081–1089.
16. Wang Y, et al. (2007) Adaptive secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) mediates imatinib and nilotinib resistance in BCR/ABL+ progenitors via JAK-2/STAT-5 pathway activation. *Blood* 109:2147–2155.
17. Weisberg E, et al. (2008) Stromal-mediated protection of tyrosine kinase inhibitor-treated BCR-ABL-expressing leukemia cells. *Mol Cancer Ther* 7:1121–1129.
18. Williams RT, den Besten W, Sherr CJ (2007) Cytokine-dependent imatinib resistance in mouse BCR-ABL+, Arf-null lymphoblastic leukemia. *Genes Dev* 21:2283–2287.
19. Yokota A, et al. (2010) Osteoclasts are involved in the maintenance of dormant leukemic cells. *Leuk Res* 34:793–799.
20. Bixby D, Talpaz M (2009) Mechanisms of resistance to tyrosine kinase inhibitors in chronic myeloid leukemia and recent therapeutic strategies to overcome resistance. *Hematology (Am Soc Hematol Educ Program)* 2009:461–476.
21. Cilloni D, Messa E, Rotolo A, Saglio G (2010) Emerging drugs for chronic myeloid leukemia. *Expert Opin Emerg Drugs* 15:175–184.
22. Krześlak A, Lipińska A (2004) Galectin-3 as a multifunctional protein. *Cell Mol Biol Lett* 9:305–328.
23. Matarrese P, et al. (2000) Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties. *Int J Cancer* 85:545–554.
24. Matarrese P, et al. (2000) Galectin-3 overexpression protects from cell damage and death by influencing mitochondrial homeostasis. *FEBS Lett* 473:311–315.
25. Nangia-Makker P, Nakahara S, Hogan V, Raz A (2007) Galectin-3 in apoptosis, a novel therapeutic target. *J Bioenerg Biomembr* 39:79–84.
26. Thijssen VL, Poirier F, Baum LG, Griffioen AW (2007) Galectins in the tumor endothelium: opportunities for combined cancer therapy. *Blood* 110:2819–2827.
27. Fukumori T, Kanayama HO, Raz A (2007) The role of galectin-3 in cancer drug resistance. *Drug Resist Updat* 10:101–108.
28. Kim SJ, et al. (2008) Increased serum 90K and Galectin-3 expression are associated with advanced stage and a worse prognosis in diffuse large B-cell lymphomas. *Acta Haematol* 120:211–216.
29. Saussez S, Camby I, Toubeau G, Kiss R (2007) Galectins as modulators of tumor progression in head and neck squamous cell carcinomas. *Head Neck* 29:874–884.
30. Shekhar MP, Nangia-Makker P, Tait L, Miller F, Raz A (2004) Alterations in galectin-3 expression and distribution correlate with breast cancer progression: Functional analysis of galectin-3 in breast epithelial-endothelial interactions. *Am J Pathol* 165: 1931–1941.
31. Wang Y, et al. (2009) Regulation of prostate cancer progression by galectin-3. *Am J Pathol* 174:1515–1523.
32. Nair RR, Tolentino J, Hazlehurst LA (2010) The bone marrow microenvironment as a sanctuary for minimal residual disease in CML. *Biochem Pharmacol* 80:602–612.
33. Roecklein BA, Torok-Storb B (1995) Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. *Blood* 85:997–1005.
34. Jackson CL, et al. (2007) Pectin induces apoptosis in human prostate cancer cells: correlation of apoptotic function with pectin structure. *Glycobiology* 17:805–819.
35. Colmone A, et al. (2008) Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science* 322:1861–1865.
36. Kuroda J, Taniwaki M (2009) Involvement of BH3-only proteins in hematologic malignancies. *Crit Rev Oncol Hematol* 71:89–101.
37. Wuillème-Toumi S, et al. (2007) Reciprocal protection of Mcl-1 and Bim from ubiquitin-proteasome degradation. *Biochem Biophys Res Commun* 361:865–869.
38. Balan V, Nangia-Makker P, Jung YS, Wang Y, Raz A (2010) Galectin-3: A novel substrate for c-Abl kinase. *Biochim Biophys Acta* 1803:1198–1205.
39. Li X, et al. (2010) c-Abl and Arg tyrosine kinases regulate lysosomal degradation of the oncoprotein Galectin-3. *Cell Death Differ* 17:1277–1287.
40. McCubrey JA, et al. (2008) Targeting survival cascades induced by activation of Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways for effective leukemia therapy. *Leukemia* 22:708–722.
41. McMillin DW, et al. (2010) Tumor cell-specific bioluminescence platform to identify stroma-induced changes to anticancer drug activity. *Nat Med* 16:483–489.
42. Balakrishnan K, Burger JA, Wierda WG, Gandhi V (2009) AT-101 induces apoptosis in CLL B cells and overcomes stromal cell-mediated Mcl-1 induction and drug resistance. *Blood* 113:149–153.
43. Akahani S, Nangia-Makker P, Inohara H, Kim HR, Raz A (1997) Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Res* 57:5272–5276.
44. Kuroda J, et al. (2006) Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. *Proc Natl Acad Sci USA* 103:14907–14912.
45. Kuroda J, et al. (2007) Apoptosis-based dual molecular targeting by INNO-406, a second-generation Bcr-Abl inhibitor, and ABT-737, an inhibitor of antiapoptotic Bcl-2 proteins, against Bcr-Abl-positive leukemia. *Cell Death Differ* 14:1667–1677.
46. Shah NP, et al. (2008) Transient potent BCR-ABL inhibition is sufficient to commit chronic myeloid leukemia cells irreversibly to apoptosis. *Cancer Cell* 14:485–493.
47. Villunger A, et al. (2003) p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 302:1036–1038.
48. Ilaria RL, Jr. (2004) Animal models of chronic myelogenous leukemia. *Hematol Oncol Clin North Am* 18:525–543, vii.
49. Kuroda J, et al. (2003) The third-generation bisphosphonate zoledronate synergistically augments the anti-Ph+ leukemia activity of imatinib mesylate. *Blood* 102:2229–2235.
50. Ren R (2002) The molecular mechanism of chronic myelogenous leukemia and its therapeutic implications: studies in a murine model. *Oncogene* 21:8629–8642.
51. Van Etten RA (2001) Models of chronic myeloid leukemia. *Curr Oncol Rep* 3:228–237.
52. Baccarani M, et al., European LeukemiaNet (2006) Evolving concepts in the management of chronic myeloid leukemia: Recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood* 108:1809–1820.
53. Streetly MJ, et al. (2010) GCS-100, a novel galectin-3 antagonist, modulates MCL-1, NOXA, and cell cycle to induce myeloma cell death. *Blood* 115:3939–3948.
54. Ito T, Tanaka H, Kimura A (2007) Establishment and characterization of a novel imatinib-sensitive chronic myeloid leukemia cell line MYL, and an imatinib-resistant subline MYL-R showing overexpression of Lyn. *Eur J Haematol* 78:417–431.
55. Kubonishi I, Miyoshi I (1983) Establishment of a Ph1 chromosome-positive cell line from chronic myelogenous leukemia in blast crisis. *Int J Cell Cloning* 1:105–117.
56. Yang RY, Hsu DK, Liu FT (1996) Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc Natl Acad Sci USA* 93:6737–6742.
57. Matsumoto Y, et al. (2004) Detection of t(14;18) in follicular lymphoma by dual-color fluorescence in situ hybridization on paraffin-embedded tissue sections. *Cancer Genet Cytogenet* 150:22–26.

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The current status of umbilical cord blood collection in Japanese medical centers: Survey of obstetricians

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ABSTRACT

As the first step of UCB banking, UCB collection has an important role in banking procedures. The aim of this study was to reveal the current status of UCB collection and discuss the management of the UCB bank. We conducted a questionnaire survey at medical centers collecting UCB, followed by semi-structured interviews with some respondents. Out of 38 institutes, 11 respondents (28.9%) thought that collection of UCB in addition to their routine medical services puts a burden on physicians. The obstetricians involved in the UCB collection are generally willing to participate in the procedure under current circumstances at medical institutes.

1. Introduction

Since the first report of successful umbilical cord blood (UCB) transplantation in 1988 there has been great interest in the use of cord blood as an alternative source of stem cells with which to treat cancer and genetic diseases [1,2]. With the increased recognition that the umbilical cord constitutes a viable source of stem cells, cord blood banks have been established worldwide to provide a large number of high-quality cord blood units to transplant centers [1,3]. In Japan there are 11 public cord blood banks, which form a network by communicating with each other and exchanging information. Comprehensive clinical summaries of UCB transplantation demonstrate that it is as effective as bone marrow transplantation, and without serious adverse events.

Organs including heart, lung, liver, kidney, and eye are defined by Organ Transplant Law as "internal organs" and are regulated by the Ministry of Health, Labour and Welfare (MHLW) in Japan. Accordingly, transplantation of these organs is strictly regulated by this law. A blood product for transfusion generally has associated medical charges that are similar to those of drugs. Such products are regulated by the Pharmaceutical Affairs Law (PAL), which includes the mandatory imposition of Good Manufacturing Practice (GMP) rules, and permission for manufacturing and market approval is issued by the MHLW. In contrast, bone marrow transplantation is defined as a medical practice, and is not regulated by the PAL, which means that GMP is not mandated for the preparation of products arising from this procedure. Although UCB shares characteristics with other blood products, the PAL does not apply to UCB.

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Generally, UCB banking involves the following procedures: (i) donor recruitment, informed consent, and testing of maternal donors; (ii) collection of the cord blood units; (iii) processing, freezing, and testing of the cord blood units; and (iv) release of cord blood units to a transplant center [1]. At many Japanese hospitals patients have the option of donating their cord blood free of charge to the public cord blood banking system. Currently there are 11 public cord blood banks in Japan, providing access to genetically unrelated cord blood. Alternatively, for a certain fee patients are able to have their cord blood stored with a commercial or private company for future use within their own family (private cord blood banking) [4,5].

Although the medical cost of UCB transplantation is covered by national health insurance, the banking procedure is not covered by such official systems in Japan. To maintain the sterility of cellular products and to manage the tracking record to deal with possible infectious diseases, Good Tissue Practice (GTP) has been introduced recently in the USA [6] and Japan [7]. Compliance to FDA 21 CFR part 1271 is obligated because UCB is regarded as a "tissue" and all cord blood banks have to follow the legislation. UCB is also defined as a tissue in the UK. Cord blood banks have been given licenses by the UK Government: the Human Tissue Authority operates under the European Union's Tissues and Cells Directive to regulate collection and use of UCB. Under GTP and also GMP regulations it is expected that cellular products remain clean and that the consistency of product characteristics are well controlled throughout preparation [8]. However, UCB are generally not processed in accordance with GTP and GMP because they are not regulated by the PAL in Japan. Thus, there are concerns about the quality of UCB stem cells and the management of infectious disease. Infection control is critical for securing the safety of cord blood transplantation [8]. UCB cells infection control is in fact secured by autonomous standards [9] of the UCB banks and by the efforts of the collecting centers. The regional cord blood banks provide both SOPs [10] and training. In Japan, pregnant women are screened for infectious diseases at their prenatal visit. Safety is secured by history taking and blood test prior to delivery, covering infections including rubella, toxoplasma, HTLV-1, chlamydiosis, HIV, hepatitis B and C, and syphilis. The results of these tests are available when screening UCB donors, and infected donors are excluded from UCB collection, even though they havenot been specially screened for UCB donation. Furthermore, SOPs require a blood test within 24 h prior to delivery or within a week after delivery. Moreover, the patient's anamnesis, including family medical history with up to three degrees of kinship, and the risks of hereditary disorder and infectious disease, are evaluated at all institutes.

To our knowledge, so far no critical issues relating to infection have been reported, and additionally the clinical outcomes of UCB stem cell transplantation seem reasonable [11]. These facts made us interested in how obstetricians involved in the collection of cord blood cells think and how they perform the procedure, since the collection of cord blood is the first step in the process of UCB transplantation, in terms of the "manufacture" of drug products. Therefore, quality is important. To investigate these issues, in this study we conducted a questionnaire survey with representative obstetricians involved in collecting UCB at medical centers that have contracts with a public cord blood bank. Subsequently, a semi-structured interview survey was performed with some respondents to further understand how they manage UCB.

2. Methods

2.1. Questionnaire survey

We first conducted an initial survey of obstetricians at medical centers collecting UCB in connection with the Japanese public cord blood bank network, by sending a confidential self-complete-type questionnaire by mail. The self-administered questionnaire was developed with reference to items used in previous studies [8], and was finalized after expert review by bank personnel. The questionnaire items were as shown in Table 1. Currently, there are 106 medical centers collecting UCB in Japan, which communicate with the 11 public cord blood banks (Fig. 1). We chose four public cord blood banks in two metropolitan areas in Japan that are supplied by a large number of medical centers (shown in bold in Fig. 1) and sent the questionnaire to 53 such centers, covering 50% of the total number of medical centers collecting cord blood for the public cord blood bank network. The medical centers contacted comprised 13 supplying UCB to Keihan cord blood bank, 17 supplying Hyogo cord blood bank, 12 supplying The Metro Tokyo Red Cross Cord Blood Bank, and 11 supplying Tokai University Cord Blood Bank. The survey was conducted in July to September 2008. We asked the medical centers for the questionnaire to be filled out by the obstetricians who mainly collected UCB in the institute.

2.2. Semi-structured interview survey

Subsequently we conducted semi-structured interviews with representatives of eight medical centers collecting cord blood, comprising five institutes supplying Hyogo cord blood bank, one institute supplying Tokyo Red Cross Cord Blood Bank, and two institutes supplying Tokai University Cord Blood Bank. We selected these medical centers because they agreed to be interviewed when the questionnaire survey was performed. A semi-structured individual interview was conducted including the following question items: (i) Standard Operating Procedures (SOPs) and training courses prior to collecting cord blood; (ii) burden and risks associated with UCB collection; (iii) infection testing for donor eligibility; and (iv) coverage of UCB banking by national health insurance.

3. Results

3.1. Questionnaire survey

A total of 38 institutes out of 53 responded to the questionnaire, a response rate of 72%. The results are as shown in Table 1.

The number of physicians collecting UCB was 10 or fewer in 90% of the institutes sampled. There were five people

Table 1
Questionnaire survey results.

Number (%) of respondents (n = 38)	Yes	No
1. How many physicians participate in UCB collection at your institute?		
5 or fewer	17(44.7)	
6–10	17(44.7)	
11–15	3(7.9)	
15 or more	1(2.6)	
2. Is UCB collected under sterile conditions?*	30(78.9)	6(15.8)
3. Are there SOPs which relate to cord blood collection?	32(84.2)	6(15.8)
In cases where SOPs exist, is there any documents relating to the method of transportation to the cord blood bank? (n = 32)*	24(75.0)	7(21.9)
4. Do you perform a training course prior to collecting cord blood?	15(39.5)	23(60.5)
In cases where no training course exists, do you think it would be appropriate to initiate such a program? (n = 23)*	11(28.9)	9(39.1)
5. How many cases do you collect in a month at your institute?		
10 or fewer	22(57.9)	
11–20	5(13.2)	
21–30	5(13.2)	
31–40	3(7.9)	
41–50	3(7.9)	
6. Do you have enough physicians involved in UCB collection at your institute?	36(94.7)	2(5.3)
7. Does the collection of cord blood put any burden on your regular clinical work as an obstetrician?*	11(28.9)	26(68.4)
8. Do you think there are any risks for the pregnant woman relating to UCB collection?	6(15.8)	32(84.2)
9. Do you perform routine screening for possible infectious disease in the pregnant woman?	34(89.5)	4(10.5)

* In cases where the number of Yes and No answers does not add up to the indicated sample size, some respondents failed to provide an answer.

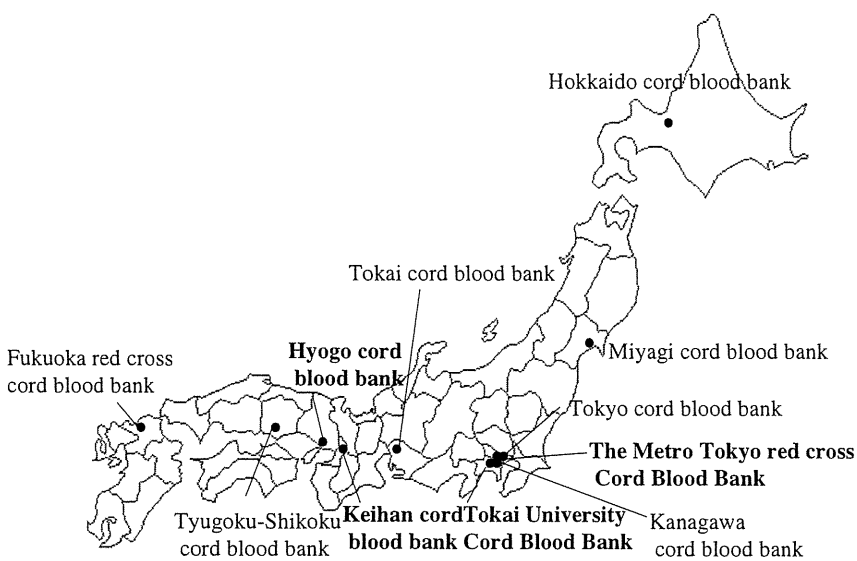

Fig. 1. Location of 11 public cord blood banks of Japan cord blood bank network.

or fewer at 17 institutes (44.7%) and six to 10 at another 17 institutes (44.7%). With regard to the sterile collection of UCB, 30 respondents (78.9%) thought that the procedure is performed in a sterile fashion. On the other hand, six respondents (15.8%) gave a negative answer and two respondents (5.3%) did not answer this question. Although 32 respondents (84.2%) indicated that there are SOPs for cord blood collection at their institute, six respondents (15.8%) did not. Fifteen respondents (39.5%) indicated that there is a training course for collecting UCB at their institute. In contrast, 23 respondents (60.5%) indicated that there is no training course at their institute; however, 11 of these 23 respondents felt that training is necessary prior to the collection of UCB.

With regard to the collection opportunities per month, 22 institutes (57.9%) indicated that there were 10 or fewer opportunities, five institutes (13.2%) were in the 11–20 range, and another five institutes (13.2%) indicated 21–30 opportunities. In addition, 36 respondents (94.7%) felt that the number of physicians participating in collection of UCB at their institute was appropriate. Twenty-six respondents (68.4%) thought that collection of UCB in addition to their routine medical services does not put a burden on physicians.

Thirty-two respondents (84.2%) indicated that UCB collection does not pose a risk to pregnant women prior to or after delivery. Screening tests for infection are performed prior to delivery at 34 institutes (89.5%).

3.2. Semi-structured interviews

The interviewees for the semi-structured interviews comprised representatives from eight medical centers, including one private university hospital, one public hospital, three private hospitals and three private clinics. Eight respondents answered the interview.

3.3. SOPs and training courses

All eight respondents answered that all SOPs documents are supplied by the related public bank and that they use the latest versions of the procedures. One institute uses documents after some modification from the original version. With regard to the sterile collection of UCB, the collection kits are sent to the institutes by the cord blood banks. Six respondents answered that if cord blood is simply collected, a training course is not mandatory. However, a training course prior to collecting UCB would be of great help for yielding large amounts of UCB cells and preventing bacterial contamination.

3.4. Burden and risks associated with UCB collection

With regard to the burden and risks associated with UCB collection, it seems that the task does not pose any burden to the respondents in this study, since the bank prepares the SOPs and supplies equipment required for collection. One respondent pointed out that, since UCB transplantation is performed on volunteer donors, it is worthwhile even though it creates extra work. In addition, all respondents suggested that neither risks nor burdens are posed to volunteer donors because collection is stopped immediately if something goes wrong with the procedure. It suffices to say that two respondents answered that the preparation of documents is a burden. Another respondent answered that it takes time to explain the procedure to the donor.

3.5. Infection testing for donor eligibility

With regard to infection testing, all the eight respondents indicated that no additional examination was required since pregnant women are screened for infectious diseases at their prenatal visit during the first trimester. Furthermore, at six of the eight institutes blood samples are tested for infection upon delivery of the baby.

3.6. Coverage of UCB banking by national health insurance

Because the UCB banking process is currently not covered by national health insurance in Japan and therefore the preparation of UCB may not be performed sustainably, we included this issue in the interview. One respondent pointed out that volunteer donation required no insurance coverage, as regular blood donation for transfusion is not covered by insurance. This respondent indicated that there is a need for the organized management of the banks because voluntary participants in cord blood banks may not always be able to manage them in a stable and reliable manner. Another respondent pointed out that since preserved UCB cells are not always used for transplantation and are sometimes destroyed, insurance coverage is inapplicable.

3.7. Other comments

Five respondents indicated that the standard of preservation is strict. Because neither UCB of insufficient volume nor UCB with any bacterial contamination is preserved, the number of collected UCB samples that are cryopreserved is much less than the total number of UCB samples collected. Five respondents answered that the UCB is not transported to the bank at weekends. Two respondents answered that transportation relies on volunteers. Two respondents indicated that the operation of the banks needs more financial support to maintain good medical performance.

4. Discussion

In this study we revealed the current status of UCB collection in Japanese medical centers and elicited opinions from the obstetricians involved in cord blood collection by conducting a questionnaire survey and semi-structured interviews. To eliminate selection bias, we choose four public cord blood banks in two metropolitan areas covering 50% of all centers. Out of a Japanese population of 130 million, the number of newborns in 2008 was 1.09 million and the number of cord blood transplants performed in that year was 928 [12]. Since the first UCB transplant was performed more than 20,000 such transplants have been reported worldwide [13] and in Japan the cumulative number of UCB transplants by 2009 was 6019. The number of cord blood provided each year is increasing every year (Fig. 2) (data from the Japan cord blood bank network). The frequency of UCB collection varied depending on the scale of the medical centers and the motivation of the obstetricians.

Several questions were suboptimal worded and answers reflect confusion. It seems that six respondents (15.8%) might have a misunderstood the SOPs or did not know what the abbreviation SOPs stands for. It might have affected that SOPs is not technical term in medicine and this abbreviation is not widely used in Japan. We found that six institutes have no SOPs, including four institutes which have only five or fewer physicians participate in the UCB collection, or ten or fewer cases are collected in a month. Responders at these institutes stated that they have no SOPs. Then six respondents (15.8%) who gave a negative answer seem to have evaluated themselves strictly about sterile conditions. In the free comment column of the questionnaire survey two respondents pointed out that the level of sterility was not clear. Recently, cell manufacturing at the GMP level has been advocated in Japan, so it seems that six respondents were considering GMP when responding to the questionnaire.

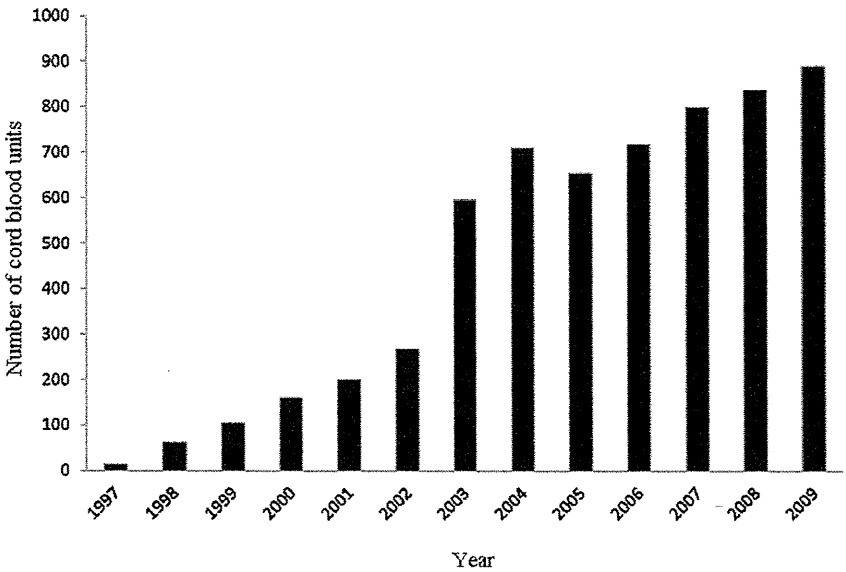

Fig. 2. The number of cord blood provided each year in Japan. Source: Japan cord blood bank network, 2010.

Cord blood banks have a minimum volume limit as the first selection criterion for UCB donations in order to store clinically useful UCB units. In our interview survey we found out that because neither UCB of insufficient volume nor UCB with any bacterial contamination was preserved, the number of collected UCB units that went forward to cryopreservation was much less than the total number collected. This indicates that quality-control standards at medical centers are strict. Actually only high-quality UCB is used in transplantation because of the strict selection criteria. Consequently, the clinical outcome of UCB transplantation in Japan seems to be above average comparing other countries. Routinely updated SOPs for UCB collection are supplied adequately to the medical centers by the cord blood banks, and this contributes to a high level of quality control for UCB collection. Judging from clinical results [14–18] it is thought that Japan cord blood banks provide UCB for transplantation within the appropriate system.

The Japanese cord blood bank service is different from the European and North American ones. The Japanese system is not a government initiative but is voluntarily established by parent organizations around the country. UCB banking is not covered by national health insurance in Japan. Only cord blood transplantation is covered by insurance and 174,000 yen is paid to the bank of parent organization by the hospital. They add up to only a tiny fraction of the price that entails a lot of costs about total banking processing, and obstetrician's extra work is not paid for. However, limited appreciation rewards are paid and the equipment is provided free of charge by the bank to the collecting center. Management of each public bank is supported by limited funds from the government, and it is very difficult to acquire extra financial support from elsewhere to improve the quality of the UCB banking procedure. We wanted to investigate physicians' views and opinions of the lack of cover by national health insurance.

Blood can be collected from the cord while the placenta is still *in utero* after vaginal delivery (*in vivo* collection) or after the placenta has been delivered (*in vitro* collection). The technique of collection varies between different cord blood banks and medical centers. In the questionnaire survey only 11 respondents (28.9%) thought that collection of UCB in addition to their routine medical services puts a burden on physicians. Thirty-two respondents (84.2%) answered that UCB collection does not pose a risk to pregnant women prior to or after delivery. In the interview survey all respondents suggested that UCB collection procedures seem to impose little burden on the obstetrician and are of almost no risk to donors because collection is stopped immediately if something goes wrong with the procedure. Two respondents in the interview survey answered that preparation of documents or providing explanation to the donor is a burden. However, many obstetricians have strong motivation, and they feel no additional burden and risks. In addition, they were satisfied with the current circumstances. They deemed UCB collection to be worthwhile despite the procedure being insufficiently paid, and they were satisfied with donors being volunteers. These results suggested that the obstetricians involved in the UCB collection are generally willing to participate in the procedure under current circumstances at medical institutes. It seems that obstetricians also feel discomfort at the proposal of collecting UCB for a fee. The banks are additionally funded by subsidies, funds from the parent organization such as Hyogo College of Medicine for Hyogo cord blood bank, and donations. In addition, some operation relies on volunteers. In the interview survey it was expressed that the reliance of some banking procedures, such as transportation of collected UCB to the banks, being partly managed by volunteers leads to the fragile operation of UCB banking.

Recently, discussions have started over whether UCB should be regulated as a medical product in Japan in line

with the regulations in foreign countries. In Japan, if UCB were to be re-defined as a blood product for transfusion, as are biopharmaceuticals derived from specific living organs, then the banking process would be covered by national health insurance and other frameworks would be in place to ensure quality control, such as GMP. UCB collection requires certain quality control; however, if UCB preparation was regulated like a drug, then UCB would be regulated by the PAL. Clinical trials are needed under Good Clinical Practice (GCP) to obtain regulatory approval under PAL. Such clinical trials would take time and have huge costs. Taken together, as the clinical outcomes of cord blood transplantation in Japan are comparable with those from other countries, it may be difficult to define UCB as a drug and for it to be regulated by the PAL. Nevertheless, steady financial support from the government is critical for operating cord blood banks and UCB collecting sites to ensure the future sustainable performance of cord blood transplantation.

The questionnaire was not designed to demonstrate potential flaws in the collection process and no data were obtained about on-site storage or shipping irregularities that would impact product quality. The result of this survey simply represent only about obstetricians' consciousness. However, if not all, this survey should help us learn about comprehension of the banking quality control. The interview survey presented here had some limitations including that it consisted of a small, select sample of the quite large number of participating obstetricians in the hospitals. Although there might have been some bias, the answers from the interview survey were very informative that could not have been obtained by questionnaire survey only. With limited time and budget these eight medical centers covered the two Japanese metropolitan areas of Kanto and Kansai evenly. The data obtained in this study could also provide a basis for future studies.

Conflict of interest

The authors declare no conflict of interest

Acknowledgments

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References

[1] Ballen KK. New trends in umbilical cord blood transplantation. Blood 2005;105:3786–92.
[2] Rubinstein P. Why cord blood? Hum Immunol 2006;67:398–404.
[3] Brunstein CG, Wagner JE. Umbilical cord blood transplantation and banking 2006. Annu Rev Med 2006;57:403–17.
[4] Sullivan MJ. Banking on cord blood stem cells. Nat Rev Cancer 2008;8:554–63.
[5] Kurtzberg J, Lyerly AD, Sugarman J. Untying the Gordian knot: policies, practices, and ethical issues related to banking of umbilical cord blood. J Clin Invest 2005;115:2592–7.
[6] [6] Food and Drug Administration: 21CFR Parts16, 1270, and 1271 Current Good Tissue Practice for Human Cell, Tissue, and Cellular and Tissue-Based Product Establishments; Inspection and Enforcement; Final Rule.
[7] Ministry of Health, Labour and Welfare, Japan. Notice for quality and safety of cellular and tissue-based pharmaceuticals and devices; 2008.
[8] McCullough J, McKenna D, Kadidlo D, Schiermqn T, Wagner J. Issues in the quality of umbilical cord blood stem cells for transplantation. Transfusion 2005;45:832–41.
[9] Japan cord blood bank network: Guidance for UCB collection.
[10] Japan cord blood bank network: Standard Operating Procedures for UCB collection.
[11] Nagamura-Inoue T, Kai S, Azuma H, Takanashi M, Isoyama K, Kato K, et al. Unrelated cord blood transplantation in CML: Japan cord blood bank network analysis. Bone Marrow Transplant 2008;42:241–51.
[12] Health and Welfare Statistics Association: Journal of Health and Welfare Statistics 2009; (9): 56
[13] Gluckman E. History of cord blood transplantation. Bone Marrow Transplant 2009;44:621–6.
[14] Laughlin MJ, Eapen M, Rubinstein P, Wagner JE, Zhang MJ, Champlin RE, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. N Eng J Med 2004;351:2265–75.
[15] Kurtzberg J, Prasad VK, Carter SL, Wagner JE, Baxter-Lowe LA, Wall D, et al. Results of the Cord Blood Transplantation Study (COBLT): clinical outcomes of unrelated donor umbilical cord blood transplantation in pediatric patients with hematologic malignancies. Blood 2008;112:4318–27.
[16] Sauter C, Barker JN. Unrelated donor umbilical cord blood transplantation for the treatment of hematologic malignancies. Curr Opin Hematol 2008;15:568–75.
[17] Advani AS, Laughlin MJ. Umbilical cord blood transplantation for acute myeloid leukemia. Curr Opin Hematol 2009;16:124–8.
[18] Atsuta Y, Suzuki R, Suzuki R, Nagamura-Inoue T, Taniguchi S, Takahashi S, et al. Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia. Blood 2009;113:1631–8.

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Antitumor effect of sunitinib against skeletal metastatic renal cell carcinoma through inhibition of osteoclast function

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We investigated the inhibitory effect of sunitinib, a newly approved multitargeted tyrosine kinase inhibitor, against the progression of renal cell cancer (RCC) bone metastases *in vivo*. *In vitro* cell proliferation was determined using the MTS assay. To investigate the inhibitory effects of sunitinib *in vivo*, we established luciferase-labeled ACHN^{Luc} cells derived from papillary RCC. Mice in which ACHN^{Luc} cells had been transplanted into the left ventricle to establish bone metastases were treated orally with 40 mg/kg/day sunitinib or vehicle control for 3 weeks. Growth of the cancer cells was monitored using an *in vivo* imaging system. In addition, 16 patients with metastatic RCC were treated with sunitinib, and serum and urine levels of amino-terminal telopeptide (NTx) were measured as markers of bone resorption. Sunitinib did not inhibit the growth of RCC cells *in vitro* at clinically or experimentally achievable serum levels (100 nM–1 μM). To investigate the inhibitory effect of sunitinib *in vivo*, we established luciferase-labeled human RCC cells (ACHN^{Luc}). Sunitinib prevented the growth of ACHN^{Luc} RCC cells in the bone metastatic mouse model. The number of osteoclasts in sunitinib-treated mice was significantly less than that in control mice. Serum and urine levels of NTx in patients with metastatic RCC declined significantly during the first 4 weeks of sunitinib treatment ($p = 0.027$). Sunitinib is a potent anticancer agent for RCC bone metastases, at least for papillary RCC.

Bone is a common site of metastasis, with the frequency of solitary or multiple metastases to bone ranging from 24 to 51% in patients with metastatic renal cell cancer (RCC).[1–3] Although bone metastasis is not an independent prognostic factor associated with poor survival, the prognosis of patients with bone metastasis is not favorable when they are treated with cytokines, with an average life expectancy of 8–16 months.[2–4] Moreover, bone metastases are associated with poor performance status due to intractable pain and pathological fractures.[5] Because treatment options for RCC patients with bone metastasis are limited, appropriate treatment strategies are desired.

Sunitinib is a newly approved, multitarget, small-molecule tyrosine kinase inhibitor for the treatment of metastatic RCC. It inhibits various receptor tyrosine kinases, including vascular endothelial growth factor (VEGF) receptors 1, 2 and 3; stem cell factor receptor (KIT) and PDGF receptors α and β.[6–8] Moreover, sunitinib has been known to inhibit the phosphorylation of colony-stimulating factor (CSF)-1R, resulting in the prevention of osteoclast function and CSF-1R-dependent osteolysis in an experimental breast cancer bone metastasis model.[9,10] These findings led us to propose the hypothesis that sunitinib may inhibit tumor growth and osteolysis in bone metastatic lesions in RCC patients.

Although establishing a treatment strategy for bone metastases from RCC is important for urologists, the assessment of inhibitory effects on the growth of bone metastases is often difficult in clinical practice. In this study, we show that sunitinib has anticancer as well as inhibitory activities against osteolysis in an experimental mouse model of bone metastasis of RCC cells.

Key words: renal cell carcinoma, bone metastases, sunitinib, *in vivo* imaging system
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Material and Methods

Animals, cell lines and reagents

Approval for these studies was obtained from the institutional review board at Akita University School of Medicine. Specific pathogen-free BALB/c *nu/nu* mice (CLEA, Kyoto, Japan) aged 7 weeks were used. The human RCC lines ACHN, CCFRC-1, CCFRC-2 and NC65 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and sunitinib was obtained from Pfizer (New York, NY).

Patients

A total of 16 native Japanese patients with metastatic RCC, who were treated at the Department of Urology at Akita University School of Medicine between 2008 and 2009, were enrolled, and the serum and urine levels of amino-terminal telopeptide (Serum NTx, normal range: 9.5–17.7 nmol/l) were measured as markers of bone resorption. The patients' characteristics are shown in Table 1. The median dose was 37.5 (25–50) mg/day and the median number of treatment cycles was 4.6 (1–21). Written informed consent was provided according to the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Akita University Graduate School of Medicine. The response was assessed by computed tomography (CT) after at least every two cycles of treatment, according to the Response Evaluation Criteria in Solid Tumors (RECIST ver. 1.0).[11]

Growth inhibitory effects of sunitinib in vitro

Cell proliferation was determined by the MTS assay using CellTiter96 (Promega Corporation, Madison) as described previously.[12]

Generation of a stable luciferase-expressing cancer cell line

Among the RCC cell lines we tested (ACHN, CCFRC-1, CCFRC-2 and NC65), ACHN was the only line that was transplanted into the left ventricle and formed bone metastases successfully. Therefore, we used $ACHN^{Luc}$ in the *in vivo* experiment. ACHN cells were stably transfected with the pGL3 control vector (Promega Corporation, Madison) and with pSV2Neo (ATCC), as described previously.[12] In brief, the cells were treated with 10 μg pGL3 control vector and 1 μg pSV2Neo vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM (Invitrogen) and selected using geneticin (400 μg/ml). Stable clones expressing luciferase were isolated and the clone with the highest level of luciferase expression (as determined by bioluminescence) was selected using luciferin (Xenogen, Alameda, CA) and an *in vivo* imaging system (IVIS; Xenogen).

In vivo effects of sunitinib

To produce bone metastasis models, RCC cell suspensions (3×10^6/100 μl phosphate-buffered saline) were injected into the left ventricle of mice under inhalation anesthesia with isoflurane (Abbott Japan, Tokyo, Japan). From 21 days after implantation, 14 mice with bone metastases were selected and divided into two matched groups on the basis of bioluminescence quantified by IVIS. On the same day, we started daily oral administration of 40 mg/kg (body weight) sunitinib or the solution used to dissolve sunitinib as vehicle control. According to the human 4 weeks on/2 weeks off schedule, mice were treated with sunitinib for 4 weeks before being sacrificed. Mice were observed by IVIS once per week.

Table 1. Patients characteristics

Factors			
Age (years old)	Median	60.5	Range: 37–80
Sex (*n*)	Male	13	(81%)
	Female	3	(19%)
Metastatic sites	Lung	12	(75%)
	Liver	4	(25%)
	Bone	5	(31%)
	Brain	3	(19%)
	Lymph node	3	(19%)
Follow-up period (month)	Median	4.5	Range: 1–37
Performance status	0 or 1	15	(94%)
	>1	1	(6%)
Diagnosis to initial treatment	>1 year	5	(31%)
	<1 year	11	(69%)
Blood hemoglobin	Normal range	7	(44%)
	ULN>	9	(56%)
Serum calcium	<10 mg/dl	15	(94%)
	≥10 mg/dl	1	(6%)
Serum LDH	<1.5 × ULN	16	(100%)
	≥1.5 × ULN	0	(0%)
MSKCC risk classification	Favorable	6	(38%)
	Intermediate	9	(56%)
	Poor	1	(6%)

ULN: upper limit of normal range.

Measurement of bone metastatic lesions by in vivo imaging

An aqueous solution of luciferin (150 mg/kg) was injected intraperitoneally 10 min before imaging. The animals were anesthetized with isoflurane and placed in the light-tight chamber of a CCD camera system (Xenogen) and photons emitted from the luciferase-expressing cells within the animal were quantified for 5 min using the software program Living Image (Xenogen) as an overlay on Igor (Wavemetrics, Seattle, WA). Using this *in vivo* imaging system, we evaluated the efficacy of sunitinib by measuring the photon counts of the metastatic lesions in the mandible and both hip joints in a blinded manner as described previously.[13]

Measurement of serum VEGF and M-CSF in the mouse bone metastasis model in vivo

The serum concentrations of VEGF and M-CSF in mice were determined using Quantikine ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. To investigate the serum concentrations of VEGF and M-CSF, sera from each of seven treated and seven untreated mice were collected and analyzed 4 weeks after $ACHN^{Luc}$ inoculation.

Histological analysis

After imaging studies, the femora of the mice were removed, frozen immediately and stored at −80°C. To detect osteoclasts, 4-μm-thick sections were stained with tartrate-resistant acid phosphatase (TRAP) using the TRAP and ALP double-stain kit (Takara Bio, Otsu, Japan), as described previously.[14] Three sections were examined in each femur. The number of TRAP-positive osteoclasts was counted per ten high-power microscope fields by two blinded examiners, as described previously.[14]

Statistical analysis

The influence of sunitinib on the growth of bone metastases was analyzed by Student's *t* test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 13.0; SPSS, Chicago, IL), and two-sided *p* values <0.05 were considered statistically significant.

Results

Effect of sunitinib on RCC growth in a mouse bone metastasis model in vivo

Injection of cancer cells via the left ventricle is an established method of inducing bone metastases, as reported previously.[12,13] In the present study, all mice that were successfully implanted with $ACHN^{Luc}$ cancer cells developed bone metastases 3 weeks after injection. Of these mice, we excluded those that showed brilliant bioluminescence in the lungs. The remaining 14 mice were then divided into two matched groups according to bioluminescence quantified by IVIS, and we administered either sunitinib or vehicle control for 4 weeks and monitored the growth of bone metastases in the lesions in the maxilla and bilateral hip joints, as described previously[12,13] (Fig. 1*b*). Metastatic bone lesions in the control group progressed during the 3 weeks. On the other hand, photon emission was significantly suppressed in the sunitinib treatment group ($p < 0.001$) (Figs. 1*a* and 1*c*). The mean body weights of the mice did not differ significantly between the two groups.

Serum VEGF and M-CSF in a mouse bone metastasis model in vivo

To examine the indirect antitumor effect of sunitinib, we measured the concentrations of VEGF and M-CSF. However, no significant difference was present in the serum concentrations of these growth factors between the two groups (Fig. 1*d*).

Effect of sunitinib on osteoclasts in a mouse bone metastasis model

Next, we investigated the efficacy of sunitinib against osteoclasts in the tumor-bearing mice. Femoral bone sections were stained with TRAP to enable counting of the number of osteoclasts, as described previously.[13] The mean number of TRAP-positive osteoclasts in mice treated with sunitinib was significantly lower than that in mice treated with vehicle control (23.1 ± 4.7 *vs.* 33.2 ± 7.9 osteoclasts/100 high-power fields, respectively; $p = 0.013$).

Sunitinib did not inhibit cell proliferation in vitro at a clinically achievable serum concentration

To assess the direct antitumor effect of sunitinib, four RCC cell lines (ACHN, CCFRC-1, CCFRC-2 and NC65) were cultured in the presence of various concentrations of sunitinib (0.1 nM–10 μM). Sunitinib inhibited the proliferation of these cell lines in a concentration-dependent manner (Fig. 2). However, sunitinib was not effective *in vitro* at the clinically achievable serum concentration (~80 nM), as demonstrated previously.[8] On the other hand, the serum concentration of sunitinib was reported to be ~100 nM on administration to mice at 40 mg/kg/day.[14] The IC50s of sunitinib for these cell lines were estimated to be >1 μM. These results suggest the involvement of an indirect growth inhibitory mechanism of sunitinib, at least partially, for bone metastatic lesions in mice.

Effect of sunitinib on serum and urine levels of NTx in patients with metastatic RCC

The characteristics and demographic data of the patients are shown in Table 1. As shown in Figure 3, both serum and urine levels of NTx significantly declined during the first 4 weeks of treatment with sunitinib ($p = 0.027$). During the holiday period when the administration was discontinued following 4 weeks of administration of sunitinib, the serum and urine levels of NTx showed gradual recovery (Fig. 3). Of these 16 patients, five had bone metastatic lesions, but we could not evaluate the efficacy of sunitinib quantitatively. Regarding the extraosseous sites, nine of 14 patients demonstrated a partial response (PR) or stable disease (SD) whereas the remaining five demonstrated progressive disease (PD). The reduction rate of the serum NTx level from the baseline in patients with favorable efficacy (PR/SD; 30.8%) was higher than that in patients with poor efficacy (PD; 22%), although the difference was not significant ($p = 0.6404$).

Discussion

In patients with metastatic RCC, bone is the major metastatic organ, second only to the lung.[1–3] Bone metastases were shown to be associated with severe bone pain, pathological fractures, spinal cord compression and a short survival

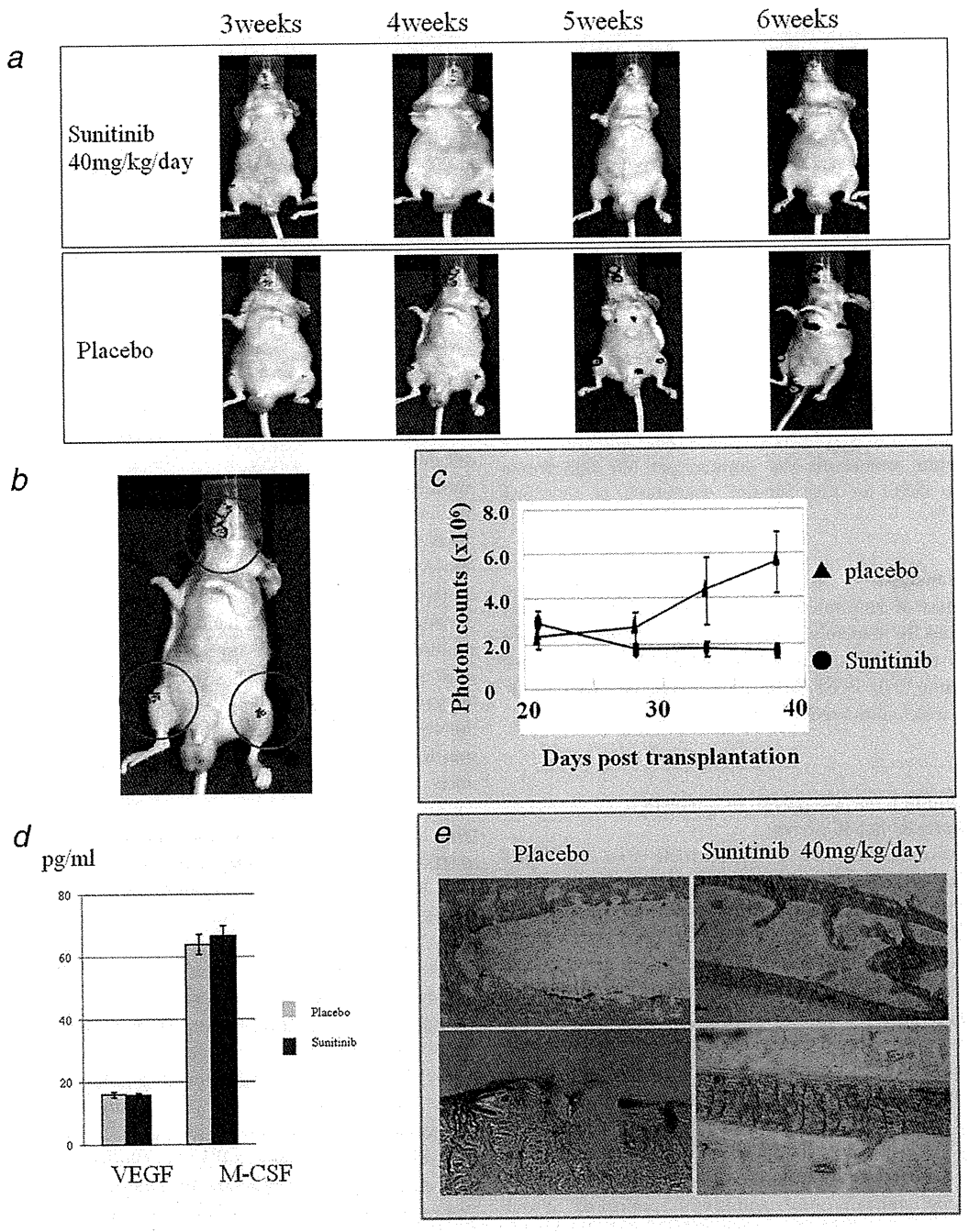

Figure 1. Growth inhibitory effect of orally administered sunitinib in an RCC bone metastatic mouse model. We established an RCC bone metastatic mouse model using the cell line ACHN^{Luc}. Images were obtained using an *in vivo* imaging system 3–6 weeks after cell transplantation by intracardiac injection (*a*). To evaluate the growth inhibitory effect of orally administered sunitinib, we selected metastatic lesions from the maxilla and bilateral hip joints as examples of bone metastasis (*b*). Average real-time growth curves of ACHN^{Luc} cells of bone metastatic lesions in sunitinib- and control vehicle-treated groups demonstrated that sunitinib significantly prevented the growth of metastatic bone lesions ($p < 0.001$; *c*). Serum levels of VEGF and M-CSF did not differ significantly between sunitinib-treated and control mice (*d*). The mean number of TRAP-positive osteoclasts in mice treated with sunitinib was significantly lower than that in mice treated with vehicle control ($p = 0.013$; *e*).

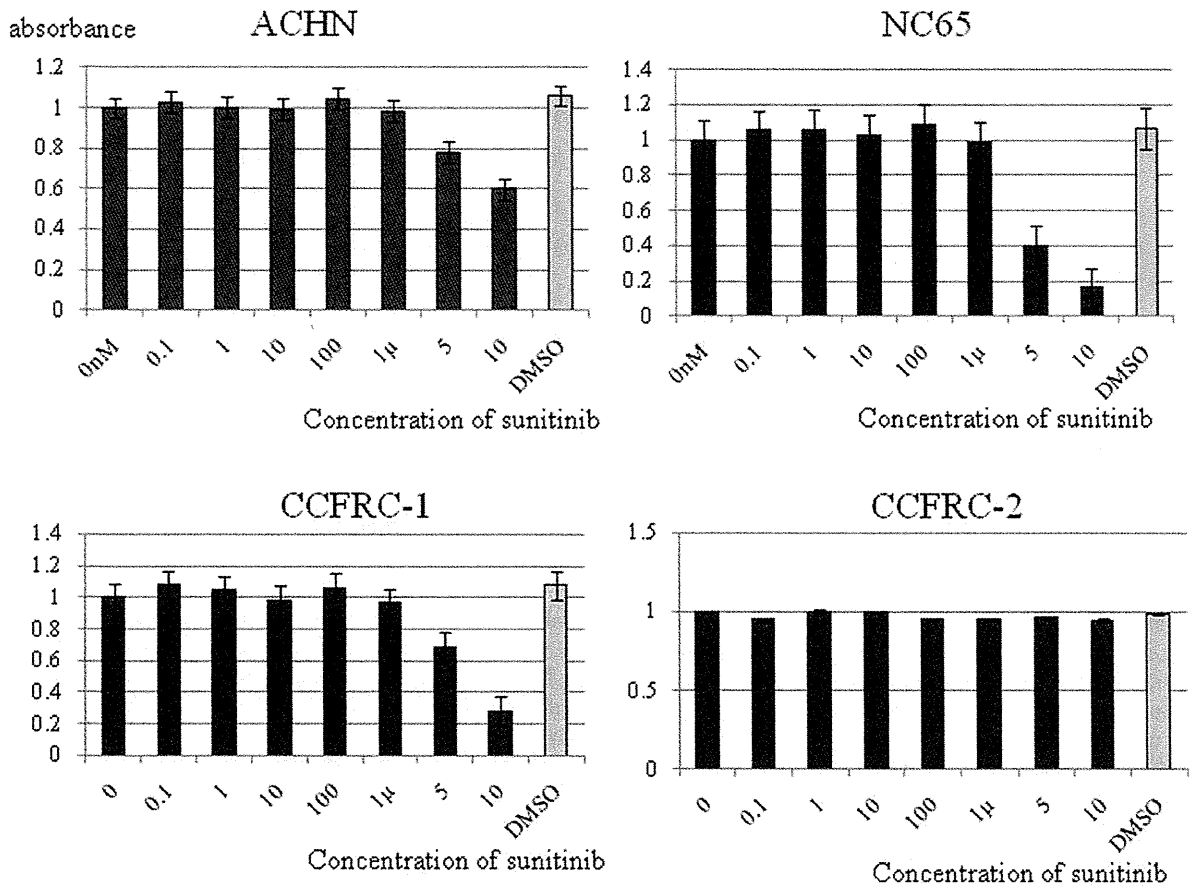

Figure 2. Sunitinib does not inhibit the growth of RCC at a clinically achievable concentration *in vitro*. Cells of the RCC lines ACHN, NC65, CCFRC-1 and CCFRC-2 were plated at 3,000 cells/well in 96-well plates, incubated for 24 hr, and then treated with various concentrations (0–100 mM) of sunitinib. After 72 hr of incubation, relative cell growth was measured in an MTS assay. Data are Mean ± SD. Sunitinib did not inhibit the growth of any of the four RCC cell lines at the clinically achievable concentration (~80 nM) *in vitro*.

period.[4,11] Several studies have demonstrated that bone metastasis is one of the risk factors for poor prognosis in the cytokine era, although it was not identified as an independent prognostic factor.[1–4] Négrier *et al.* investigated the prognostic factors of 782 metastatic RCC patients treated with cytokines and found that 32% (248/776) had bone metastases, and that these patients had a significantly worse prognosis than those without bone metastases ($p = 0.008$).[2] Recently, Naito *et al.* retrospectively analyzed the prognosis of 1,463 Japanese metastatic RCC patients in the cytokine era and demonstrated that 24.6% (320/1,302) had bone metastases, and that these patients also had a significantly worse prognosis than those without bone metastases ($p = 0.003$).[3] Accumulated evidence suggests that systemic immunotherapy is not effective in the management of bone metastasis of RCC.

The efficacy of sunitinib against RCC bone metastasis, however, remains to be established and is difficult to evaluate in clinical practice. Thus, we sought to investigate the efficacy of sunitinib against bone metastatic RCC in the preclinical setting. The dose of sunitinib used in this study (40 mg/kg/day) was intended to provide a serum level of sunitinib similar to that attained in the clinical setting.[8,15] Pharmacokinetic and pharmacodynamic analyses showed that the clinical dose of 50 mg/day led to plasma concentrations ranging from 50 to 100 ng/ml in humans.[8] This dose is equivalent to the plasma concentration in mice administered sunitinib at 40 mg/kg/day.[15] Data from VEGF-induced vascular permeability assays also support 50–100 ng/ml as the range, including the minimum plasma concentrations required to inhibit VEGFR and PDGFR *in vivo*.[8] Therefore, our results obtained in the RCC bone metastatic model used in this study might be reflective of those obtained in the clinical setting.

Similar to several other *in vitro* analyses, our results showed that sunitinib at concentrations of 50–100 ng/ml did not inhibit the proliferation of RCC cells *in vitro*.[10,16] Therefore, we sought an indirect mechanism for this *in vivo* growth inhibition of RCC bone metastases. Bone is an abundant repository for immobilized growth factors, including