vectors are highly efficient delivery systems and they are the most powerful tools for transfection so far. However, their clinical application is limited by toxic immune responses and random integration into host cells' DNA followed by insertional mutagenesis [49, 50, 163]. Thus, we will discuss non-viral DDS, especially lipid-based carriers and polymers below.

Cationic liposomes represent one of the most attractive carriers used for the delivery of nucleic acid-based drugs. Positively charged liposomes facilitate complex formation with negatively charged siRNAs and bind to cell membranes. After binding to the cell membrane, a siRNA/liposome complex enters the cytoplasm via endocytosis after which the complex escapes from the endosome and releases its siRNA to the RNAi machinery [164, 165] (Fig. (**3**)). N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), a synthetic cationic lipid, was first used to transfer plasmid DNA into mammalian cells [166]. Since then, numerous cationic liposomes have been generated for DDSs of DNAs and siRNAs. LIC contains the cationic lipid analogue 2-O-(2-DAME)-carbamoyl-1,3-O-dioleoylglycerol and phosphatidylcholine derived from egg. LIC nanoparticles are uniform in size, and the diameter of the siRNA/LIC complex is around 200 nm [167]. siRNA/LIC complexes were shown to inhibit the progression of urinary bladder cancer tumors and lung cancer tumors in xenograft mouse models [9, 64].

To protect the therapeutic nucleic acid in the siRNA/liposome complex from nuclease degradation in the bloodstream and to deliver the complex to the targeted sites, several liposome modifications have been tested. A modified cationic liposome comprising polyethylene glycol (PEG) residues was formulated [63]. Treatment using this pegylated liposome resulted in increased concentrations of siRNA in plasma as well as in the targeted tumors, and consequently the progression of tumors was inhibited and the survival rates of cancer-bearing mice were prolonged.

A stable nucleic-acid-liposome particle (SNALP) has been generated for systemic *in vivo* delivery of siRNAs. SNALPs are composed of a lipid bilayer containing a mixture of cationic and fusogenic lipids that enables cellular uptake and endosomal release of siRNAs. The SNALP surface is coated with a diffusible PEG-lipid conjugate that provides a neutral, hydropholic exterior and stabilizes the particle during formation [168]. Complexes formed with SNALPs and siRNAs against targeted mRNAs show excellent outcomes in several preclinical studies using mice and nonhuman primates [168-170], and it is expected that SNALPs will be suitable for application in cancer therapy.

Active, targeted delivery of siRNAs is necessary to obtain maximum benefits and minimum adverse effects. However, hematopoietic cells are intractable to the delivery methods, and to overcome this difficulty, Antibody (Ab)-combined DDSs have been developed. Nanometer-scale (around 80 nm in diameter) liposomes were formed from neutral phospholipids, and hyauronan was attached to the liposome outer surface and then modified with monoclonal Abs. The lyophilized lipid nanoparticles were rehydrated with water containing protamine-condensed siRNAs. Following intravenous administration of β7-integrin Ab-conjugated nanoparticles containing Cyclin D1 siRNAs, leukocyte proliferation and the expression of T helper cell 1 cytokines were suppressed in mice with colitis [171]. Ab-mediated delivery carriers are formed by generating a fusion protein containing cationic protamine and single-chain Ab fragment, and represent another type of DDS. HIV-1 envelope (gp160) Ab-loaded fusion protein combined with targeted siRNAs inhibited the growth of gp160-expressing B16 tumors [172]. These Ab-combined nanoparticles are expected to be applied to clinical settings.

In contrast to many kinds of cationic liposomes, anionic liposomes bound by Sialyl Lewis X (SLX) have been developed [173]. SLX is a sugar chain expressed on the surface of leukocytes that binds with E-selectin expressed on the surface of endothelial cells. Because SLX-combined liposomes (SLX-Lipo) are negatively charged, as are vascular endothelial cells and cells such as erythrocytes and leukocytes, these liposomes are sparred from the reticulo-endothelial system, phagocytosis by macrophages, and non-specific absorption with endothelial cells. In addition, due to the hydrophlilization of the liposome surface, retention of these liposomes in the bloodstream is prolonged. Consequently, Cy5.5-conjugated SLX-Lipo was shown to accumulate in tumors and at the sites of inflammation such as arthritis [173]. This unique liposome will be useful as a systemic delivery carrier for siRNAs.

Linear or branched cationic polymers can bind and condense large nucleic acids and have been used as transfection agents [174, 175]. Polymer complexes with siRNAs are taken up by cells into the cytoplasm *via* endocytosis and escape from the endosome. siRNAs are released into the cytoplasm by action of the proposed "proton sponge effect" mechanism in which polymers buffer the low endosomal pH through enhanced influx of protons and water, resulting in endosomal rupture [176]. Polyethyleneimine (PEI) is a synthetic polymer used broadly as a delivery carrier for nucleic acids, including DNAs, siRNAs, and oligonucleotides [177-179]. The intraperitoneal treatment of PEI/human epidermal growth factor 2 siRNA complex showed an inhibition of subcutaneous tumors [178]. Arg-Gly-Asp peptide-conjugated PEGylated PEI successfully delivered VEGFR2 siRNA to subcutaneous tumors by intravenous injection [180]. Cyclodextrin polymers have also been used as effective DDSs. The growth of Ewing's sarcoma tumors in a metastatic model was inhibited by the administration of EWS-FL11 siRNA in a cyclodextrin-containing polycation (CDP) assembly [181]. In another study, CDP nanoparticles with transferrin and PEG were loaded with the M2 subunit of ribonucleotide reductase (RRM2) siRNA and administered intravenously to monkeys in escalating doses. The results demonstrated the efficacy of CPDs self-assemble in a non-human primate [182], and in other studies it was shown that this particle is effective in knocking down *RRM2* mRNA in mice [183].

Atelocollagen is a natural polymer and its efficacy as an *in vivo* siRNA delivery system has been reported in many studies (Table **2**). Atelocollagen is type I collagen obtained from calf dermis. Amino acid sequences at the N- and C-termini of the collagen molecules are called telopeptide, and they have antigenecity of collagen molecules. As the telopeptide is removed from collagen molecules by pepsin treatment, atelocollagen shows low immunogenicity. There-

fore, atelocollagen has been shown to be a suitable biomaterial with an excellent safety profile and it is used clinically for a wide range of purposes. The molecular weight of atelocollagen is approximately 300,000 and the length is 300 nm. It forms a helix of 3 polypeptide chains [184]. Atelocollagen is positively charged, which enable binding to nucleic acid molecules. Moreover, at low temperature atelocollagen exists in liquid form, which facilitates easy mixing with nucleic acid solutions. The size of the atelocollagen-nucleic acid complex can be varied by altering the ratio of siRNA to atelocollagen. Because atelocollagen naturally forms a fiber-like structure under physiological conditions, particles formed a high concen-tration of atelocollagen persist for an extended period of time at the site of introduction, which is advantageous to achieve a sustained release of the associated nucleic acid (Table **2**). It has been confirmed that atelocollagen is eliminated through a process of degradation and absorption similar to the metabolism of endogenous collagen [184]. Alternatively, particles formed under conditions of low atelocollagen concentrations result in siRNA/atelocollagen complexes approximately 300 nm in size that are suitable for systemic delivery by intravenous administration (Table **2**). As atelocollagen accumulates in the liver, such atelocollagen could be used as a liver-directed DDS [10]. Atelocollagen complexes protect siRNA from degradation by nucleases and are transduced efficiently into cells, resulting in long-term gene silencing. Atelo llagen is an attractive non-viral DDS for siRNAs.

In the development of nucleic acids for use combination with such delivery carriers, many efforts h been made to identify siRNA modifications to overcome following problems: (1) nuclease degradation in bloodstream, (2) rapid uptake by reticuloendotherial syste and (3) nonspecific activation of the immune syst mediated by Toll-like receptors (TLRs) (described belo Modification of the sugar structures found in nucleotides incorporation of 2'-O-methyl, 2'-O-fluoro, or 2'-O-metho etyl has been tested [185-188]. These modifications im resistance to endonuclease activity and avoid the activat of nonspecific immunity. A reduction in exonuclease susc tibility was demonstrated when the 3'-end phosphodie group was replaced with phosphorothioate [188]. A loc nucleic acid (LNA) is a bicyclic nucleic acid in whic ribonucleoside is linked between the 2'-oxygen and the carbon with a methylene unit. This modification was sho to increase serum stability and reduce off-target effects [1 189].

Conjunction of siRNA with small molecules or pepti is another form of nucleic acid modification. Conjugation cholesterol to the sense strand of the siRNA duplex increa binding to serum albumin and improves the distribution

Table 2. Experimental RNAi Therapies Using Atelocollagen

Target genes	Cancers	Models	Administration	References
VEGF	prostate cancer	subcutaneous	local	[253]
FGF-4	testicular cancer	orthotopic	local	[254]
EZH2, p100-α	bone metastatic tumor (prostate cancer)	orthotopic	systemic (i.v.)	[255]
EGFR	head and neck cancer	subcutaneous	local	[256]
HPV18E6E7	cervical cancer	subcutaneous	local	[257]
Midkine	prostate cancer	subcutaneous	local	[258]
GST-pi	prostate cancer (androgen-independent)	subcutaneous	local	[259]
HPV16E6E7	cervical cancer	subcutaneous	local	[260]
PAR-2	pancreatic cancer	subcutaneous	local	[261]
TS	salivary gland cancer	subcutaneous	local	[262]
RPN2*	breast cancer	subcutaneous	local	[233]
PLK-1	liver metastatic tumor (non-small cell lung cancer)	orthotopic	systemic (i.v.)	[10]
syndecan-1 (CD138)	prostate cancer	subcutaneous	local	[263]
β-catenin	multiple myeloma	subcutaneous	local	[105]
miR-16	bone metastatic tumor (prostate cancer)	orthotopic	systemic (i.v.)	[264]
Bcl-$_X$L	prostate cancer	subcutaneous	local, systemic (i.v.)	[234]
VEGF-A	Ewing's sarcoma	subcutaneous	local	[265]
mutant p53 promoter/enhancer	fibrosarcoma (chemical-induced)	orthotopic	local	[266]

VEGF-A; vascular endothelial growth factor-A, FGF-4; fibroblast growth factor-4, EZH2; enhancer of zeste homolog 2, p100-α; phosphoinositide hydroxykinase p100α subunit, EGFR; epidermal growth factor receptor, HPV; Human Papillomavirus, GST-Pi; glutathione S-transferase-Pi, PAR proteinase-activated receptor-2, TS; thymidylate synthase, RPN2; ribophorin II, PLK-1; polo-like kinase-1, *RPN2 expression induces resistance to docetax Although RPN2 siRNA alone does not induce apoptosis in breast cancer cells, RPN2 siRNA treatment increases the susceptibility of docetaxel on bre cancer cells.

siRNA to the targeted organ including the liver. Cholesterol-conjugated apolipoprotein B siRNAs were used to knockdown apolipoprotein B expression in liver and jejunum of mice, resulting in a decrease in cholesterol levels [190]. In another study, cholesterol-conjugated siRNAs against mRNAs associated with Huntington's disease delayed the abnormal behavioral phenotype observed in a rapid-onset mouse model [191].

BIOLUMINESCENT *IN VIVO* IMAGING ANIMAL MODELS

With an aim to establish RNAi strategies for cancer therapy, researchers have investigated the therapeutic effects in animal models. Previously, novel antineoplastic compounds have been usually screened using mouse models implanted human tumor cells subcutaneously. However, as these subcutaneous xenograft models do not clearly represent the primary sites of cancers or the metastatic sites, these models do not sufficiently represent the essential features of clinical cancers and surgical orthotopic models have been developed [192-194]. Whereas surgical orthotopic cancer models mimic the clinical situations, the assessment of cancer therapy and tumor growth required the sacrifice of the animals at a certain time point after the implantation of cancer cells and the evaluation of tumor load at death. Advances in molecular biology and medical technology have brought us non-invasive *in vivo* imaging of tumor load in small animals [195-197]. Several non-invasive imaging approaches including magnetic resonance imaging, computerized tomography, positron emission tomography, fluorescence imaging (FLI), and bioluminescence imaging (BLI) enable us to evaluate tumor sizes at serial time points from the same individual subject.

The BLI signals are produced by the reactions of luciferases with their substrate luciferin. Luciferases compromise a family of photoproteins isolated from various species such as firefly and sea pansy and they release photons by the reaction of luciferin under the existence of ATP [198]. Cancer cells are transfected with the gene for luciferase (Luc) and the bioluminescence signal from tumor-bearing mice is detected with a sensitive charge-coupled device (CCD) camera. Whereas FLI detects approximately 1,000-10,000 GFP expressing tumor cells inoculated intraperitoneously in mice [199], BLI can detect approximately 400-1,000 Luc-labeled cells inoculated subcutaneously or intraperitoneously and 1,000-10,000 cells inoculated intravenously [197, 200]. BLI is the most sensitive non-invasive approach to date. Moreover, there is much lower level of background noise in BLI compared with that in FLI because autofluorescence of non-labelled cells increases noise in FLI [195]. After luciferin is injected intraperitoneally in intracranial tumor-bearing mice, BLI signals of luciferase-bearing cancer cells increase during the first minutes, reaching a plateau after 10-15 minutes, and remains stable for additional 15-20 minutes [201]. Because intensity of BLI signals is correlated with tumor load, this approach is suitable for real time spatiotemporal analysis of tumor growth that reveals the dynamics of cancers and the therapeutic effects in experimental mouse models [151, 200, 202, 203]. However, BLI has some disadvantages. It is necessary to modify cancer cells transgenically for monitoring BLI as FLI. BLI shows anatomical low-resolution and it is difficult to detect signals released from deeper regions. Therefore, it is not likely to be applied to a clinical setting [195, 196].

PRECLINICAL APPLICATION OF RNAI THERAPY AGAINST PLK-1 IN MURINE ORTHOTOPIC MODELS OF CANCER

Here we introduce two applications of PLK-1 siRNA against urinary bladder cancer and metastatic liver tumors. As described above, PLK-1 is overexpressed in tumors of urinary bladder and in non-small cell lung cancer. PLK-1 expression levels are tightly correlated with histological grades of tumors, clinical stages, and prognosis of the patients. Superficial urinary bladder cancers, which compromise approximately 70% of urinary bladder cancers at initial diagnosis, are usually managed with transuretheral resection, followed by intravesical administration of agents such as Bacillus Calmette-Guerin (BCG), mitomycin C, and adriamycin. Although intravesical administration of BCG is considered to be the most effective strategy for the eradication and prophylaxis of the recurrent superficial cancers, this strategy causes irritation voiding symptoms, and life-threatening BCG sepsis. Therefore, other options of intravesical therapy for bladder cancer treatment are under investigation [9, 204]. Clinical trials of siRNA therapeutics often rely upon localized drug delivery [205], because maintenance of higher siRNAs concentrations is necessary for efficacy against the targeted diseases. Therefore, we investigated the efficacy of intravesical therapy of PLK-1 siRNA against urinary bladder cancers by using an orthotopic mouse model. Bladder cancer-bearing mice were established by the implantation of Luc-labeled UM-UC-3 bladder cancer cells into the murine bladder cavity through the urethra. Four days after the implantation, engraftment in the bladder was evaluated by using the *In vivo* Imaging System (IVIS) of BLI [151]. Mice were treated by intravesical administration, with 100 µl of PLK-1 siRNA/LIC-101 liposome complex (6, 0.6 µM) for 5 days, and progression of urinary bladder cancer was successfully inhibited in a dose-dependent manner [9].

Liver metastasis is one of the most important prognostic factors in lung cancer patients. However, despite the development of new chemotherapeutic agents, current therapies are not sufficient to inhibit liver metastasis. We investigated the effects of PLK-1 siRNA on the liver metastasis of lung cancers. We first established a mouse model of liver metastasis. Spleens were exposed to allow direct intrasplenic injections of Luc-labeled A549 lung cancer cells. Ten minutes after injections of tumor cells, the spleens were removed. After confirmation by IVIS visualization of Luc-labeled A549 cell engraftment, 200 µl of PLK-1 siRNA/atelocollagen complex (25 µg of siRNA), nonsense siRNA/atelocollagen complex (25 µg of nonsense siRNA), or PBS/atelocollagen complex was administered by intravenous injection for 10 consecutive days following day 1 of transplantation. The final concentration of atelocollagen was 0.05%. On day 35, mice treated with nonsense siRNA/atelocollagen complex or PBS/atelocollagen complex showed extensive metastases in the liver when compared to mice treated with PLK-1 siRNA/atelocollagen complex. More-

over, on day 70 after the inoculation of tumor cells, livers of mice treated with nonsense siRNA/atelocollagen or PBS/atelocollagen complex had numerous large tumor nodules, whereas the livers of mice treated with PLK-1 siRNA/atelocollagen complex showed a much lower number of smaller nodules. These findings indicate that PLK-1 siRNA/atelocollagen complex is an attractive therapeutic tool for further development as a treatment against liver metastasis of lung cancer [10]. Consequently, our preclinical applications suggest that PLK-1 siRNA is a promising tool for cancer therapy.

ADVERSE EFFECTS OF RNAi IN *IN VIVO* APPLICATION

Although RNAi shows excellent specificity in gene-silencing, several adverse effects are brought in *in vivo* application. One probable adverse effect is activation of immune reaction [189, 206]. Mammalian immune cells express family of TLRs, which recognize pathogen-associated molecules including unmethylated CpG DNA and viral dsRNA. Among 13 TLRs, TLR7 and TLR8 recognize ssRNA sequence-dependently and produce IFNs and inflammatory cytokines such as IL-12 and TNF-α through the activation of NF-κB and IFN regulatory factor (IRF) -7 [207]. Furthermore, these TLRs recognize uridine- or guanosine-rich ssRNA [208, 209]. TLR3 recognizes unmethylated CpG DNA but not ssRNA [207]. dsRNA is directly binds to TLR3 [210] and this signaling pathway is activated sequence-independently [207, 211]. The size of ssRNA is also important for the immune reaction. Twelve nt ssRNAs containing the immunostimulatory motif (5'-GUCCUUCA A-3') merely induced IFN-α, however, 16 to 19 nt ssRNA restored IFN production [186]. The administration of siRNAs into mammalian cells activates the immune systems also sequence-independently. siRNAs induce dsRNA-activated protein kinase (PKR) autophosphorylation and PKR produces IFNs through the activation of NK-κB and IRF-3 [212]. One possible strategy to prevent the immune activation is chemical modification of siRNA. The 2' position of nucleotides is within TLR-7-interacting sequences, and 2' O-methyl or 2' fluoro- modification abrogate immune response. Furthermore, the uridine or guanosine modification is most effective [213]. LNA can also reduce the immunostimulatory effects [188]. Although the cationic lipid-based DDSs are efficient for the trasnduciton of siRNAs into cells, the complexes of siRNAs with cationic lipids induce the production of inflammatory cytokines [186, 187]. siRNAs conjugated to cholesterol have no significant activation of immune system and improve the distribution of siRNA to the targeted organ including the liver. Cholesterol-conjugated apolipoprotein B siRNAs induce a decrease of apolipoprotein B expression in liver and jejunum of mice, resulting in a decrease in cholesterol levels without the activation of immune systems [190].

Although RNAi mediated gene silencing is induced by perfect complenetarity in target RNA sequence, partially complementary sequences in unintended RNAs induce gene silencing (off-target effect). This off-target effect is induced by the sequence complementarity in the seed region of siRNAs or shRNAs [214]. Moreover, the 7 nt motif complimentary to 2-8 nt at the 5' end of antisense strands of siRNAs has been shown to be a key determinant in direc off-target effects because an siRNA with 1 nt mutated ir 7 nts region failed to induce off-target effects [215]. T are several ways to control the off-target effects. Method *in silico* screening of siRNA constructs are useful optimization to prevent the off-target effects and sev groups have been developing algorithm [216-218]. Chem modification is also useful. The O-methyl modificatio the 2'-position of the ribose within the seed region of siF sense strands reduces sense strand-mediated off-ta activity [219]. Asymmetrically designed siRNAs reduce target effects compared to symmetric siRNAs [2 Asymmetric siRNAs with 15 nt of the sense strand in sequence-specific gene silencing without silencing of target genes. The incorporation of the sense strand into R can also cause off-target effects [221, 222]. It is specul that the asymmetric siRNAs enter RISC with high efficie and persist in Ago 2 protein longer compared to siRN Asymmetric siRNAs reduce off-target silencing by redu incorporation of the sense strand into RISC.

Although shRNAs can induce stable gene silencing, t is a possibility that long-term silencing by shR overexpression causes fatal adverse effects. As shRN processed through the miRNA pathway, the miR maturation is blocked in response to shRNA concentrat The sustained high-level shRNA expression in the live mice by AAV vector downregulated liver-derived miRN resulting in hepatic injury and death [223]. One explana is that saturation of Exportin-5 whose function is nuc transport inhibited the miRNA maturation pathv Overexpression of Exportin-5 can tolerate higher dose shRNA without its toxicity [224]. However, another st shows that the administration of synthesized siRN induced acute and long-term gene silencing wit interrupting the endogenous miRNA biogenesis [2 Considering these findings, careful modification formulation of siRNAs could avoid the competition betw siRNA and miRNA. Usage of inducible siRNA vectors [2 227] or selection of promoter such as tissue-spec polymerase II promoters [224] would be other option avoid this adverse effect.

CLINICAL TRIALS OF RNAi TOWARDS CANC THERAPIES

The first systemic administration of an siRNA drug humans was performed in a patient with CML in experimental setting of a traditional clinical study [228] 47-year-old, Ph-positive and IM resistant, female patie with CML received allogeneic bone marrow transplantati However, an extramedullary relapse of pleural effusions subcutaneous nodes occurred. The patient received furt intravenous or subcutaneous administration of siR against *Bcr-Abl* mRNA. In combination with IM cytosine-arabinoside, the siRNA was delivered us liposomes at doses of 10 or 30 μg/kg as a systemic treatm and at 300 μg as a local treatment. Levels of *Bcr-Abl* mR expression were dramatically decreased following the f administration; however, this effect was not observed a further more administrations of the siRNA. The siR treatment was well tolerated without any adverse effects.

siRNA cancer therapies have been conducted in clinical settings, but few clinical trials for cancer therapy are ongoing (http://clinicaltrials.gov/ct2/home) as many of the current siRNA therapeutics in advanced clinical trials rely on localized drug delivery [205]. Alnylam Pharmaceuticals is developing ALN-VSP01 targeting kinase spindle protein and VEGF, and conducting a Phase I study in patients with advanced tumors with liver involvement. Calando Pharmaceuticals is conducting a Phase I study of CALAA-01 in patients with solid tumors refractory to standard-of-care therapies. CALAA-01 is composed of RRM2 siRNA and CDP nanoparticles called Rondel™, and CALAA-01 has been proven safe and effective in mice and nonhuman primates studies [182, 183]. Clinical studies using LNAs are also ongoing. Santaris Pharma has developed LNA against Bcl-2, SPC2996, for use in an ongoing Phase I/II study in patients with relapsed or refractory chronic lymphocytic leukemia is ongoing. Enzon Pharmaceuticals has developed a LNA against hypoxia-inducible factor-1α and a Phase I/II study in patients with advanced solid tumors or lymphoma is ongoing.

CONCLUSION

Studies to establish the pharmacokinetics and pharmacodynamics of siRNAs on the administration are necessary step in the potential approval of siRNA as a tool for cancer therapy. Moreover, to maximize efficacy and to minimize adverse effects of RNAi, it should be determined whether siRNAs are best administered alone or in combination with chemotherapeutic agents [77, 229], and whether it is better to administer a single specific siRNA or multiple specific siRNAs [144, 230-234]. In conclusion, RNAi therapy represents a powerful strategy against cancers and may offer a novel and attractive therapeutic option. The success of RNAi depends on the suitable selection of target genes and the development of DDSs. We anticipate that the continued development of effective DDSs and the accumulation of evidence further proving the success of siRNA treatment will advance siRNA as a promising strategy for cancer therapy.

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GUIDELINE

Guidelines for safety management of granulocyte transfusion in Japan

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

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

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

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

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

1 Introduction

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

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

2 Text of the guidelines

2.1 Purpose of the guidelines

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

2.2 Indications for GTX therapy

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

2.3 Appropriateness of medical institutions

2.3.1 Transfusion service

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

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

2.3.2 Role of the hospital transfusion committee

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

2.3.3 Area for collection of granulocyte concentrates

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

2.4 Management of granulocyte donors

2.4.1 Selection of granulocyte donors

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

2.4.2 Age of granulocyte donors

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

2.4.3 Blood group of granulocyte donors

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

2.4.4 Collection from the same donor

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

2.4.5 Cytomegalovirus (CMV) serology

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

2.4.6 Alloimmunization

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

2.4.7 Medical examinations and laboratory testing

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

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

2.4.8 Informed consent

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

2.4.9 Post-donation care

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

2.5 Quality assurance of granulocyte concentrates

2.5.1 Collection of granulocyte concentrates

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

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

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

2.5.2 Methods of granulocyte collection

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

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

2.5.3 *Preparation of granulocyte concentrates*

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

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

2.6 Administration of granulocyte concentrates

2.6.1 *Infusion of granulocyte concentrates*

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

2.6.2 *Premedication*

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

2.7 Evaluation of effectiveness of GTX therapy

2.7.1 *Success of GTX therapy*

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

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

2.7.2 Discontinuation of GTX therapy

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

2.8 Complications of GTX therapy

2.8.1 Donor-associated side effects

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

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

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

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

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

2.8.2 Recipient-associated side effects

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

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

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

3 Conclusion

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

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

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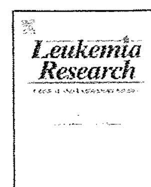

Osteoclasts are involved in the maintenance of dormant leukemic cells

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ABSTRACT

Osteoclasts (OCs) are specialized cells for the resorption of bone matrix that have also been recently reported to be involved in the mobilization of hematopoietic progenitor cells. When Ba/F3 cells expressing wild-type *bcr-abl* were co-cultured with osteoblasts (OBs), OCs, and bone slices, their proliferation was significantly suppressed, and the Ki-67 negative population, which is believed to be in G_0 phase, was increased. The results of our *in vitro* experiments suggest that OCs could be involved in the maintenance of dormant leukemic cells in the bone marrow (BM) microenvironment through the release of soluble factors, one of which could be TGF-β.

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1. Introduction

It has been suggested that the bone marrow (BM) microenvironment might play an important role in regulating hematopoiesis, and is considered a hematopoietic stem cell (HSC) niche. HSCs are thought to reside in the BM niche and respond to various signals relayed by soluble factors, adherent molecules, or the extracellular matrix that regulate their self-renewal, proliferation, or differentiation. Osteoblasts (OBs) are bone-derived stromal cells that line the surface of the endosteum and mineralize the bone matrix. These cells are destined to form the HSC niche in the endosteum and regulate HSC number or differentiation [1–4]. It is also recognized that the HSC niche is a hypoxic environment, thus HSCs are protected from oxidative stress and are maintained in a senescent state in the niche [5,6]. Osteoclasts (OCs) are terminally differentiated cells of the monocyte/macrophage lineage that resorb mineralized bone matrix and degrade the extracellular matrix of the endosteum in trabecular bone. The direct interaction of OBs and OC precursors is essential for OC differentiation and is mediated by the binding of the RANK ligand (RANKL) on OBs to the receptor activator of NF-κB (RANK) on OC precursors [7–9]. Recently, both OBs and OCs have been reported to be involved in regulating hematopoiesis in the BM niche through the degradation of the niche components that anchor HSCs to the niche [10,11]. Another group has speculated that OCs induce the release of calcium ions from the bone matrix at the endosteum and the resulting calcium gradient directs calcium-sensing receptor (CaR)-expressing HSCs to engraft and localize to the BM niche [12]. We have previously reported that the third-generation bisphosphonate, zoledronic acid (ZOL), exerts anti-tumor effects in a murine model of chronic myelogenous leukemia (CML) [13–15]. ZOL induces apoptosis in OCs and is used as a therapeutic agent in the treatment of osteoporosis or hypercalcemia of malignancy. We hypothesized that ZOL suppresses the progression of leukemia not only by directly inducing apoptosis in leukemic cells, but also by reducing the number of OCs. To test this hypothesis, we investigated whether OCs influence the growth of leukemic cells by using an *in vitro* co-culture system. In the present study, we demonstrate that OCs suppress the growth of leukemic cells and induce a dormant state *in vitro*. We also present evidence that OCs might maintain leukemic cells in a quiescent state in the BM microenvironment which could act as both a normal hematopoietic and leukemic stem cell niche, potentially contributing to the incidence of relapse and the onset of acquired drug resistance in leukemias.

2. Materials and methods

2.1. Reagents and cell lines

Ba/F3 cells expressing wild-type (wt) *bcr-abl*^{p210} were generated as previously described [16]. Cells were maintained at 37 C in a fully humidified atmosphere of 5% CO_2 as suspension cultures in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/mL

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penicillin–streptomycin and 292 μg/mL L-glutamine (Gibco, Paisley, Scotland). Recombinant human TGF-β_1 and monoclonal anti-TGF-β antibody were purchased from R&D systems (Minneapolis, MN, USA). A Quantikine Mouse/Rat/Porcine/Canine TGF-β_1 immunoassay (R&D systems) was used for the detection of murine TGF-β_1 in culture supernatants.

2.2. Primary murine osteoblasts

Primary murine OBs were enzymatically isolated from the calvaria of 1–2-day-old BALB/cA mice (CLEA Japan, Osaka, Japan) by sequential digestion with 0.1% collagenase (Wako) and 0.2% dispase (Sanko Junyaku, Tokyo, Japan) solution. Harvested cells were plated in 100 mm dishes in alpha-MEM (Sigma–Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated FBS, 100 μg/mL penicillin–streptomycin and 292 μg/mL L-glutamine. After incubation for 24 h, the culture medium was changed, and cells were collected on the subsequent day by trypsinization with 0.05% trypsin–EDTA (Invitrogen, Carlsbad, CA) and expanded. Six to seven days after the initial harvest, cells were collected and stored at −80 C until use.

2.3. Osteoclast induction in vitro

BM cells were flushed out with 10% FBS alpha-MEM from both tibias and femurs of 5–10-week-old BALB/cA mice. Each well of a culture plate was coated with 500 μL of a mixture of collagen gel (Cellmatrix Type I-A, Nitta gelatin, Osaka, Japan), 5× alpha-MEM (GIBCO) and $NaHCO_3$/HEPES buffer following the manufacturer's instructions. First, bone slices derived from adult murine femurs were placed on the collagen gel. Next, OBs were seeded at a concentration of 1×10^5 cells/well, and then 2×10^6 cells/well of BM cells were seeded alongside the OBs. Vitamin D_3 (Calcitriol, Wako) and prostaglandin E_2 (Wako) were added to each well at a final concentration of 0.02 μM and 2 μM, respectively. Six to seven days after the start of co-culture, OBs and OCs were used for co-culture with Ba/F3 wt *bcr-abl* cells.

2.4. Indirect co-culture of leukemic cells with osteoblasts, osteoclasts and bone slices

Ba/F3 wt *bcr-abl* cells were seeded at 1×10^4 cells/well into transwell chambers (Transwell #3401, Corning Incorporated, Acton, MA, USA) in which the membrane pore size is 0.4 μm, thereby creating an indirect co-culture condition. Viable cells were counted by trypan blue dye exclusion at 48 and 72 h after the co-culture was initiated. At 72 h, co-cultured Ba/F3 wt *bcr-abl* cells were harvested and fixed with cold 70% ethanol, and stored at −20 C until cell cycle analysis.

2.5. TRAP staining

Cells were fixed with 10% neutral buffered formaldehyde (Wako) at room temperature for 10 min and fixed again with ethanol–acetone (50:50, vol/vol) for 1 min. Cells were then incubated with the TRAP staining solution: 5 mg of naphthol AS-MX phosphate (Sigma) was dissolved in 0.5 mL of N,N-dimethyl formamide (Wako), and 30 mg of fast red violet LB salt (Sigma) and 0.1 M sodium acetate buffer, pH 5.0, containing 50 mM sodium tartrate, were added to the mixture. After incubation for 5 min at room temperature, cells were washed with diluted water.

2.6. Cell cycle analysis

Fixed Ba/F3 wt *bcr-abl* cells were washed twice with wash buffer (PBS containing 1% FBS) and the pellet was resuspended to a concentration of 1×10^6 cells/100 μL. 20 μL of FITC anti-human Ki-67 antibody or FITC isotype control (BD Pharmingen, San Diego, CA, USA) was added to 100 μL of cell suspension and incubated at room temperature for 30 min in the dark. Cells were then washed with wash buffer and resuspended in 500 μL of wash buffer, followed by the addition of 20 μL of BD Via-Probe™ Cell Viability Solution (BD Pharmingen). The samples were analyzed using FACSCanto II flow cytometer and BD FACSDiva software.

2.7. Western blot analysis

Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane (GE Healthcare Bio-Sciences, Tokyo, Japan). The membranes were saturated with 5% (wt/vol) non-fat dry milk in TBST (25 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 7.8, 140 mM NaCl, 0.1% [vol/vol] Tween20), and incubated overnight at 4 C with rabbit monoclonal anti-smad2/3 antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal anti-phospho-smad2 (1:1000; Cell Signaling Technology), or rabbit monoclonal anti-actin antibodies (1:1000; Sigma). The membranes were washed thoroughly with TBST and incubated for 1 h at room temperature with anti-rabbit IgG coupled to horseradish peroxidase (1:1000; GE Healthcare Biosciences). Detection was performed with enhanced chemiluminescence kits (GE Healthcare Biosciences).

2.8. Measurement of TGF-β_1 in the culture supernatants by ELISA

The culture supernatants of OBs, OCs, and/or bone slices were collected and centrifuged for particle removal. Samples were stored at −80 C until use. ELISA for murine TGF-β_1 was performed according to manufacturer's instructions. TGF-β_1 concentrations were calculated by subtracting the background of FBS in the supernatants from the actual values.

2.9. Statistical analysis

P values were derived from 2-sided tests, and values of less than 0.05 were considered statistically significant.

3. Results

3.1. OCs suppress the growth of Ba/F3 wt bcr-abl cells in the presence of bone slices

We indirectly co-cultured Ba/F3 wt *bcr-abl* cells with OBs, OCs, and/or bone slices using transwell chambers. As shown in Fig. 1A, OBs enhanced the growth of Ba/F3 wt *bcr-abl* cells ("OB") and this effect was observed even in the presence of OCs ("OB + OC"). Interestingly, when bone slices were added to the OB + OC well ("OB + OC + bone"), the proliferation of Ba/F3 wt *bcr-abl* cells was significantly suppressed compared to OB + OC. Thus, we hypothesized that OCs might suppress the proliferation of Ba/F3 wt *bcr-abl* cells through the activity of soluble factors that are released from the bone slices or produced in the presence of the bone slices by OCs. When Ba/F3 cells were cultured in the presence of bone slices alone, no increase in the proliferation rate was observed,

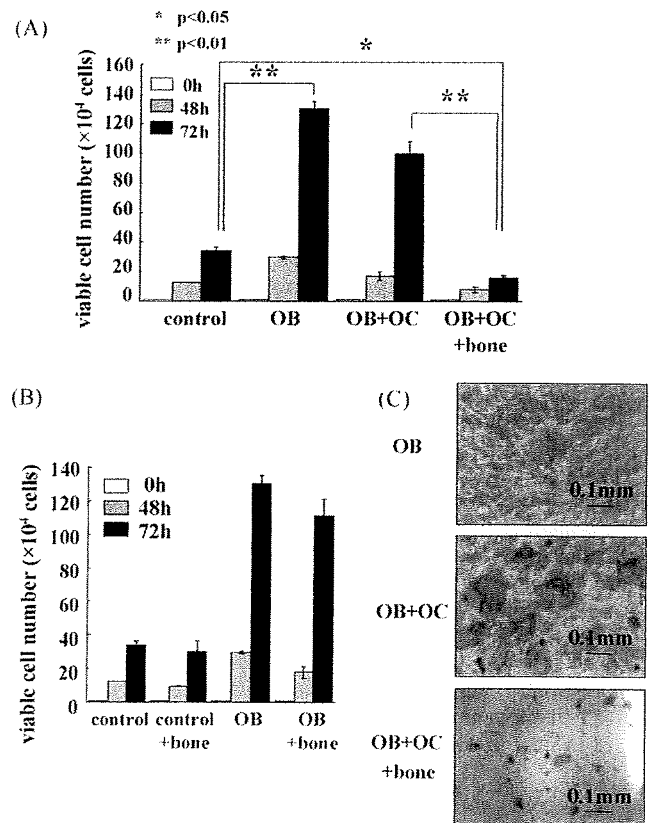

Fig. 1. OCs suppress the growth of Ba/F3 wt *bcr-abl* cells in the presence of bone slices. (A and B) Each bar indicates the viable cell number at 0 h (white), 48 h (gray) and 72 h (black) after the co-culture was initiated. The representative results shown are the means ± standard deviations (SD) of duplicated counts from two independent wells. (C) The images of TRAP-stained wells of OBs only, OB + OC, and OB + OC + bone 72 h after the co-culture were initiated.