brain tumors, and in preclinical studies significant efficacy in a severe model of glioma was observed [14•].

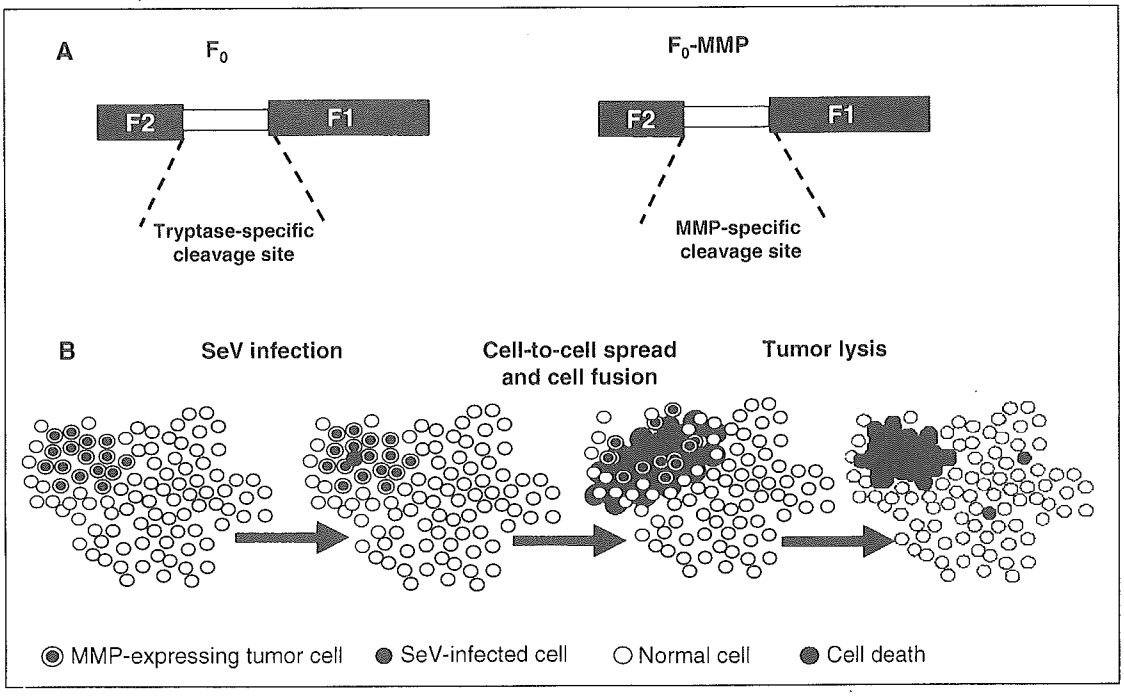
Tumor cells

The research group at Chiba University, Japan has administered recombinant ΔFΔM/SeV carrying the *lacZ* or human IL-2 (hIL-2) gene into established rat 9L brain tumors (gliosarcoma) *in vivo*, simultaneously with peripheral vaccination using irradiated 9L cells [14•]. Sequential monitoring with magnetic resonance imaging (MRI) was used to evaluate the therapeutic efficacy. Extensive transduction of the *lacZ* gene into the tumors was observed, as well as high-level production of hIL-2 *in vivo*. The intracerebral injection of ΔFΔM/SeV-hIL-2 brought about significant reduction of tumor growth, including complete elimination of tumor (in three out of ten tumor-bearing treated rats) in this severe model (100% mortality in untreated animals). Significant amounts of 9L-specific cytotoxic T-cells were induced by the peripheral vaccination, and CD4+ and CD8+ T-cells in the target tumors.

Selective transfection and destruction of tumor cells has long been a 'holy grail' in cancer gene therapy. Kinoh *et al* utilized specific features of SeV to address this problem [15••]. DNAVEC used reverse genetics to generate a variety of SeV backbones, and has previously reported generation of an M gene-deleted SeV (ΔM/SeV; Figure 1C) [16].

The M protein is essential for organization of all viral components at the cell membrane after replication and for efficient budding [17]. Interestingly, ΔM/SeV does not bud from infected cells, but instead spreads extensively from cell to cell, leading to formation of large syncytia followed by rapid cell death. To generate a vector suitable for selective killing of cancer cells, Kinoh *et al* made use of the F protein in the virus envelope [15••]. Virus entry into cells is only possible if the immature F_0 precursor is cleaved by specific proteases at a sequence-specific site in the protein. *In vivo*, a tryptase secreted only by Clara cells (tryptase Clara) is responsible for wild-type virus entry and infection in the rodent lung and likely explains the restricted virus tropism, despite broad expression of SeV receptors on many cell types throughout the body. *In vitro* trypsin also cleaves the F_0 protein. Most aggressive tumors express high levels of matrix metalloproteases (MMPs) that allow tumor cells to destroy extracellular matrix and metastasize through the circulation and lymphatic system. By removing the tryptase Clara-specific cleavage sites in the F_0 protein and replacing it with an MMP-specific cleavage site, Kinoh *et al* generated a vector (MMP-ΔM/SeV) that only entered MMP-expressing cells and then spread from cell to cell inducing cell death (Figure 2) [15••]. When tested *in vitro*, this vector was no longer activated by trypsin, but efficiently spread in MMP-expressing tumor cell lines. Importantly in nude mice *in vivo*, the MMP-ΔM/SeV, but not a control virus, spread efficiently and reduced tumor volume in tumors (3 to 8 mm) that expressed high levels of MMP, but not in low-expressing tumors. Although data were not reported, the researchers claim that the MMP-ΔM/SeV did not infect other organs, including the lung, skin and muscle [15••].

Figure 2. Tumor cell-specific Sendai virus.



(**A**) The fusion protein (F) is important for cell entry, but the precursor (F_0) has to be cleaved by tryptase Clara into F1 and F2 at a specific cleavage site to enable virus entry via cell fusion. To restrict virus transduction to tumor cells, the tryptase-specific cleavage site was exchanged with a matrix metalloprotease (MMP)-specific cleavage site. MMPs are expressed predominantly by tumor cells and, thus, cell entry becomes tumor specific. (**B**) Following transduction of MMP-expressing tumor cells, the matrix (M) protein-deleted (MMP-ΔM/SeV) virus selectively transfects MMP-expressing tumor cells, spreads from cell to cell and causes cell fusion and ultimately cell lysis.

Fetus

The first study describing the prenatal use of both primitive and new-generation SeVs has been published. Waddington *et al* assessed SeV- and ΔF/SeV-mediated gene transfer in mouse fetuses and neonates, following intra-amniotic and prenatal intraperitoneal, intravascular and intramuscular injection [18]. Not surprisingly, fetal mortality was higher with first- than with second-generation virus (although numbers were low) likely due to accumulation of higher virus concentration after injection of transmission-competent first-generation virus. Gene expression was widespread, but distribution depended on the delivery route with, for example, high expression in lung and peritoneum after intra-amniotic and intraperitoneal injection, respectively. Although duration of gene expression was not extensively assessed in this study, preliminary data indicated that expression was transient.

Ex vivo stem cell transduction

SeV is a cytoplasmic virus that does not require nuclear entry or a DNA intermediate in its life cycle. Compared with integrating vectors, there is no risk of SeV-induced insertional mutagenesis and SeV is therefore an interesting candidate for *ex vivo* stem cell transduction. In addition, homologous recombination between genomes of negative-stranded RNA viruses is low, which greatly reduces the risk of generating wild-type virus after transduction.

SeV transfects hematopoietic stem cells of all linages efficiently, and the capacity of SeV to transduce non-human primate embryonic stem cells was assessed [19]. Sasaki *et al* reported efficient, and, importantly, stable SeV-mediated gene transfer for at least one year, indicating that the replicating viral genomes are passed on to daughter cells [19]. Interestingly, SeV transduction did not inhibit cell differentiation and the cells were able to form fluorescent teratomas when transplanted into immunodeficient mice [19].

Inactivated SeV and individual viral components utilized for gene transfer

Hemagluttinating virus of Japan-liposomes

The capacity of SeV to enhance fusion of lipid vesicles with cell membranes has been utilized in the form of hemagluttinating virus of Japan (HVJ)-liposomes. These consist of liposome/plasmid DNA complexes mixed with ultraviolet-inactivated wild-type SeV.

Even in the comparatively restricted time-period that this review aims to cover, there are too many HVJ-liposome related papers to quote each one. Briefly, HVJ-liposomes carrying HGF or IL-10 cDNA have been injected intramuscularly to treat diabetic neuropathy and atherosclerosis, respectively [20,21]. HGF/HVJ-liposomes have also reduced ischemic injury in the brain [22]. When complexed to DNA/RNA oligonucleotides, these liposomes have been injected intravenously to induce gene repair in the liver [23], although this has not been successful. Short interfering RNA against RAD51 has been complexed to HVJ-lipsomes and enhanced the anticancer effects of cisplatin *in vivo* [24]. After intrarenal injection, HVJ-liposomes appear to transfect glomerular cells [25]. In addition, first attempts have also been made to use these formulations for vaccination purposes [26,27] and protein delivery [28].

Self-replicating ribonucleoprotein complexes

Iida and Hasegawa have recently generated virus-like particles (VLPs), based on SeV self-replicating ribonucleoprotein complexes [29]. These VLPs are F-protein deficient because the fusion protein is not supplied *in trans* during virus production in cell culture. VLPs are therefore non-infectious and are rendered infectious by the addition of non-viral gene transfer agents such as Lipofectamine 2000. These complexes transfected a variety of cell lines efficiently and, importantly, transfection was not inhibited by the addition of anti-SeV antibodies directed against the HN protein. This may be an important advantage over standard SeV vectors, which are inhibited by anti-SeV antibodies, preventing repeated administration in mouse models *in vivo*. We are currently assessing transfection efficiency and repeat administration of VLPs in airway epithelial cells (AECs) *in vivo*. AECs are terminally differentiated non-dividing cells and a cytoplamic expression vector may increase transfection efficiency because it bypasses the nuclear membrane, which presents a significant barrier for non-viral gene transfer agents. Finally, DNAVEC has recently shown that RNPs isolated from VLPs and wild-type or genetically engineered SeVs are also promising in combination with gene transfer agents [29].

F/HN pseudotyping of lentivirus

The HN and F proteins are critical for SeV uptake into cells, binding to sialic acid residues on the cell membrane and triggering cell/virus fusion, respectively. This has been applied to construct a new pseudotyped simian immunodeficiency virus (SIV) vector for use in airway gene transfer. SeV F and HN proteins were successfully incorporated into the African green monkey SIV (SIVagm) vector by truncation of the cytoplasmic tail of the F protein and addition of the cytoplasmic tail of SIVagm transmembrane envelope protein to the N terminus of the HN protein. As with the vesicular stomatitis virus G glycoprotein (VSVG)-pseudotyped vector, the SeV F/HN-SIVagm vector transduced various animal and human cell lines. Furthermore, the vector transfered reporter genes into polarized epithelial cell cultures of rat trachea from the apical and basolateral sides [30] and DNAVEC recently succeeded in increasing the vector production titer [K Mitomo, personal communication]. The new vector is quite stable during purification steps, such as during concentration by ultracentrifugation. This virus may be more efficient than the commonly used VSVG-pseudotyped lentiviral vectors in certain tissues. For example, AECs are refractory to VSVG-SIV transduction via the apical membrane, requiring tight junction permeabilization with detergents such as lysophosphatidylcholine to access the basolateral surface. In contrast, however, F/HN-SIV can directly transfect airway epithelial cells via the apical membrane dose dependently *in vivo*.

SeV has been used in a variety of vaccination protocols

HIV immunization

It has long been recognized that DNA vaccines, and vaccines based on recombinant viral vectors, or combinations of both, (DNA priming plus virus booster) induce cellular immune responses that may protect against subsequent viral infection. Several publications describe the use of

recombinant SeV expressing the HIV GAG protein as booster after plasmid DNA priming, and report generation of high levels of virus-specific cytotoxic T-lymphocytes (CTLs) and inhibition of replication of SIV (strain SHIV89.6P) that causes acute T-cell depletion in non-human primates [31]. More recently, these studies were extended to pathogenic immunodeficiency viruses that induce chronic, more clinically relevant disease progression (strain SIVmac239) [32•]. Matano *et al* showed that five out of eight macaques vaccinated with DNA-prime/GAG-expressing SeV-booster experienced controlled viral replication after challenge with SIVmac239 5 weeks after infection [32•]. Similar to previous studies, vaccine-induced CTLs appeared crucial in containing virus replication. Interestingly, although virus replication was not contained in three out of eight animals, vaccine-induced CTLs seemed to select for viruses with reduced replication ability. Although DNA-prime/GAG-expressing SeV-booster vaccination appears to be effective in the early stages of infection, the same research group reported that viral load increased in vaccinated macaques in later phases of infection [33]. Interestingly, SIV-specific CD8 T-cells remained high when control of virus replication was lost. When comparing SIV-specific CD4 T-cells in animals that lost virus control and those that retained control in the chronic phase, the researchers noted that increases in viral load were accompanied by a decrease in SIV-specific CD4 T-cells, supporting an important role of CD4 T-cells in the control of virus infection. Most recently, Kato *et al* extended the use of SeV-GAG vaccines to non-human primates with chronic HIV infection and reported successful induction of SeV-specific T-cell responses[34••].

RSV immunization

Respiratory syncytial virus (RSV) infection can cause serious respiratory problems in children, but currently no effective vaccine exists. An SeV-based vaccine has previously been developed against human para-influenza virus type 1 (hPIV1), which is closely related to RSV. Takimoto *et al* engineered an SeV expressing the RSV G glycoprotein [35••]. Following intranasal infection of rats, the animals developed RSV-specific antibodies and were protected from RSV when challenged 4 weeks after vaccination, but cellular immune reponses were not analyzed [35••]. Although promising, further long-term studies are required to assess the suitability of SeV vaccination against RSV.

HPIV1 immunization

Similar to RSV, there is no effective vaccine against hPIV1 infections, which cause bronchiolitis and pneumonia in children. SeV has high sequence homology and antigenic cross-reactivity with hPIV1 and is therefore a good candidate for xenotropic vaccination. In non-human primates, SeV immunization generated virus-specific antibodies and protected against hPIV1 challenge. The first SeV trial has been carried out in healthy human adults [36]. Nine volunteers were enrolled for a dose-escalation study and SeVs (5×10^5, 5×10^6 and 5×10^7 egg infectious doses/individual, n = 3/dose) were delivered by dropper into each nostril (0.5 ml total volume). SeV administration was well tolerated during the one-year follow-up and there was no evidence of virus shedding. Interestingly, all volunteers had some degree of pre-existing anti-SeV antibodies likely due to cross-reactivity of anti-hPIV1 antibodies generated in response to previous hPIV1 infection. An increase in post-vaccination anti-SeV antibodies, and therefore evidence of immunogenicity of the SeV-vaccine, was only detected in three out of nine individuals. The investigators speculated that pre-existing antibodies may have prevented efficient virus replication, which may not be a problem in immunologically naive children, the ultimate target population for an SeV vaccine [36].

Modifications to the vector backbone will further improve utilization of SeV gene transfer vectors

A common trend in adenovirus and adeno-associated virus (AAV) vectorology has been the removal of as many viral genes as possible to improve the safety and toxicity profile of the viruses. Adenovirus and AAV vectors completely depleted ('gutted') of any viral genes have been generated. For SeV, the NP, P and L proteins are essential parts of the viral structure/function and cannot be deleted. However, viruses without the F or M protein have already been produced, by supplying these proteins *in trans* during vector production in F or M stably expressing cell lines, respectively, and these modifications changed the behavior of SeV after infection [3••,15••]. DNAVEC has constructed SeV with double deletions of F and HN, and recombinant SeVs deleted for both the F and M gene (ΔF/ΔM-SeV; Figure 1E) have been generated at high titers [13••]. Furthermore, triple deletion for F, HN and M has been reported (Figure 1F) using helper cells expressing these deleted gene products *in trans* [M Inoue, personal communication]. Bernloehr *et al* reported production of an HN protein-deleted SeV, although in this study the HN protein was provided through co-transfection with an adenovirus carrying the HN protein *in trans* [37].

Importantly, these newly designed vectors display a number of interesting properties; formation of virus-like particles after infection is reduced and reduction in cell toxicity without a reduction in gene expression. Currently, most of the characterization of the double-deleted viruses has been carried out *in vitro* and it remains to be evaluated how they will perform in different organs *in vivo*.

In general, wild-type SeV infection in cell culture is transient. However, occasionally a subset of cells survives and is subsequently persistently infected with SeV. The SeV π strain was isolated from persistently infected cells and Nishio *et al* sequenced the L and P protein, which comprise the SeV polymerase, and identified six amino acid substitutions (three in each of the L and P genes) in the SeV π strain [38]. A combination of careful molecular and functional analysis revealed that amino acid 1618 in the L gene is crucial in regulating persistence of expression. It is currently unknown whether changing this amino acid may also allow persistent expression *in vivo*.

Conclusions

Wild-type and recombinant SeV may have wide-ranging applications for gene therapy and vaccination. They offer a range of advantages, including high levels of expression,

lack of genotoxicity and for the non-transmissible type of SeV vectors, a relative lack of concern over viral shedding. Though gene expression is transient, high-level expression of SeV in a large range of tissues may offer therapeutic value and perhaps advantages over more persistent expression systems. Following infection, SeV elicits a potent immune response, which is beneficial in vaccination and perhaps cancer applications. However, similar to other viral gene transfer vectors, SeV can currently not be repeatedly administered in rodent experiments at intervals of shorter than several months, which may suggest limitation in human use for chronic diseases that require repeated administration at short intervals. As for other gene transfer agents, it is therefore necessary to carefully design the protocols to be used, and the diseases to be targeted when using SeV. Clearly, it is also important to continue the improvements in vector design described in this review.

Acknowledgements

We thank Stefano Ferrari for critical reading of this manuscript and Luci Somerton for help with the artwork. UG is funded by a CF Trust Senior Fellowship.

References

•• of outstanding interest
• of special interest

1. Pinkenburg O, Vogelmeier C, Bossow S, Neubert WJ, Lutz RB, Ungerechts G, Lauer UM, Bitzer M, Bals R: **Recombinant Sendai virus for efficient gene transfer to human airway epithelium.** *Exp Lung Res* (2004) **30**(2):83-96.

2. Li HO, Zhu YF, Asakawa M, Kuma H, Hirata T, Ueda Y, Lee YS, Fukumura M, Iida A, Kato A, Nagai Y, Hasegawa M: **A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression.** *J Virol* (2000) **74**(14):6564-6569.

3. Ferrari S, Griesenbach U, Shiraki-Iida T, Shu T, Hironaka T, Hou X, Williams J, Zhu J, Jeffery PK, Geddes DM, Hasegawa M, Alton EW: **A defective nontransmissible recombinant Sendai virus mediates efficient gene transfer to airway epithelium *in vivo*.** *Gene Ther* (2004) **11**(22):1659-1664.

•• *This paper reports the first proof-of-principle that F protein-deleted SeV transfects airway epithelium efficiently.*

4. Griesenbach U, Boyton R, Somerton L, Escudero Garcia S, Ferrari S, Owaki T, Tsugumine S, Geddes DM, Hasegawa M, Altmann D, Alton EWFW: **Tolerisation of mice to allow repeated administration of recombinant Sendai virus vector to the airways.** *Mol Ther* (2005) **9**(Suppl 1):S274.

5. Griesenbach U, McLachlan G, Somerton L, Collie D, Inoue M, Hironaka T, Owaki T, Geddes DM, Hasegawa M, Alton EWFW: **Repeated administration of Sendai virus into lung.** *Mol Ther* (2005): in press.

6. McLachlan G, Griesenbach U, Owaki T, Hillery L, Williams J, Yafeng Z, Boyd C, Gill D, Hasegawa M, Alton EW, Collie D: **Recombinant Sendai virus vector to evaluate vector distribution in a large animal model of lung gene transfer.** *Mol Ther* (2005) **9**(Suppl 1):S188.

7. **Clinical trial protocol.** Kyushu University, Fukuoka, Japan (2005). *http://www.med.kyushu-u.ac.jp/pathol1/*

8. Shirakura M, Inoue M, Fujikawa S, Washizawa K, Komaba S, Maeda M, Watabe K, Yoshikawa Y, Hasegawa M: **Essential role of PDGFRα-p70S6K signaling in mesenchymal cells during therapeutic and tumor angiogenesis *in vivo*.** *Circulation Res* (2004) **9**(5):1186-1194.

9. Masaki I, Yonemitsu Y, Yamashita A, Sata S, Tanii M, Komori K, Nakagawa K, Hou X, Nagai Y, Hasegawa M, Sugimachi K, Sueishi K: **Angiogenic gene therapy for experimental critical limb ischemia: Acceleration of limb loss by overexpression of vascular endothelial growth factor 165 but not of fibroblast growth factor-2.** *Circulation Res* (2002) **90**(9):966-967.

10. JingJing J: **Biomedicine breakthroughs.** *China Business Weekly* (2004): November 23. *http://www.chinadaily.com.cn/english/doc/2004-11/23/content_394610.htm*

11. Shirakura M, Fukumura M, Inoue M, Fujikawa S, Maeda M, Watabe K, Kyuwa S, Yoshikawa Y, Hasegawa M: **Sendai virus vector-mediated gene transfer of glial cell line-derived neurotrophic factor prevents delayed neuronal death after transient global ischemia in gerbils.** *Exp Anim* (2003) **52**(2):119-127.

12. Shirakura M, Inoue M, Fujikawa S, Washizawa K, Komaba S, Maeda M, Watabe K, Yoshikawa Y, Hasegawa M: **Postischemic administration of Sendai virus vector carrying neurotrophic factor genes prevents delayed neuronal death in gerbils.** *Gene Ther* (2004) **11**(9):784-790.

13. Inoue M, Tokusumi Y, Ban H, Shirakura M, Kanaya T, Yoshizaki M, Hironaka T, Nagai Y, Iida A, Hasegawa M: **Recombinant Sendai virus vectors deleted in both the matrix and the fusion genes: Efficient gene transfer with preferable properties.** *J Gene Med* (2004) **6**(10):1069-1081.

•• *Double-deleted SeVs can be produced and, importantly, these deletions alter the properties of the virus.*

14. Iwadate Y, Inoue M, Saegusa T, Tokusumi Y, Kinoh H, Hasegawa M, Tagawa M, Yamaura A, Shimada H: **Recombinant Sendai virus vector induces complete remission of established brain tumors through efficient interleukin-2 gene transfer in vaccinated rats.** *Clin Cancer Res* (2005) **11**(10):3821-3827.

• *This study demonstrated the efficient gene transfer and therapeutic effects of SeV in brain tumors.*

15. Kinoh H, Inoue M, Washizawa K, Yamamoto T, Fujikawa S, Tokusumi Y, Iida A, Nagai Y, Hasegawa M: **Generation of a recombinant Sendai virus that is selectively activated and lyses human tumor cells expressing matrix metalloproteinases.** *Gene Ther* (2004) **11**(14):1137-1145.

•• *This paper provides proof-of-principle that minor changes in the F protein alter SeV tropism.*

16. Inoue M, Tokusumi Y, Ban H, Kanaya T, Shirakura M, Tokusumi T, Hirata T, Nagai Y, Iida A, Hasegawa M: **A new Sendai virus vector deficient in the matrix gene does not form virus particles and shows extensive cell-to-cell spreading.** *J Virol* (2003) **77**(11):6419-6429.

17. Sugahara F, Uchiyama T, Watanabe H, Shimazu Y, Kuwayama M, Fujii Y, Kiyotani K, Adachi A, Kohno N, Yoshida T, Sakaguchi T: **Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein.** *Virology* (2004) **325**(1):1-10.

18. Waddington SN, Buckley SM, Bernloehr C, Bossow S, Ungerechts G, Cook T, Gregory L, Rahim A, Themis M, Neubert WJ, Coutelle C *et al*: **Reduced toxicity of F-deficient Sendai virus vector in the mouse fetus.** *Gene Ther* (2004) **11**(7):599-608.

19. Sasaki K, Inoue M, Shibata H, Ueda Y, Muramatsu SI, Okada T, Hasegawa M, Ozawa K, Hanazono Y: **Efficient and stable Sendai virus-mediated gene transfer into primate embryonic stem cells with pluripotency preserved.** *Gene Ther* (2005) **12**(3):203-210.

20. Kato N, Nakanishi K, Nemoto K, Morishita R, Kaneda Y, Uenoyama M, Ikeda T, Fujikawa K: **Efficient gene transfer from innervated muscle into rat peripheral and central nervous systems using a non-viral haemagglutinating virus of Japan (HVJ)-liposome method.** *J Neurochem* (2003) **85**(3):810-815.

21. Namiki M, Kawashima S, Yamashita T, Ozaki M, Sakoda T, Inoue N, Hirata K, Morishita R, Kaneda Y, Yokoyama M: **Intramuscular gene transfer of interleukin-10 cDNA reduces atherosclerosis in apolipoprotein E-knockout mice.** *Atherosclerosis* (2004) **172**(1):21-29.

22. Shimamura M, Sato N, Oshima K, Aoki M, Kurinami H, Waguri S, Uchiyama Y, Ogihara T, Kaneda Y, Morishita R: **Novel therapeutic strategy to treat brain ischemia: Overexpression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model.** *Circulation* (2004) **109**(3):424-431.

23. Ino A, Yamamoto S, Kaneda Y, Kobayashi I: **Somatic gene targeting with RNA/DNA chimeric oligonucleotides: An analysis with a sensitive reporter mouse system.** *J Gene Med* (2004) **6**(11):1272-1280.

24. Ito M, Yamamoto S, Nimura K, Hiraoka K, Tamai K, Kaneda Y: **Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin.** *J Gene Med* (2005):doi:10.1002/jgm.753.

25. Ito K, Chen J, Asano T, Vaughan ED Jr, Poppas DP, Hayakawa M, Felsen D: **Liposome-mediated gene therapy in the kidney.** *Hum Cell* (2004) **17**(1):17-28.

26. Qiang L, Yi J, Fu-De C: **Melanoma vaccine based on the vector of membrane fusogenic liposomes.** *Pharmazie* (2004) **59**(4):263-267.

27. Takeda S, Shiosaki K, Kaneda Y, Nakasatomi T, Yoshizaki H, Someya K, Konno Y, Eda Y, Kino Y, Yamamoto N, Honda M: **Hemagglutinating virus of Japan protein is efficient for induction of CD4+ T-cell response by a hepatitis B core particle-based HIV vaccine.** *Clin Immunol* (2004) **112**(1):92-105.

28. Owada-Makabe K, Tsubota Y, Yukawa K, Kakimoto N, Liang XM, Ichinose M, Maeda M: **Direct *in vivo* protein transduction into a specific restricted brain area in rats.** *Neurosci Lett* (2005) **378**(1):18-21.

29. Iida A, Hasegawa M: **SeV RNA replicon vector: An efficient cytoplasmic expression system derived from Sendai virus self-replicating ribonucleoprotein complexes.** *Mol Ther* (2005) **9**(Suppl 1):S275.

30. Shirohzu H, Mitomo K, Tabata T, Griesenbach U, Hyde SC, Alton EFEW, Ueda Y, Hasegawa M: **Efficient *in vivo* transduction of mouse airway epithelial cells by simian immunodeficiency virus vector pseudotyped with Sendai virus F and HN proteins.** *Mol Ther* (2005) **9**(Suppl 1):S188.

31. Matano T, Kano M, Nakamura H, Takeda A, Nagai Y: **Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen.** *J Virol* (2001) **75**(23):11891-11896

32. Matano T, Kobayashi M, Igarashi H, Takeda A, Nakamura H, Kano M, Sugimoto C, Mori K, Iida A, Hirata T, Hasegawa M *et al*: **Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial.** *J Exp Med* (2004) **199**(12):1709-1718.

• *This study demonstrated that SeV is a useful vector in AIDS vaccination strategies.*

33. Lun WH, Takeda A, Nakamura H, Kano M, Mori K, Sata T, Nagai Y, Matano T: **Loss of virus-specific CD4+ T cells with increases in viral loads in the chronic phase after vaccine-based partial control of primary simian immunodeficiency virus replication in macaques.** *J Gen Virol* (2004) **85**(7):1955-1963.

34. Kato M, Igarashi H, Takeda A, Sasaki Y, Nakamura H, Kano M, Sata T, Iida A, Hasegawa M, Horie S, Higashihara E *et al*: **Induction of Gag-specific T-cell responses by therapeutic immunization with a Gag-expressing Sendai virus vector in macaques chronically infected with simian-human immunodeficiency virus.** *Vaccine* (2005) **23**(24):3166-3173.

•• *This study demonstrated that SeV is a useful vector in AIDS vaccination strategies.*

35. Takimoto T, Hurwitz JL, Coleclough C, Prouser C, Krishnamurthy S, Zhan X, Boyd K, Scroggs RA, Brown B, Nagai Y, Portner A, Slobod KS: **Recombinant Sendai virus expressing the G glycoprotein of respiratory syncytial virus (RSV) elicits immune protection against RSV.** *J Virol* (2004) **78**(11):6043-6047.

•• *This study demonstrated that SeV is a useful vector in RSV vaccination strategies.*

36. Slobod KS, Shenep JL, Lujan-Zilbermann J, Allison K, Brown B, Scroggs RA, Portner A, Coleclough C, Hurwitz JL: **Safety and immunogenicity of intranasal murine parainfluenza virus type 1 (Sendai virus) in healthy human adults.** *Vaccine* (2004) **22**(23-24):3182-3186.

37. Bernloehr C, Bossow S, Ungerechts G, Armeanu S, Neubert WJ, Lauer UM, Bitzer M: **Efficient propagation of single gene deleted recombinant Sendai virus vectors.** *Virus Res* (2004) **99**(2):193-197.

38. Nishio M, Nagata A, Tsurudome M, Ito M, Kawano M, Komada H, Ito Y: **Recombinant Sendai viruses with L1618V mutation in their L polymerase protein establish persistent infection, but not temperature sensitivity.** *Virology* (2004) **329**(2):289-301.

Venture

センダイウイルスベクターによる細胞質遺伝子治療

Sendai Virus Vector-based Cytoplasmic Gene Therapy

井上　誠
ディナベック株式会社

Key words
gene therapy, Sendai virus,
negative-strand RNA virus

Abstract

「細胞質遺伝子治療」という新しい概念でのベクター開発を行っている。センダイウイルスベクターのような「細胞質型RNAベクター」は，全生活環においてDNAへの変換がなく宿主細胞染色体との相互作用がないことから，従来使用されてきた染色体組み込み型あるいは核内で増殖し低頻度ながら染色体への組込みが生じるベクターとは異なり，全く遺伝毒性がないベクターである。このような特徴あるベクターを開発するにあたって，われわれはセンダイウイルスゲノムから遺伝子を欠失していく手法をとってきた。F遺伝子欠失型，M遺伝子欠失型あるいは複数の遺伝子を同時に欠失したベクターを開発しているが，遺伝子を欠失することで安全性を高めるとともに，治療対象疾患に則したベクターへ改良することに成功している。重症の虚血下肢や各種癌に対する遺伝子治療および遺伝子ワクチンとしての適用が検討されている。また，遺伝子解析への応用も期待される。

はじめに

治療用遺伝子を核内に入れたり，染色体遺伝子に組み込むことなく，細胞質において直接発現する遺伝子治療法が開発されている。この方法は，われわれが開発しているセンダイウイルスベクターをはじめとする「細胞質型RNAベクター」を利用することで可能になるが（図1），遺伝子治療として大変新しい概念となるので，われわれは「細胞質遺伝子治療」という言葉を使用している。現在までに臨床応用に至っているウイルスベクターは，レトロウイルスベクターやアデノ随伴ウイルスベクターなどのような染色体組み込み型のベクター，あるいは，アデノウイルスベクターのように核内で増殖し，低頻度ながら染色体への組込みが生じるベクターである。また，リポソームなどを用いてプラスミドを導入する非ウイルスベクターの場合も，染色体への組込みにより治療用遺伝子を発現する。このようなベクターによる染色体への組込みについては，以前からその危険性が指摘されていたが，現実にフランスでの小児の重症複合免疫不全症(X-SCID)治療で，レトロウイルスベクターにより白血病を引き起こす副作用が報告され，問題点が改めて浮き彫りになった[1]。X-SCID治療については遺伝子治療による劇的な治療効果があることから，risk and benefitの観点により治療が再開される可能性があるが，遺伝子治療の普及のためには，染色体への組込みのない，すなわち遺伝毒性のないベクターの開発が強く望まれている。

1．センダイウイルスベクターの特徴

われわれがベクターとして開発しているセンダイウイルスは，一本鎖の非分節型マイナス鎖RNAをゲノムとして持つ。ゲノムRNAは，ヌクレオカプシッド(NP)蛋白と非常に強く結合しており，この状態でのみRNA合成の鋳型活性を有する。野生型ウイルス（全長15,384塩基）のゲノムRNAには主要な6個の遺伝子がコードされ，3'端から順にNP蛋白

Makoto Inoue
DNAVEC Corporation
〒305-0856　茨城県つくば市観音台1-25-11　Fax: 029-856-4286　E-mail: inoue@dnavec-corp.com

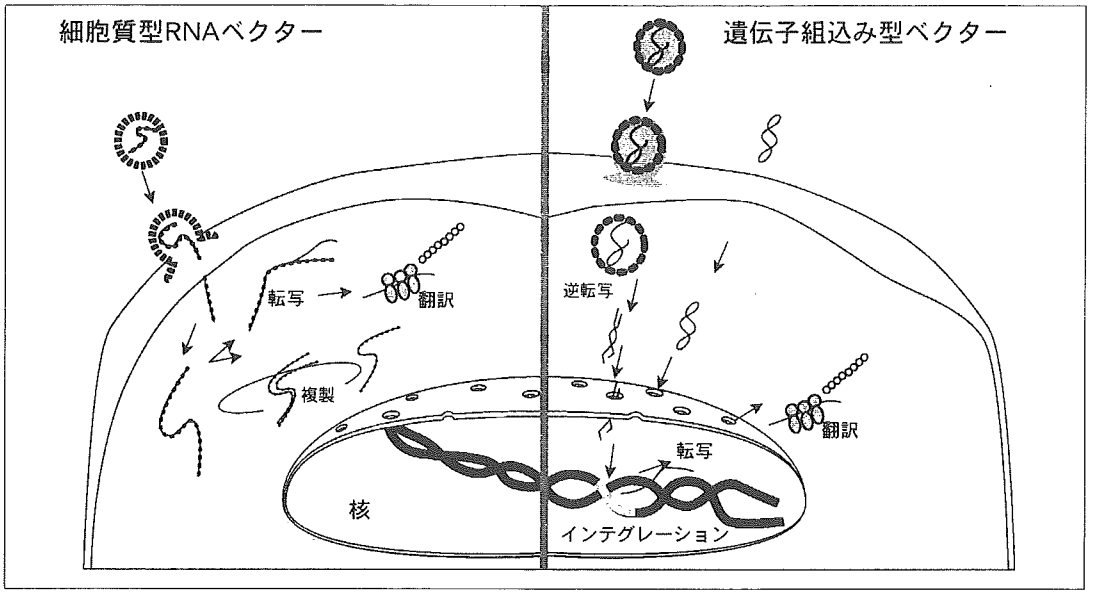


図1　細胞質型RNAベクターと遺伝子組込み型ベクターの遺伝子発現様式
センダイウイルスベクターのような細胞質型RNAベクターは，細胞質内で複製と転写翻訳を行い，核内へは移行しない。また，DNAへの変換もない。一方，遺伝子組込み型ベクターは，DNAとして核内に移行し，染色体にインテグレートされ，遺伝子は転写，翻訳される。

質遺伝子，RNAポリメラーゼの小サブユニットであるリン酸化蛋白質(P)遺伝子，ウイルス粒子の殻の構造を内側から維持し，ウイルス粒子のアセンブリーと出芽に関与するマトリックス蛋白質(M)遺伝子，そして宿主細胞への侵入にかかわる膜融合蛋白質(F)遺伝子とその際の細胞結合に関わる赤血球凝集素／ノイラミニダーゼ(HN)遺伝子，最後にRNAポリメラーゼの大サブユニットである巨大(ラージ)蛋白質(L)遺伝子が直線的に並んでいる（図2）。各遺伝子がコードするタンパク質のうち，NP, P及びLの3種類のタンパク質は，ウイルスのゲノムRNAとともに，転写・複製の鋳型となり，かつ自律複製可能なレプリコンであるゲノムRNA-タンパク複合体 (ribonucleoprotein complex, RNP)を形成する。それぞれの遺伝子は個々の転写制御ユニットを形成しているため，単独のmRNAとして転写され，それぞれから一個の蛋白質が翻訳される。例外的に，P遺伝子からはP蛋白質以外に異なる蛋白質読み枠を利用して翻訳される非構造蛋白質(C)とP mRNAの読み取り途中でのRNA編集により読み枠が変えられてできる蛋白質(V)の3つが翻訳される。このような方法により，総計8種類の蛋白質が産生される。

センダイウイルスは，その全生活環においてDNAへの変換がなく，転写ならびにゲノムの複製は細胞質内で，自前のRNAポリメラーゼ（PおよびL蛋白質）を利用して行われるため，宿主細胞の染色体との相互作用がない。これらの特徴は，同じRNAウイルスであるがプロウイルスDNAへ変換された後，宿主細胞染色体へ組込まれるレンチウイルスベクターやレトロウイルスベクターとは全く異なる。また，センダイウイルスは，HN蛋白がN-アセチルシアル酸を受容体として認識して結合する。シアル酸糖鎖はほとんどの哺乳動物細胞で発現しているため，宿主域が広く，*in vitro*においてはほとんどの細胞に感染可能である。このベクターの感染効率も高く，多くの細胞において MOI (Multiplicity of infection)=3 ～ 10でほぼ100%近い細胞に感染を成立することができる。例えば，各種細胞株，初代肝細胞，初代肺上皮細胞，初代平滑筋細胞，$CD34^+$血液幹細胞，大脳皮質初代神経細胞および後根神経節初代神経細胞などへの効率的な感染と遺伝子発現が確認されている。感染後（遺伝子導入後），直ちにゲノムの転写・複製を開始し，以後遺伝子発現は対数的に増加し，2日程で遺伝子発現は最大となり，多くの培養細胞において以後安定な持続感染（持続的遺伝子発現）に至る。実験動物(*in vivo*)においては，気道上皮細胞，筋肉，血管内皮細胞，脳室上衣細胞，鼻粘膜上皮細胞及び網膜下神経細胞などへの効率的な感染と遺伝子発現が確認されている（図3）[3]。

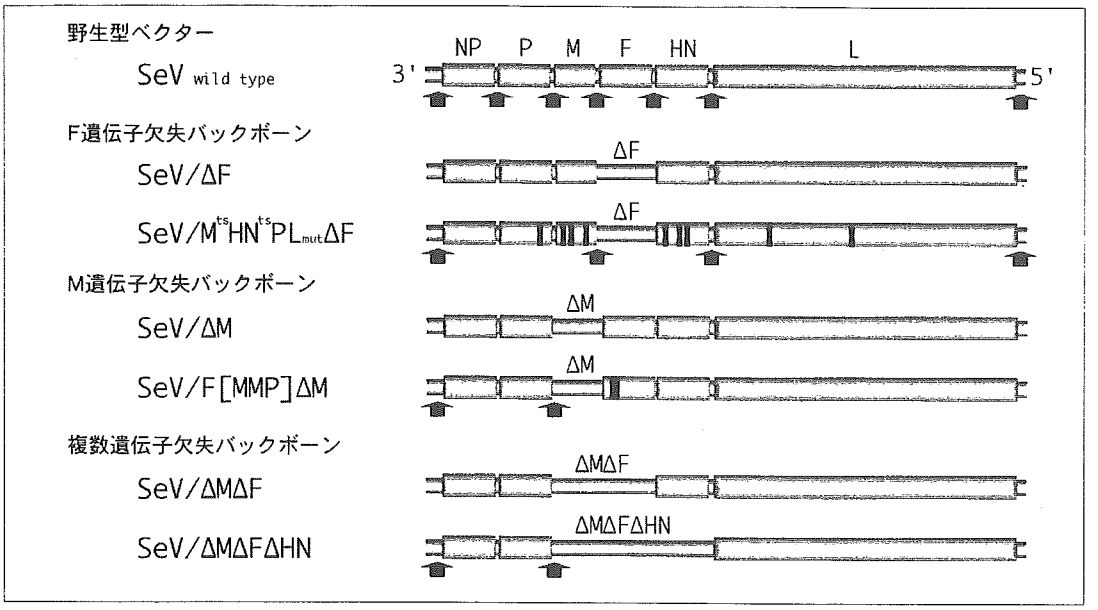


図2　各種遺伝子欠失型センダイウイルス(SeV)ベクター
目的によって各種遺伝子欠失型ベクターを使い分ける。また，目的遺伝子(GOI)の期待する発現量により，遺伝子搭載位置（矢印）を変更してベクターを構築する。

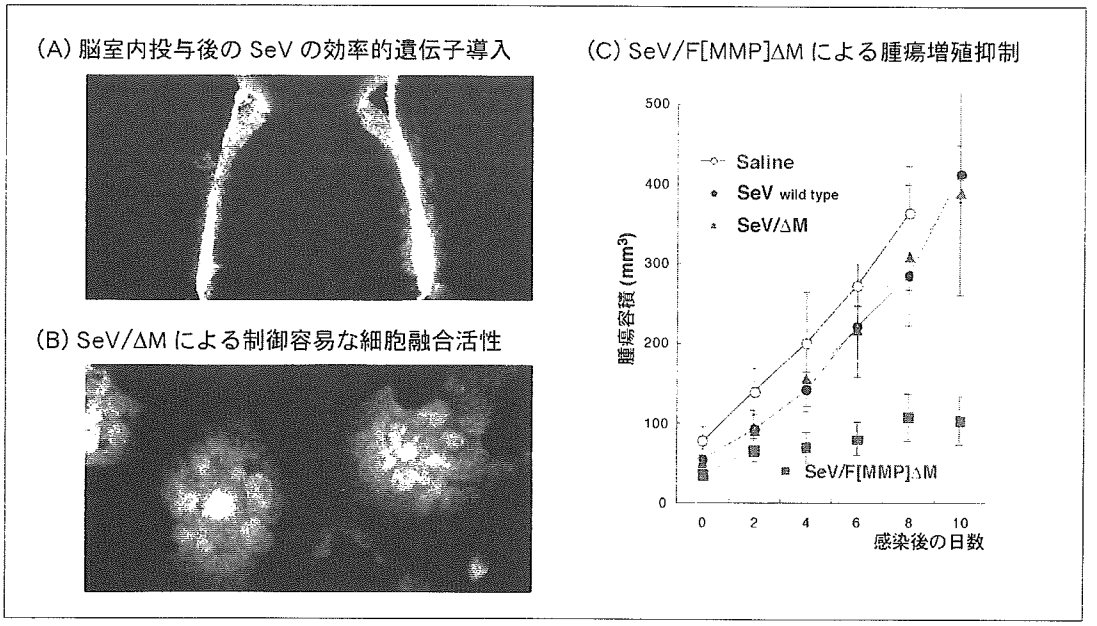


図3　センダイウイルスベクターの特徴的遺伝子導入と活性

2．センダイウイルスベクターの改良

このような特徴のあるセンダイウイルスのベクター化を進めるにあたって，われわれはセンダイウイルスゲノムから遺伝子を欠失していく手法をとってきた（図2）。遺伝子を欠失することで安全性を高めるとともに，治療対象疾患に則したベクターへ改良することに成功している。遺伝子欠失型ベクター粒子を再構成するためには，欠失した遺伝子産物をトランスに供給するパッケージング細胞（ヘルパー細胞）の作出が必須であり，その性能こそが遺伝子欠失型ベクターの再構成および生産の成否を決定する。

1）F遺伝子欠失バックボーン

F蛋白質は，宿主細胞への侵入にかかわる膜融合蛋白質であるが，われわれはゲノムからこのF遺伝子を欠失させることにより，二次感染性のない，非伝播型ベクターへ改良することに成功した[3]。F蛋白発現パッケージング細胞は，サル腎由来細胞LLC-MK$_2$を元に，F遺伝子を発現誘導後に持続発現細胞として樹立する方法により構築した。再構成されたF遺伝子欠失型ベクターは，wild typeと同等の感染性（遺伝子導入能）・遺伝子発現能を有しており，元のウイルスの組織傷害性などはかなり減弱していた。このような非伝播型ベクターへの改良による安全性能向上の成功を受けて，人への適用を実施することが可能になり，実際に，九州大学付属病院において臨床研究が計画され，現在，厚生労働省での審議が行われている。その計画では，FGF-2 (fibroblast growth factor 2：塩基性線維芽細胞増殖因子）遺伝子を搭載したF遺伝子欠失型ベクターを利用して，慢性動脈硬化症などを原因とする重症の虚血下肢（重症虚血肢）に対する血管新生を目的とした遺伝子治療が実施される。ベクター選択とともに，搭載遺伝子選択にも工夫が凝らされた計画である。この臨床研究は国内開発ベクターによる初めての細胞質遺伝子治療となるが，臨床研究実施に向けてGMP生産にも成功している。つまり，この新しいベクターの臨床レベルの基本技術は出来上がっている状況である。

われわれは，このF遺伝子欠失に加えてアミノ酸変異を導入することでさらに安全なベクターへ改良することにも成功している[4]。センダイウイルスの温度感受性変異株および持続感染変異株で同定された合計9種のアミノ酸変異を導入することで，さらに大巾に細胞傷害性を減弱させることができた。このベクターは，先に述べたF蛋白発現パッケージング細胞を利用して生産することが可能であり，GMP生産への移行も容易である。

2）M遺伝子欠失バックボーン

M蛋白質は，ウイルス粒子のアセンブリーと出芽の中心的役割を担っており，ゲノムからM遺伝子を欠失することで，感染細胞からの粒子放出が原理的にも抑えられる。非常に興味あることに，われわれが構築に成功したM遺伝子欠失型ベクターは，感染細胞を隣接した細胞と融合させるため，最終的に数十個以上の細胞が融合したシンシチウム(syncytium)を形成する（図3-B）[5]。これは，感染細胞の表面にベクターからFおよびHN両蛋白が発現し，センダイウイルスと細胞が膜融合を生じるのと同じメカニズムで，感染細胞に隣接した非感染細胞と融合したためである。ベクターとして重要な点は，この融合がF蛋白質の活性化依存的であるため，このシンシチウム形成を制御できるという点である。そこで，このシンシチウム形成を癌特異的に生じるようなベクターへの改変を試みた。F蛋白質は不活性型であるF$_0$蛋白として合成され，生理的には気道で分泌されるトリプターゼクララによりF$_1$およびF$_2$に切断されて活性化型となる[6]。ベクター生産時には培地中にトリプシンを添加しているため，活性化されたベクターが生産される。このF$_0$の切断部位を，浸潤転移性癌細胞で分泌が亢進しているマトリックスメタロプロテアーゼ(matrix metallo-proteinase: MMP)が認識する特異的基質配列に変更し，癌細胞特異的に細胞融合が生じるようなベクターへの改良を行った[7]。MMPによる切断効率と，切断後のF蛋白の活性の両者を満たす必要があり，「PLGMTS」という新規の配列へ置換することによって初めてMMP依存的なF蛋白の活性化が可能であった。構築されたF改変M遺伝子欠失型ベクターは，MMP発現を亢進している癌細胞でのみ細胞融合活性を示し，ヒト線維肉腫細胞であるHT1080の担癌モデルマウスにおいて，顕著な癌増殖抑制活性を示した(図3-C)。癌細胞特異的にシンシチウムを形成する新しいタイプのoncolyticベクターが構築できたことになる。特筆すべきは，治療用遺伝子を搭載せずに，ベクターのみの作用で治療効果が確認されていることであり，治療用遺伝子搭載（サイトカインあるいは自殺遺伝子など）を含め，現在さらなる改良検討を実施している。

3）複数遺伝子欠失バックボーン

ウイルスベクターを*in vivo*に直接投与した場合には，*in vitro*における感染（遺伝子導入）のような持続感染には至らないことが多く，染色体組み込み型ベクター以外のものでは遺伝子発現は数日～数週間の場合が多い。これはベクターに対する免疫反応が惹起されるためであり，F遺伝子欠失型センダイウイルスベクターでも同様である。センダイウイルスベクター由来の遺伝子を，出来るだけゲノムから

欠失していくことによりその減弱がはかられると期待され，中和抗体産生に関わるエンベロープ関連遺伝子の欠失は重要な意味を持つと考えられる。われわれはすでに，MおよびFの2遺伝子欠失型ベクターの構築に成功しており，wild typeおよびF遺伝子欠失型ベクターと同等の感染性（遺伝子導入能）・遺伝子発現能を有しており，組織傷害性などはさらに減弱していることを確認した[8]。現在，M, F, HN 3遺伝子欠失型ベクターも実験レベルでの調製が可能になっている。このタイプのベクターでは，エンベロープ関連遺伝子全てをゲノムから欠失しており，下に述べる細胞質型RNAベクターのデザインでは最も進化したものになっている。

3．細胞質型RNAベクター全体の開発状況

「細胞質型RNAベクター」を遺伝子治療用ベクターとして開発する試みは，その後数多く実施されており，そのほとんどがoncolytic virusとしての癌治療への応用である。具体的には，mumps virus, Newcastle disease virus, measles virus, vesicular stomatitis virus, influenza virus, reovirus, およびpoliovirusなどで実施されている[9]。この中で，Newcastle disease virusのワクチン株(PV701)を利用した臨床試験が先行しており，比較的良好な結果が得られている[10]。また，measles virusを用いた，卵巣癌や多発性骨髄腫に対する臨床試験も近々に実施される予定である。但し，癌治療以外への応用に関しては今までのところ報告がない。また細胞質型RNAベクターの分野において，唯一われわれのみが遺伝子欠失型の再構成と大量生産に成功しており，安全性の高い非伝播型ベクターへの改良により，癌以外への適用が実施可能になっていると言える。センダイウイルスのヒトへの投与実績としては，野生型ウイルスをヒトパラインフルエンザウイルスⅠ型ウイルスへの生ワクチンとして使用した米国での臨床試験が報告されている[11]。このような野生型センダイウイルスをヒトへ投与した場合でも重篤な副作用は検出されておらず，非伝播型センダイウイルスベクターであれば，理論的により安全に投与できるものと期待され，臨床研究の実施が待たれる。

表　センダイウイルスベクター利用の方向性

1．遺伝子治療用ベクター
・重症虚血肢など良性疾患の治療
・各種癌など悪性疾患の治療
・遺伝子ワクチン
2．遺伝子解析用ベクター
・遺伝子機能解析
・蛋白質機能解析(*in vitro*, *in vivo*)
3．汎用的利用
・スクリーニング用ベクター
・蛋白質生産

おわりに

遺伝子治療は大きく2種類に分類することができる。第一は生まれながらにして遺伝子に欠陥があるために発症する病気に対して，欠陥遺伝子そのものを正常な遺伝子と入れ替えあるいは補うことによって，その病気を治療する治療法である。他方は，欠陥遺伝子そのものでなくとも，治療用遺伝子を薬として利用し，病気の進行を抑え，ベクターは一種のdrug delivery法として利用するものである。センダイウイルスベクターのような「細胞質型RNAベクター」の利用は，現時点では後者に属することが多いが，前者の利用法への試みも実施している。他のベクターにも共通する免疫原性の問題を十分に解析し，さらなる改良を推進していきたい。また，われわれは，非伝播型センダイウイルスベクターの外部供給体制が整い，一般的な受注生産が可能な状況にある。遺伝子治療分野のみではなく，遺伝子解析分野においても有効に機能することが期待され（表），今まで，遺伝子導入効率の面で諦めていた実験も，この新しいベクターにより可能になる可能性が高い。他のベクターにはない特徴を有する，「細胞質型RNAベクター」を是非一度利用して性能を見極めていただきたい。

文　献

1) Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al.*: Science. 302: 415-419, 2003.
2) Bitzer, *et al.*: J Gene Med. 74: 6564-6569, 2003.
3) Li HO, Zhu YF, Asakawa M, *et al.*: J Virol. 74: 6564-6569, 2000.
4) Inoue M, Tokusumi Y, Ban H, *et al.*: J Virol. 77: 3238-3246, 2003.
5) Inoue M, Tokusumi Y, Ban H, *et al.*: J Virol. 77: 6419-6429, 2003.
6) Nagai Y.: Trends Microbiol. 1: 81-87, 1993.
7) Kinoh H, Inoue M, Washizawa K, *et al.*: Gene Ther. 11: 1137-1145, 2004.
8) Inoue M, Tokusumi Y, Ban H, *et al.*: J Gene Med. 6: 1069-1081, 2004.
9) Russell SJ.: Cancer Gene Ther. 9: 961-966, 2002.
10) Lorence RM, Pecora AL, Major PP, *et al.*: Curr Opin Mol Ther. 5: 618-624, 2003.
11) Slobod KS, Shenep JL, Lujan-Zilbermann J, *et al.*: Vaccine. 22: 3182-3186, 2004.

Anne Spinewine, MPharm MSc*
School of Pharmacy
Didier Schoevaerdts, MDGimbada B. Mwenge, MD
Christian Swine, MD
Department of GeriatricsMont-Godinne Medical Center

Alain Dive, MD
Intensive Care Unit
Mont-Godinne Medical Center
Université catholique de Louvain
Louvain, Belgium

*Anne Spinewine is Aspirant of the Belgian Fonds National de la Recherche Scientifique.

REFERENCES

1. Juurlink DN, Mamdani MM, Kopp A et al. Drug-induced lithium toxicity in the elderly: A population-based study. J Am Geriatr Soc 2004;52:794–798.
2. Finley PR, Warner MD, Peabody CA. Clinical relevance of drug interactions with lithium. Clin Pharmacokinet 1995;29:172–191.
3. Stockley IH. Stockley's Drug Interactions, 6th Ed. London: Pharmaceutical Press, 2002.
4. Dunner DL. Drug interactions of lithium and other antimanic/mood-stabilizing medications. J Clin Psychiatry 2003;64(Suppl. 5):38–43.
5. Dawson AH, Whyte IM. Therapeutic drug monitoring in drug overdose. Br J Clin Pharmacol 1999;48:278–283.
6. Rochon PA, Gurwitz JH. Optimising drug treatment for elderly people: The prescribing cascade. BMJ 1997;315:1096–1099.
7. Hanlon JT, Schmader KE, Ruby CM et al. Suboptimal prescribing in older inpatients and outpatients. J Am Geriatr Soc 2001;49:200–209.

CARDIAC DYSFUNCTION WITH SEVERE ANEMIA IN AN AGED CASE

To the Editor: On April 16, 2002, an 88-year-old woman was admitted to our hospital with complaints of easy fatigability and exertional dyspnea. Anemic conjunctiva palpebrae and pretibial edema were noticed on admission. Laboratory findings on admission showed white blood cell count 4,880/μL, red blood cells 131×10^4/μL, hemoglobin (Hb) 4.4 g/dL, hematocrit 12.9%, platelets 25.7×10^4/μL, reticulocytes 0.1%, serum creatinine 0.9 mg/dL, and serum iron 171 microgram/dL. Erythropoietin was highly elevated (2,860 mU/mL). Bone marrow examination showed severe hypoplasia specific for erythroid line (erythroid 0%), suggesting a diagnosis of pure red cell aplasia. Chest roentgenogram showed an enlarged heart with a cardiothoracic ratio of 60% and presence of pleural effusion, suggesting impaired cardiac function by severe anemia. Electrocardiogram (ECG) showed no abnormality except low amplitude of T-waves. Ultrasonocardiogram revealed reduced left ventricular ejection fraction and left ventricular dilatation at diastolic phase. Chest computerized tomography revealed relatively enlarged thymic mass (3 × 3 cm) for her age. As blood transfusion was performed to raise Hb level to 7 g/dL, pleural effusion and exertional dyspnea disappeared. Thymectomy was performed for treatment of pure red cell aplasia. After the thymectomy, cyclosporine A was administered. Hb gradually rose and reached 8.5 g/dL to 9.5 g/dL, accompanying normalization of erythropoietin (EPO) level (~20 IU/L). Cardiac function was promptly recovered, as shown by chest roentgenogram, ECG, and ultrasonocardiogram (Figure 1). Pretibial edema decreased gradually but did not completely disappear at 10 g/dL of Hb. EPO injection of 6,000 IU per week was started. About a month later, Hb level reached 14.0 g/dL, at which point she revealed no edema at all. During the additive EPO treatment, Hb level was within a range of 11 g/dL to 14 g/dL, and the improved cardiac function was maintained.

Although anemia is prevalent in old age, the minimum physiologically required value of Hb is potentially modifiable. Anemia is defined according to World Health Organization (WHO) criteria as a concentration below 12 g/dL in women and below 13 g/dL in men. Some studies report a particularly notable increase in prevalence of anemia in the oldest subjects (≥85).[1–7] Even though normal Hb level can be deduced from mean value of Hb level of a healthy aged population, it is still unclear whether the mean value reflects a physiologically sufficient Hb level for aged organs. The present case showed that cardiac function was mostly normalized at 8.5 g/dL of Hb but sufficiently recovered and maintained in a range from 11 g/dL to 14 g/dL. Eleven g/dL of Hb seemed to be the minimum required Hb level for maintenance of normal cardiac function in this patient. In the present case, cardiac malfunction under severe anemic condition was evidenced clearly using ultrasonocardiogram. Anemia is a known risk factor for ischemic heart disease. The reduction in oxygen delivery by erythrocytes with anemia may be a cause of more severe cardiovascular diseases.[7,8] Nevertheless, in this case with severe anemia, ECG did not seem to reveal characteristic changes for presumptive tissue hypoxia such as ST depression or inverted T-wave. It has been reported that anemic condition accompanies ST-T depression or inverted T-wave,[9] but correlation between T-amplitude and Hb has not been reported. In the present case, the ratio of amplitude of T/QRS complex (T/QRS ratio) increased as Hb level increased. Although the other ECG parameters, including heart rate, RR difference, QT interval, and QT dispersion, did not correspond to the

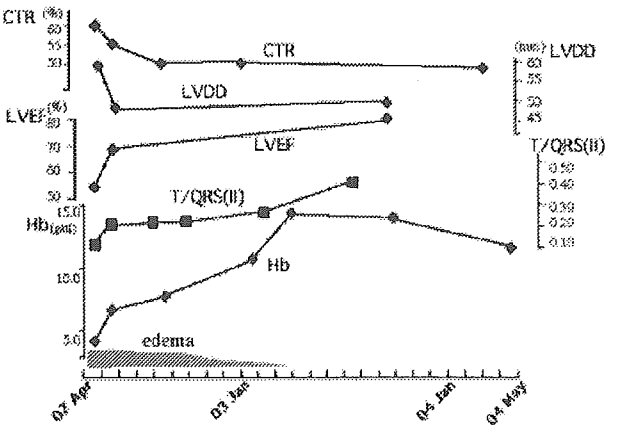


Figure 1. Cardiac function and hemoglobin (Hb) level. CTR = cardiothoracic ratio; LVDD = left ventricular diastolic diameter; LVEF = left ventricular ejection fraction; T/QRS(II) = T-wave amplitude/QRS complex amplitude in lead II of the electrocardiogram.

improving cardiac function, the observed recovery of T-amplitude revealed a good correlation with that of cardiothoracic ratio, left ventricular ejection fraction, and left ventricular dilatation at diastolic phase (Figure 1). This observation implies that the T/QRS ratio is a good indicator of cardiac malfunction caused by severe chronic anemia. Accumulation of further evidence is necessary to confirm the correlation between T-amplitude and cardiac dysfunction in chronic severe anemia.

In the present case, prolonged pretibial edema was observed even at 10 g/dL of Hb after a prompt recovery of cardiac function. This implies that pretibial edema in older patients caused by cardiac malfunction with severe anemia is prone to persist and that the edema can be erased by raising Hb to more than 11 g/dL. In cases of anemia in older patients, low T-wave and a cardiothoracic ratio greater than 50% may be a hint of the presence of anemia. Although 12g/dL of Hb is the limit value of WHO criteria in women, 11 g/dL can be regarded as a candidate value for the goal of treatment of anemia to keep sufficient cardiac function even in cases aged 85 and older.

Toyoki, Maeda, MD, PhD
Junichi Oyama, MD, PhD
Yuki Mukai, MD
Shinji Satoh, MD, PhD
Masahiro Sugano, MD, PhD
Naoki Makino, MD, PhD

Department of Molecular and Cellular Biology
Kenzaburo Tani, MD, PhD
Department of Molecular Genetics
Division of Molecular and Clinical Genetics
Medical Institute of Bioregulation
Kyushu University
Oita, Japan

REFERENCES

1. Balducci L. Anemia, cancer, and aging. Cancer Control 2003;10:478–486.
2. Chassagne P, Verdonck A, Druesne L et al. Normocytic anemia in the elderly. Should the cause of anemia systematically investigated independently of hemoglobin concentration? Rev Med Intern 2004;25:189–194.
3. Cesari M, Penninx BW, Lauretani F et al. Hemoglobin levels and skeletal muscle: Results from the InCHIANTI study. J Gerontol A Biol Sci Med Sci 2004;59A:M249–M254.
4. Izaks GJ, Westendorp RG, Knook DL. The definition of anemia in older persons. JAMA 1999;281:1714–1717.
5. Nilsson-Ehle H, Jagenburg R, Landahl S et al. Blood haemoglobin declines in the elderly: Implications for reference intervals from age 70 to 88. Eur J Haematol 2000;65:297–305.
6. Penninx BW, Pahor M, Cesari M et al. Anemia is associated with disability and decreased physical performance and muscle strength in the elderly. J Am Geriatr Soc 2004;52:719–724.
7. Beghe C, Wilson A, Ershler WB. Prevalence and outcomes of anemia in geriatrics: A systematic review of the literature. Am J Med 2004;116(Suppl 7A):3S–10S.
8. Zeidman A, Fradin Z, Blecher A et al. Anemia as a risk factor for ischemic heart disease. Isr Med Assoc J 2004;6:16–18.
9. Stanojevic M, Stankov S. Electrocardiographic changes in patients with chronic anemia. Srp Arh Celok Lek 1998;126:461–466.

Leukemia & Lymphoma, August 2005; 46(8): 1217–1222

Taylor & Francis
Taylor & Francis Group

Sustained molecular remission by non-myeloablative stem cell transplantation after autologous hematopoietic stem cell transplantation in a patient with multiple myeloma

YASUHIRO NAKASHIMA[1], MOTOAKI SHIRATSUCHI[1], YASUNOBU ABE[2], KOICHIRO MUTA[2], KENZABURO TANI[3], SATOSHI SHIOKAWA[1] & JUNJI NISHIMURA[1]

[1]*Division of Clinical Immunology, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan,* [2]*Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan,* [3]*Division of Molecular and Clinical Genetics, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan*

Abstract

Multiple myeloma (MM) is refractory to conventional chemotherapy. To achieve a sustained complete remission, we performed planned non-myeloablative allogeneic stem cell transplantation (NST) after autologous hematopoietic stem cell transplantation (HSCT) in a patient with stage III MM. Autologous HSCT was performed using high-dose melphalan after conventional chemotherapy, followed by NST from an HLA-identical sibling using low-dose total body irradiation (200 cGy) for conditioning. Cyclosporine and mycophenolate mofetil were used for graft-vs-host disease (GVHD) prophylaxis. Acute GVHD was transiently seen in the skin and intestine, while, in addition, mild chronic GVHD was seen in the oral mucosa and skin. Complete donor chimerism was achieved and the disappearance of tumor-derived monoclonal B cells was confirmed based on an analysis of immunoglobulin light chain messenger signals on day 156 when chronic GVHD occurred. The clinical course in this case strongly suggested the existence of a graft-vs-myeloma effect.

Keywords: *Autologous hematopoietic stem cell transplantation, graft-vs-myeloma effect, multiple myeloma, non-myeloablative hematopoietic stem cell transplantation*

Introduction

Multiple myeloma (MM) is a neoplasm of the plasma cells, which is characterized by the production of monoclonal protein. MM is refractory to conventional chemotherapy and the duration of survival ranges from a few months to many years, with a median survival of 3 years [1]. The efficacy and problems of high-dose therapy followed by autologous or allogeneic hematopoietic stem cell transplantation (HSCT) have been demonstrated [1–4].

The effectiveness of single high-dose chemotherapy with autologous HSCT remains controversial [5–9]. Although the graft-vs-myeloma effect associated with graft-vs-host disease (GVHD) was reported either after the withdrawal of immunosuppression or donor lymphocyte infusion [10–12], the treatment-related mortality (TRM) was reported to be much higher in allogeneic bone marrow transplantation than autologous HSCT [13]. To reduce transplantation-related mortality, allogeneic HSCT with non-myeloablative conditioning has been widely performed for hematological and non-hematological malignancies, including MM [14–17]. Recently, some groups reported promising results of autologous HSCT followed by non-myeloablative allogeneic stem cell transplantation (NST) in which they achieved both a low TRM and a prolonged disease-free survival [18–21].

To evaluate the disease status after HSCT, a method to perform the molecular monitoring of residual myeloma cells has been developed [22]. The cumulative risk of relapse at 5 years after myeloablative allogeneic HSCT was 0% for persistently polymerase chain reaction (PCR)-negative patients and 100% for persistently PCR-positive patients. Although the efficacy of molecular monitor-

Correspondence: Junji Nishimura, Division of Clinical Immunology, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu 874-0838, Japan. E-mail: jnjn@tsurumi.beppu.kyushu-u.ac.jp

Received for publication 3 March 2005.

ISSN 1042-8194 print/ISSN 1029-2403 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10428190500096708

ing after NST is still unclear, some case reports have shown molecular remission to be correlated with a sustained clinical complete remission (CR) [20,21]. We herein report a case of MM treated with scheduled NST after autologous HSCT. In this case, we were able to achieve complete donor chimerism and molecular remission of MM after the occurrence of chronic GVHD.

Case report

A 64-year-old female was admitted to our hospital in October 2001 for an evaluation of monoclonal gammopathy and Bence Jones proteinuria. A peripheral blood analysis revealed anemia (hemoglobin 9.3 g/dl). The serum concentration of total protein was high at 13.2 g/dl with gammopathy (γ-gl 51.4%). The IgG concentration was also high at 9025 mg/dl. The blood plasma electric fractions revealed monoclonal gammopathy of IgG-λ. Bone marrow aspiration revealed an increase in atypical plasma cells (53% of nucleated cells). No bone lesions were found in an X-ray survey and bone scintigraphy. A diagnosis of stage III MM was thus made. Because a poor prognosis was predicted, we planned to perform NST after autologous HSCT. The protocol of this treatment was approved by the ethics committee of Kyushu University Hospital, and written informed consent was obtained from the patient.

After the patient received 2 courses of VAD therapy (vincristine, doxorubicin, dexamethasone), peripheral blood stem cell mobilization with cyclophosphamide (4 g/m^2) and granulocyte colony-stimulating factor (G-CSF) was performed. In March 2002, she was treated with 2 doses of melphalan (100 mg/m^2) followed by autologous stem cell support (CD34$^+$ cells 4.5 $\times$ 10^6/kg). The recovery of neutrophils ($>$ 500/μl) and platelets ($>$ 50,000/μl) was performed on days 11 and 13, respectively. No infections or organ dysfunction were observed. In April, the serum concentration of IgG and the percentage of atypical plasma cells in bone marrow had decreased to 1888 mg/dl and 1.8%, respectively (Figure 1). However, monoclonal protein was still detectable.

In June 2002, she received low-dose total body irradiation (200 cGy) followed by an infusion of G-CSF-mobilized allogeneic peripheral blood stem cells from an HLA-identical sibling (day 0). The number of infused CD34$^+$ cells was 8.4 $\times$ 10^6/kg. Prophylaxis for GVHD was started with cyclosporine (CSP) on day -3 and mycophenolate mofetil (MMF) on day 0. Myelosuppression after transplantation was mild and a blood transfusion was not performed. On

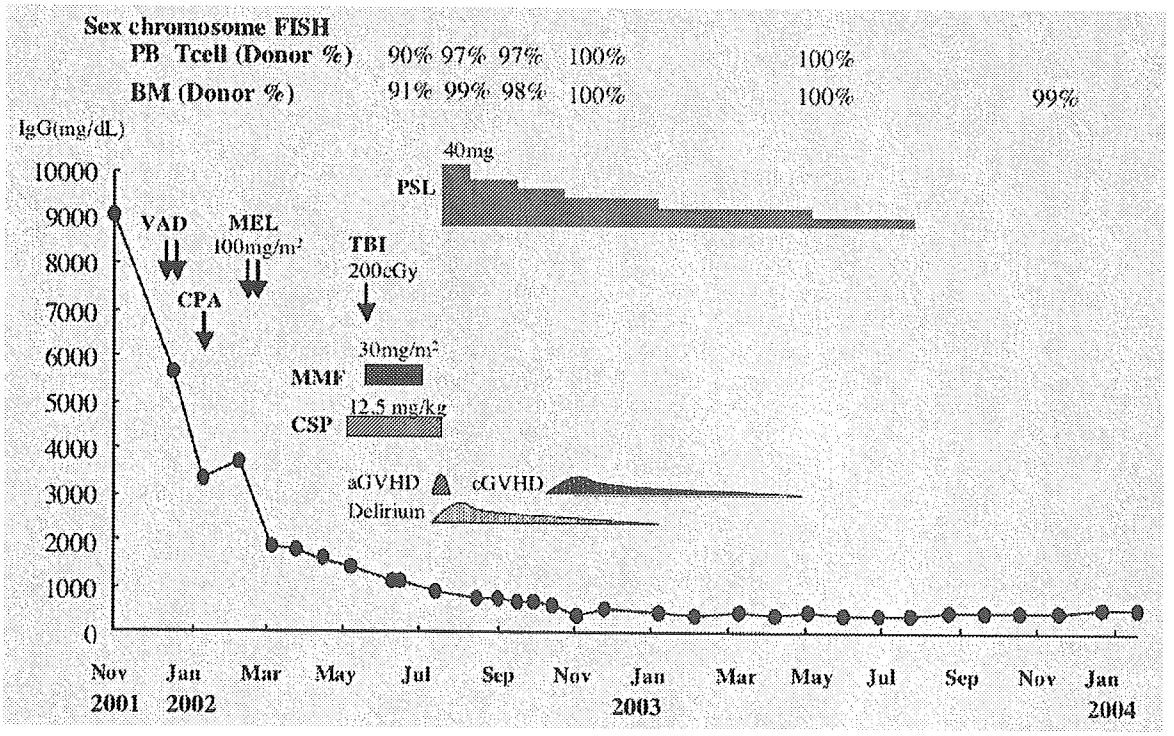


Figure 1. Clinical course. VAD, vincristine, doxorubicin, dexamethasone; CPA, cyclophosphamide; MEL, melphalan; CSP, cyclosporine; MMF, mycophenolate mofetil; TBI, total body irradiation; PSL, prednisolone; aGVHD, acute graft-vs-host disease; cGVHD, chronic graft-vs-host disease.

day 28, the percentage of plasma cells in the bone marrow decreased to 0.6%. An analysis of chimerism by fluorescence in situ hybridization (FISH) of sex chromosomes revealed that the percentages of donor cells in the peripheral blood T cells and bone marrow cells were 90 and 91%, respectively (Figure 1). Mild acute GVHD of the skin and the intestine observed in this period improved until day 35 without any changes in the immunosuppressive therapy. On day 30, she began to suffer from delirium. Brain magnetic resonance imaging in T2W1 revealed multiple high-signal areas in the white matter of the bilateral cerebral hemisphere, which was considered to be compatible with encephalopathy induced by CSP. Prednisolone was therefore used for immunosuppression after the discontinuation of CSP on day 36. The patient's delirium gradually improved by the administration of quetiapine fumarate. On day 90, the monoclonal protein of IgG-λ disappeared by immunoelectrophoresis and the serum concentration of IgG decreased to 741 mg/dl. After day 140, chronic GVHD of the skin and oral mucosa was observed. On day 156, complete donor chimerism was achieved in both the peripheral blood and bone marrow by FISH analysis. Although the skin lesions of chronic GVHD disappeared, a clinical CR was sustained until December 2004.

To evaluate the presence of minimal residual disease, we performed reverse transcriptase-PCR (RT-PCR) and a subsequent single-strand conformation polymorphism (SSCP) analysis of the complementarity determining region (CDR) 3 of immunoglobulin λ light chain messenger signals, as previously described [2]. Mononuclear cells (MNCs) from serial bone marrow and peripheral blood samples were used as the materials. In an RT-PCR SSCP analysis, monoclonal tumor B cells were detected as a single band and a clonal identity of tumor B cells in different samples could be determined by comparing the mobility of each sample. Serial dilution experiments, in which the MNCs from B-chronic lymphocytic leukemia (CLL) were serially diluted into the MNCs from a healthy subject, revealed that an RT-PCR SSCP analysis detected 1 malignant cell in 100–1000 MNCs [2]. Because each myeloma cell has a much higher level of RNA content than a B-CLL tumor cell, the sensitivity of RT-PCR SSCP analysis is expected to be higher in the detection of myeloma cells than that of B-CLL cells.

As shown in Figure 2, a dominant monoclonal band was observed in bone marrow on admission and on day 30 after NST. These bands were considered to be derived from myeloma cells. Faint

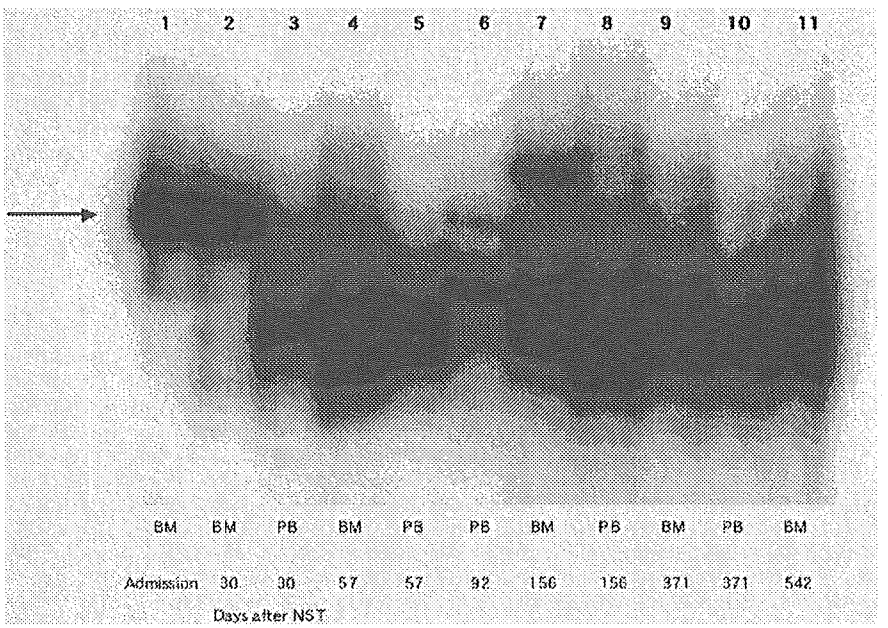


Figure 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) single-strand conformation polymorphism (SSCP) analysis of complementarity determining region 3 (CDR3) of immunoglobulin light chain messenger RNA. Lane 1, bone marrow (BM) on admission; lane 2, BM on day 30 after non-myeloablative allogeneic stem cell transplantation (NST); lane 3, peripheral blood (PB) on day 30 after NST; lane 4, BM on day 57 after NST; lane 5, PB on day 57 after NST; lane 6, PB on day 92 after NST; lane 7, BM on day 156 after NST; lane 8, PB on day 156 after NST; lane 9, BM on day 371 after NST; lane 10, PB on day 371 after NST; lane 11, BM on day 542 after NST. An arrow indicates a myeloma cell-derived monoclonal band. A dominant monoclonal band observed in lanes 1 and 2 became faint in lanes 3–6 and disappeared in lanes 7–11.

bands, which had an identical mobility with a dominant band in bone marrow on admission and on day 30, were observed in peripheral blood on day 30, bone marrow on day 57, peripheral blood on day 57, and peripheral blood on day 92. However, on days 156, 372, and 542, no myeloma cell-derived bands were detected in the bone marrow and peripheral blood samples. Interestingly, the transient clonal bands with a different mobility from that of myeloma cells were detected in all samples except bone marrow on admission and on day 30 after NST.

Discussion

Many large-scale studies have demonstrated a high CR rate to be achieved by autologous HSCT, but it seems to be difficult to maintain the CR status because of a high relapse rate [5–9] (Table I). In 2 large-scale randomized analyses conducted in France and England, the median overall survival (OS) and event-free survival (EFS)/progression-free survival (PFS) in an autologous HSCT group were significantly longer than those in a standard therapy group [5,6]. On the other hand, Segeren et al. [7] reported that EFS and OS did not improve after autologous HSCT in comparison with the use of high-dose melphalan therapy (140 mg/m^2). Barlogie et al. [8] described an improvement in PFS and OS after tandem autologous HSCT based on an intention-to-treat analysis. A randomized study in France showed a significant advantage in the tandem transplantation group based on 7 year EFS and OS against single transplantation [9].

Although the graft-vs-myeloma effect can eliminate myeloma cells, most MM patients are unable to receive allogeneic HSCT with high-dose conditioning therapy because of their advanced age, organ dysfunction, or immunodeficiency. NST can be performed more safely than myeloablative allogeneic HSCT. Badros et al. [17] reported 31 patients with MM who underwent NST. Although most of their patients were either refractory to chemotherapy or high-risk cases, they achieved a low TRM and a high CR or near CR rate. Interestingly, the patients who received autologous HSCT before NST achieved a high EFS and OS. That is why it is natural to consider that the combination therapy of autologous HSCT and NST can cause low TRM and long PFS. The study of autologous HSCT followed by NST has been reported by 2 groups. Kröger et al. [18] used fludarabine, melphalan, and anti-thymocyte globulin as a conditioning regimen, and the 2 year OS and disease-free survival were 74 and 56%, respectively. Maloney et al. [19] reported 32 patients treated with total body irradiation (200 cGy) as conditioning and CSP and MMF as an immunosuppressant. The day 100 TRM and CR rates were 6 and 53%, respectively. Only 3 patients developed severe GVHD, and OS was 81% with a median follow-up of 20 months after autologous HSCT.

At the time of diagnosis, the patient presented herein had some poor prognostic factors. Because she had an HLA-identical sibling, we scheduled NST after autologous HSCT for this patient. Even after autologous HSCT, monoclonal protein could be detected by a less sensitive immunoelectrophoresis method. For the detection of residual myeloma cells,

Table I. Results of hematopoietic stem cell transplantations in multiple myeloma.

Reference		Number	Conditioning	TRM (%)	PFS, EFS	OS
[5]	auto	100	MEL 140 + TBI	2.7	28% (5 years)	52% (5 years)
	conventional	100	VMCP, BVAP	NA	10% (5 years)	12% (5 years)
[6]	auto	201	MEL 200	4	31.6 months*	54.1 months*
	conventional	200	ABCM	NA	19.6 months*	42.3 months*
[7]	auto	132	CY + TBI	3.9	22 months*	47 months*
	conventional	129	MEL 140	NA	21 months*	50 months*
[8]	tandem auto	231	MEL 200 → MEL 200 or TBI, CY	5	42% (5 years)	58% (5 years)
[9]	single auto	199	MEL 140 + TBI	4	10% (7 years)	21% (7 years)
	tandem auto	200	MEL 140 → MEL 140 + TBI	6	20% (7 years)	42% (7 years)
[13]	auto	189	MEL ± TBI ± CY	13	18 months*	34 months*
	allo	189	TBI + CY ± MEL etc.	41	10 months*	18 months*
[17]	NST	31	MEL 100	10	31% (2 years)	31% (2 years)
[18]	auto → NST	17	MEL 200 → Flu + MEL + ATG	11	56% (2 years)	74% (2 years)
[19]	auto → NST	54	MEL 200 → TBI 2Gy	6	55% (2 years)	78% (2 years)

*For 50% survival.

TRM, treatment-related mortality; PFS, progression-free survival; EFS, event-free survival; OS, overall survival; MEL, melphalan; TBI, total body irradiation; VMCP, vincristine, melphalan, cyclophosphamide, prednisone; BVAP, vincristine, carmustine, doxorubicin, prednisone; NA, not available; ABCM, doxorubicin, carmustine, cyclophosphamide, melphalan; CY, cyclophosphamide; Flu, fludarabine; ATG, anti-thymocyte globulin; NST, non-myeloablative allogeneic stem cell transplantation.

we performed an RT-PCR SSCP analysis instead of a PCR analysis using patient-specific primers. By using the RT-PCR SSCP analysis, we could detect transient oligoclonal B-cell expansion after a clinical CR had been achieved. Monoclonal or oligoclonal gammopathy can be recognized in more than 50% of all patients after HSCT [23,24]. Mitus et al. [23] reported that the appearance of the M component was closely correlated with the development of GVHD. We considered the tumor-derived monoclonal B cells to be eliminated after chronic GVHD occurred and therefore benign oligoclonal B-cell expansion is thus considered to be related in some manner with the development of chronic GVHD.

In conclusion, autologous HSCT followed by NST for elderly patients could be safely performed. In addition, we were also able to reduce tumor burden by high-dose chemotherapy, and thereby eliminate residual myeloma cells by a graft-vs-myeloma effect. For monitoring minimal residual disease, the RT-PCR SSCP method was thus found to be sufficiently sensitive and useful for the detection of tumor-derived monoclonal protein. To investigate the efficacy of this treatment strategy, a large-scale randomized protocol study comparing this treatment with NST alone will be needed.

Acknowledgements

We thank Ms Eriko Kohno and Ms Kyoko Fujimoto for their excellent technical support.

References

1. Tricot G. Treatment advances in multiple myeloma. Br J Haematol 2004;125:24–30.
2. Bensinger WI. Recent developments in hematopoietic stem cell transplantation for multiple myeloma. Int J Hematol 2003;77:232–238.
3. Kyle RA, Rajkumar V. Multiple myeloma. N Engl J Med 2004;351:1860–1873.
4. Sirohi B, Powles R. Multiple myeloma. Lancet 2004;363:875–887.
5. Attal M, Harousseau JL, Stoppa AM, Sotto JJ, Fuzibet JG, Rossi JF, Casassus P, Maisonneuve H, Facon T, Ifrah N et al. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. N Engl J Med 1996;335:91–97.
6. Child JA, Morgan GJ, Davies FE, Owen RG, Bell SE, Hawkins K, Brown J, Drayson MT, Selby PJ. High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. N Engl J Med 2003;348:1875–1883.
7. Segeren CM, Sonneveld P, van der Holt B, Vellenga E, Croockewit AJ, Verhoef GEG, Cornelissen JJ, Schaafsma MR, van Oers HJ, Wijermans PW et al. Overall and event-free survival are not improved by the use of myeloablative therapy following intensified chemotherapy in previously untreated patients with multiple myeloma: a prospective randomized phase 3 study. Blood 2003;101:2144–2151.
8. Barlogie B, Jagannath S, Desikan KR, Mattox S, Vesole D, Siegel D, Tricot G, Munshi N, Fassas A, Singhal S et al. Total therapy with tandem transplants for newly diagnosed multiple myeloma. Blood 1999;93:55–65.
9. Attal M, Harousseau JL, Facon T, Gulhot F, Doyen C, Fuzibet JG, Monconduit M, Hulin C, Calliot D, Bouabdallah R et al. Single versus double autologous stem-cell transplantation for multiple myeloma. N Engl J Med 2003;349:2495–2502.
10. Libura J, Hoffmann T, Passweg JR, Gregor M, Fvre G, Tichelli A, Gratwohl A. Graft-versus-myeloma after withdrawal of immunosuppression following allogeneic peripheral stem cell transplantation. Bone Marrow Transplant 1999;24:925–927.
11. Tricot G, Vesole DH, Jagannath S, Hilton J, Munshi N, Barlogie B. Graft-versus-myeloma effect: proof of principle. Blood 1996;87:1196–1198.
12. Bertz H, Burger JA, Kunzmann R, Mertelsmann R, Finke J. Adoptive immunotherapy for relapsed multiple myeloma after allogeneic bone marrow transplantation (BMT): evidence for a graft-versus-myeloma effect. Leukemia 1997;11:281–283.
13. Bjorkstrand BB, Ljungman P, Svensson H, Hermans J, Alegre A, Apperleym J, Blade J, Carlson K, Cavo M, Ferrant A et al. Allogeneic bone marrow transplantation versus autologous stem cell transplantation in multiple myeloma: a retrospective case-matched study from the European Group for Blood and Marrow Transplantation. Blood 1996;88:4711–4718.
14. Giralt S, Estey E, Albitar M, van Besien K, Rondon G, Anderlini P, O'Brien S, Khouri I, Gajewski J, Mehra R et al. Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft versus leukemia effect without myeloablative therapy. Blood 1997;89:4531–4536.
15. Slavin S, Nagler A, Naperstek E, Kapelushnik Y, Aker M, Cividalli G, Varadi G, Kirschbaum M, Ackerstein A, Samuel S et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant diseases. Blood 1998;91:756–763.
16. McSweeney PA, Niederwieser D, Shizuru JA, Sandmaier BM, Molina AJ, Maloney DG, Chauncey TR, Gooley TA, Hegenbart U, Nash RA et al. Hematopoietic cell transplantation in older patients with hematological malignancies: replacing high-dose cytotoxic therapy with graft versus tumor effects. Blood 2001;97:3390–3400.
17. Badros A, Barlogie B, Siegel E, Cottler-Fox M, Zangari M, Fassas A, Morris C, Anaissie E, van Rhee F, Tricot G. Improved outcome of allogeneic transplantation in high-risk multiple myeloma patients after nonmyeloablative conditioning. J Clin Oncol 2002;20:1295–1303.
18. Kröger N, Schwerdtfeger R, Kiehl M, Sayer HG, Renges H, Zabelina T, Fehse B, Tögel F, Wittkowsky G, Kuse R et al. Autologous stem cell transplantation followed by a dose-reduced allograft induced high complete remission rate in multiple myeloma. Blood 2002;100:755–760.
19. Maloney DG, Molina AJ, Sahebi F, Stockerl-Goldstein KE, Sndmaier BM, Bensinger W, Storer B, Hegenbart U, Somlo G, Chauncey T et al. Allografting with nonmyeloablative conditioning following cytoreductive autografts for the treatment of patients with multiple myeloma. Blood 2003;102:3447–3454.
20. Mitterbauer M, Kalhs P, Keil F, Prinz E, Moser K, Mannhalter C, Mtterbauer G, Brugger S, Gisslinger H, Lechner K et al. Continuous complete clinical and molecular remission in two patients with refractory lymphoid malignancies after autografting followed by allogeneic stem cell transplantation with reduced intensity conditioning. Br J Haematol 2002;118:132–135.

21. Dodero A, Perfetti V, Ciceri F, Corradini P. Clinical and molecular remission following reduced intensity conditioning and allogeneic transplantation in a patient with refractory multiple myeloma. Haematologica 2002;87:ECR41.
22. Corradini P, Cavo M, Lokhorst H, Martinelli G, Terragna C, Majolino I, Valagussa P, Boccadoro M, Samson D, Bacigalupo A et al. Molecular remission after myeloablative allogeneic stem cell transplantation predicts a better relapse-free survival in patients with multiple myeloma. Blood 2003;102:1927–1929.
23. Mitus AJ, Stein R, Rappeport JM, Weinstein HJ, Alper CA, Smith BR. Monoclonal and oligoclonal gammopathy after bone marrow transplantation. Blood 1989;75:2764–2768.
24. Nagashima T, Muroi K, Kawano-Yamamoto C, Komatsu N, Ozawa K. Paraproteinemia after hematopoietic stem cell transplantation. Leuk Lymphoma 2004;45:135–137.

Oncogene (2006) 25, 493–502

www.nature.com/onc

ORIGINAL ARTICLE

Rb plays a role in survival of Abl-dependent human tumor cells as a downstream effector of Abl tyrosine kinase

K Nagano[1], C Itagaki[1], T Izumi[1], K Nunomura[1], Y Soda[2], K Tani[3], N Takahashi[4], T Takenawa[5] and T Isobe[1]

[1]*Division of Proteomics Research, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan;* [2]*Division of Molecular Therapy, Advanced Clinical Research Center, University of Tokyo, Minato-ku, Tokyo, Japan;* [3]*Department of Advanced Molecular and Cell Therapy, Medical Institute of Bioregulation, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan;* [4]*Department of Applied Biological Science, Tokyo University of Agriculture and Technology, Saiwai-cho, Fuchu-shi, Tokyo, Japan;* [5]*Division of Biochemistry, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan*

The retinoblastoma (Rb) gene product is a tumor suppressor that is mutated or inactivated in many types of human cancers. Although Rb is known to be an upstream negative regulator of Abl protein tyrosine kinase, we propose here that Rb also functions as a downstream effector of Abl that plays a positive role in survival of Abl-dependent human tumor cells, including Bcr/Abl-positive chronic myelogenous leukemia (CML). We show that Rb is constitutively phosphorylated at tyrosine in Abl-dependent tumor cells, and that Abl phosphorylates Rb specifically at Y805 within the C-terminal domain of the molecule. We also show that ectopic expression of Rb induces apoptosis in Abl-dependent tumor cells by inhibiting the Abl tyrosine kinase activity, and that Rb-induced apoptosis is compromised by Abl-catalysed phosphorylation of Rb at Y805. Furthermore, the silencing of endogenous Rb by RNA interference induced apoptosis in Abl-dependent tumor cells. Thus, our findings suggest that Abl-catalysed tyrosine phosphorylation of Rb is necessary for survival of Abl-dependent human tumor cells, and raises the possibility that this phosphorylated Rb can be a molecular target for cancer therapy aimed at inducing apoptosis of Abl-dependent tumor cells, such as Bcr/Abl-positive CML.

Oncogene (2006) **25**, 493–502. doi:10.1038/sj.onc.1208996; published online 19 September 2005

Keywords: Rb; Abl; tyrosine phosphorylation; chronic myelogenous leukemia; apoptosis

Introduction

The retinoblastoma (Rb) gene product is a tumor suppressor that regulates multiple cellular processes such as growth, differentiation, cell fate specification, and apoptosis, and is mutated or inactivated in many types of human cancers (Horowitz *et al.*, 1989; Onadim *et al.*, 1992; Weinberg, 1995; Sherr, 1996; Harbour and Dean, 2000; Zhang *et al.*, 2004). The tumor suppressor activity of Rb derives from its ability to inhibit cell cycle transit by repressing the transcription of genes required for the G1–S-phase transition, such as E2F/Dp1-dependent transcription (Nevins, 1992; Helin *et al.*, 1993; Sellers *et al.*, 1995; Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Rb also regulates apoptosis and is cleaved by caspases in apoptotic cells (Janicke *et al.*, 1996; Tan and Wang, 1998; Harbour and Dean, 2000). Expression of caspase-resistant Rb in mice yields cell-type and signal-dependent protection from apoptosis (Harbour and Dean, 2000; Chau *et al.*, 2002). In contrast, it has also been reported that restoration of Rb expression in Rb-negative prostate cancer cells sensitizes the cells to apoptosis induced by γ-irradiation (Bowen *et al.*, 2002), and phosphorylation of Rb at S567 triggers apoptosis (Ma *et al.*, 2003). The reason for these apparent contradictory observations regarding the role of Rb in tumor cell apoptosis is unknown, but it has been postulated that Rb function might depend on the tumor cell type and its regulation by site-specific modification, such as phosphorylation (Tan and Wang, 1998; Harbour and Dean, 2000; Ma *et al.*, 2003).

The nonreceptor tyrosine kinase Abl resembles Src family kinases, but has unique features, including the ability to shuttle between the cytoplasmic and nuclear compartments (Baltimore *et al.*, 1995; Wen *et al.*, 1996; Taagepera *et al.*, 1998; Wang, 2000). Activation of Abl by DNA damage can induce apoptosis in the nucleus (Baskaran *et al.*, 1997; Shafman *et al.*, 1997; Wang, 2000). In contrast, Abl also has oncogenic potential that is activated in the Bcr/Abl hybrid protein of chronic myelogenous leukemia (CML). The specific inhibitor of Abl, imatinib, has been proved to be a useful therapeutic agent for Bcr/Abl-positive CML (Druker *et al.*, 2001; Calabretta and Perrotti, 2004; Harata *et al.*, 2004). Bcr/Abl activates a number of cytoplasmic signal transduction pathways that are normally controlled by receptor tyrosine kinases (Druker *et al.*, 2001), while the downstream

Correspondence: Dr T Isobe, Division of Proteomics Research, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.
E-mail: isobe-toshiaki@c.metro-u.ac.jp
Received 22 April 2005; revised 21 June 2005; accepted 5 July 2005; published online 19 September 2005

effectors of Bcr/Abl are not well understood. The C-terminal domain of Rb binds the kinase domain of Abl and inhibits its catalytic activity (Guo *et al*., 1999; Wang, 2000), and thus Rb is considered to be an upstream negative regulator of Abl. However, the physiological link between Rb and Abl in cancer cells still remains obscure.

Here, we demonstrate that Rb functions as a downstream effector of Abl that plays a positive role in survival of Abl-dependent human tumor cells, including Bcr/Abl-positive CML. Based on these data, we propose that Abl-catalysed tyrosine phosphorylation of Rb is necessary for survival of Abl-dependent human tumor cells.

Results

Constitutive tyrosine phosphorylation of Rb in tumor cells

Treatment with genistein, an inhibitor of protein tyrosine kinases, induced apoptosis in HeLa S3 cells (data not shown), suggesting that constitutive tyrosine phosphorylation is necessary for survival of HeLa S3 cells. To gain more insight into this observation, we prepared a fraction containing tyrosine-phosphorylated proteins from HeLa S3 cell extracts by affinity-capture with an antibody to phosphotyrosine (Figure 1a, upper panel). The tumor suppressor protein, Rb, was one of many proteins found in the fraction that was potentially phosphorylated at tyrosine within the cells (lower panel). Interestingly, we found that Rb was tyrosine-phosphorylated in certain cell types including Bcr/Abl-positive CML cell lines such as KU-812 and K562, whereas other human tumor cell lines, including MCF-7, A549, and 293-T cells, did not contain the tyrosine-phosphorylated form of Rb (Figure 1b). HeLa S3 is not typical from this viewpoint, as the cell expresses wild-type Abl instead of Bcr/Abl; however, Abl activity appears to be essential for this cell because imatinib, a specific inhibitor of Abl, induced apoptosis (Figure 2). Thus, we postulated that Rb might be constitutively phosphorylated at tyrosine in Abl-dependent tumor cells.

Tyrosine phosphorylation of Rb by Abl tyrosine kinase

It has been reported that Rb is an upstream negative regulator of Abl tyrosine kinase (Guo *et al*., 1999; Wang, 2000). To explore whether Rb is a substrate of Abl and serves as a downstream effector of Abl, N-terminal (Rb-N), A/B pocket (Rb-A/B), and C-terminal (Rb-C) domains of Rb were expressed in bacteria with a GST tag and subjected to an *in vitro* kinase assay. Of the three segments, only Rb-C was strongly phosphorylated with a constitutively active (W118K), but not an inactive (kinase dead (KD)), mutant of Abl (Supplementary Figure 1a and b). To identify the phosphorylation sites, Rb-C was incubated with or without Abl and subjected to mass spectrometry (MS) analysis. A doubly charged peptide ion ($m/z = 676.25$) was only detected after Rb-C was incubated with Abl (Figure 3a, lower panel,

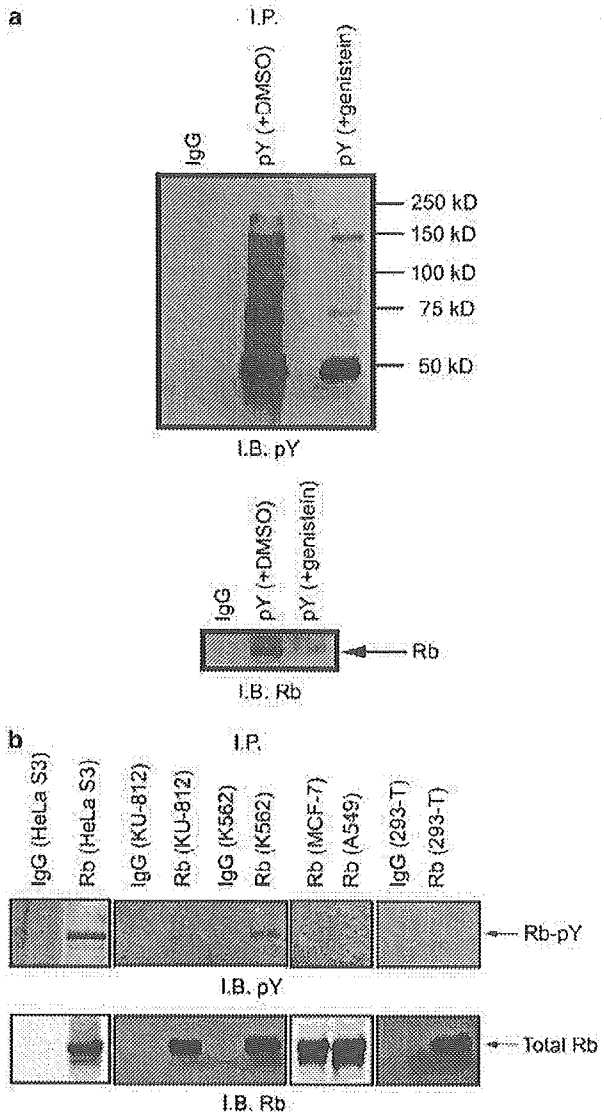


Figure 1 Rb is constitutively phosphorylated at tyrosine. (**a**) HeLa S3 cells were treated with either DMSO or 250 μM genistein for 3 days. The cell lysates were affinity-purified on a column immobilized either with anti-phosphotyrosine or anti-IgG, and immunoblotted with anti-phosphotyrosine (upper panel) or anti-Rb (lower panel). (**b**) Lysates from human tumor cell lines, HeLa S3, KU-812, K562, MCF-7, A549, and 293-T cells were immunoprecipitated with anti-Rb, followed by immunoblotting with anti-phosphotyrosine (upper panel) or anti-Rb (lower panel).

indicated by an arrow). This ion was assigned to the peptide fragment spanning Rb residues 799–810 having the sequence IPGGNIpYISPLK, including phosphorylated Y805 (Figure 3b, lower panel). We found the equivalent unphosphorylated peptide fragment ($m/z = 636.22$) in the Rb-C samples incubated with and without Abl (Figure 3a and b, indicated by arrowheads). Although we detected an additional, minor phosphopeptide spanning Rb residues 768–787, which was probably phosphorylated at Y771 (Supplementary Figure 2c),