Expert Opinion

Update on non-viral delivery methods for cancer therapy: possibilities of a drug delivery system with anticancer activities beyond delivery as a new therapeutic tool

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Importance of the field: Cancer is the most formidable human disease. Owing to the heterogeneity of cancer, a single-treatment modality is insufficient for the complete elimination of cancer cells. Therapeutic strategies from various aspects are needed for cancer therapy. These therapeutic agents should be carefully selected to enhance multiple therapeutic pathways. Non-viral delivery methods have been utilized to enhance the tumor-selective delivery of therapeutic molecules, including proteins, synthetic oligonucleotides, small compounds and genes.

Areas covered in this review: As non-viral delivery methods, liposomes and polymer-based delivery materials to target tumors mainly by systemic delivery, physical methods including electroporation, sonoporation, and so on, to locally inject therapeutic molecules, and virosomes to use the viral infectious machinery for the delivery of therapeutic molecules are summarized.

What the reader will gain: This article aims to provide an overview of the characteristic properties of each non-viral vector. It will be beneficial to utilize appropriately the vector for cancer treatment.

Take home message: Efficient and minimally invasive vectors are generally considered to be the ideal drug delivery system (DDS). However, against cancer, DDS equipped with antitumor activities may be a therapeutic choice. By combining therapeutic molecules with DDS having antitumor activities, enhancement of the multiple therapeutic pathways may be achieved.

Keywords: antitumor immunity, drug delivery, gene transfer, liposome, oncolysis, physical method, polymer, virosome

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1. Introduction

Cancer is still an uncontrollable disease, although many therapeutic treatments have been developed. Although cancer rates have decreased recently in the US [1], cancer is still a formidable disease with high rates of morbidity and mortality. The first line of cancer treatment is the surgical removal of cancer lesions along with the surrounding normal tissues. With the aid of cancer imaging technologies, the removal becomes more tumor-selective [2]. However, in many cases the cancer cells are not completely eliminated from the body, which allows the tumors to recur. Disease recurrence is the most difficult problem in cancer treatment. To suppress the growth of residual or inoperable tumors, numerous anticancer reagents have been

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Article highlights.

- Liposome is a representative non-viral delivery system and has been improved for efficient delivery by using cationic lipids and helper lipids. Polymeric micelles, atelocollagen, cationized gelatin, chitosan and polyethyleneimine are also used for the delivery of anticancer reagents.
- Those nanoparticles have been utilized to target tumor tissues by systemic administration. To enhance retention of vectors in tumor tissue, small-sized and polyethylene glycol-conjugated particles are developed. Moreover, particles are also decorated with tumor-targeting molecules such as transferrin and folate.
- Physical methods including electroporation, sonoporation, hydrodynamic gene delivery method and particle bombardment are also used for the delivery of therapeutic molecules. These methods are limited to local injection of therapeutic molecules mainly plasmid DNA.
- Virosomes that mimic viral infection machinery have been developed for enhancing the delivery of therapeutic molecules. One is the construction of synthetic vectors with viral component. Another is the use of pseudovirion derived from enveloped viruses such as HVJ and hepatitis B virus.
- Although the general concept for the ideal drug delivery system is the construction of efficient and minimally invasive methods, limited to cancer therapy, drug delivery system equipped with antitumor activities may be useful for the enhancement of the multiple therapeutic pathways.

This box summarizes key points contained in the article.

developed. The identification of molecular targets for cancer therapy has led to the specific destruction of neoplastic cells in several types of cancer [3]. However, many types of cancer still require effective anticancer drugs. To enhance anticancer activity while minimizing adverse effects, the cancer-selective delivery of therapeutic molecules is desired. The concept of targeted drug delivery was proposed by Paul Ehrlich as a 'magic bullet' in 1906 [4], and a drug delivery system (DDS) for cancer was developed more than a century later when Bangham defined liposomes [5]. Drug delivery vectors are classified as viral or non-viral. Viral vectors such as retroviral vectors, adenoviral vectors, adeno-associated viral vectors, herpes viral vectors and vaccinia viral vectors have been used in gene therapy. Each type of viral vector has advantages and limitations [6]. Viral vectors are generally more efficient than non-viral vectors for gene delivery and gene expression both *in vitro* and *in vivo*. However, serious side effects have been reported in clinical trials [7,8]. The therapeutic molecules in viral vectors are restricted to DNA and RNA, whereas non-viral vectors, as non-viral DDS, can deliver proteins, synthetic oligonucleotides and small compounds as well as DNA and RNA. Various types of non-viral DDS have been developed [9-11].

This review summarizes the recent progress of non-viral DDS to deliver drug and genes for cancer treatment and includes a discussion on the possibility of DDS with intrinsic anticancer activities.

2. Non-viral DDS for cancer therapy

2.1 Liposomes

Liposomes have been widely used to introduce macromolecules into cells. The original concept of liposomes is the incorporation of water-soluble molecules into vesicles consisting of a lipid bilayer. To increase DNA transfer efficiency, cationic lipids were synthesized. Using cationic lipids, liposome/DNA complexes or lipoplexes were developed [12]. In lipoplexes, cationic liposomes are associated with negatively charged DNA without incorporation, as performed in classical liposomes (**Figure 1A**). Numerous cationic lipids have been synthesized to improve further the transfection efficiency and to reduce the cytotoxicity of the lipoplex [10,12-14]. Felgner *et al.* first used a synthetic cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) (**Figure 1B**) [12]. In cancer gene therapy, lipoplex with *HLA-B7* and *β-2 microglobulin* genes induced antitumor immunity in HLA-B7-negative melanoma patients, and the liposomal drug (Allovectin-7) has been clinically tested in multiple institutions to treat metastatic melanoma [14]. 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-cholesterol) (**Figure 1B**) was used as a cationic lipid in that trial [15]. Cationic liposomes incorporating DNA inside the particle were also developed. The *IFN-β* gene delivered by cationic liposomes composed of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG) (**Figure 2B**) [16], dilauroyl phosphatidylcholine, and dioleoyl phosphatidylethanolamine in a molar ratio of 1:2:2 was used to treat glioblastoma patients in Japan. Cationic liposomes can also be used for the delivery of small interfering RNA (siRNA).

One of the problems with non-viral DDSs that are incorporated into cells by endocytosis is that the endosomal membrane fails to penetrate into the cytoplasm [17]. In endocytosis-mediated delivery, therapeutic molecules tend to be degraded in the endosome or lysosome. Lipids may protect the molecules of interest from degradation prior to reaching the cytoplasm [18]. A neutral lipid, DOPE (dioleoylphosphatidylethanolamine) [19] or DMRIE-C (a 1:1 mixture of *N*-[1-(2,3-dimyristyloxy)propyl]-*N,N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide (DMRIE) and cholesterol) [13], destabilizes the endosomal membrane. These helper lipids are used as an important component of liposomes.

As a therapeutic drug, the systemic administration of polymeric nanoparticles has been desired. Tumor-targeting particles have been developed based on two approaches, passive and active targeting [20-22] Tumor tissues develop blood vessels by secreting angiogenic factors. However, these vessels have immature and leaky structures [23] – a characteristic that can be utilized when designing therapies. Small drug delivery

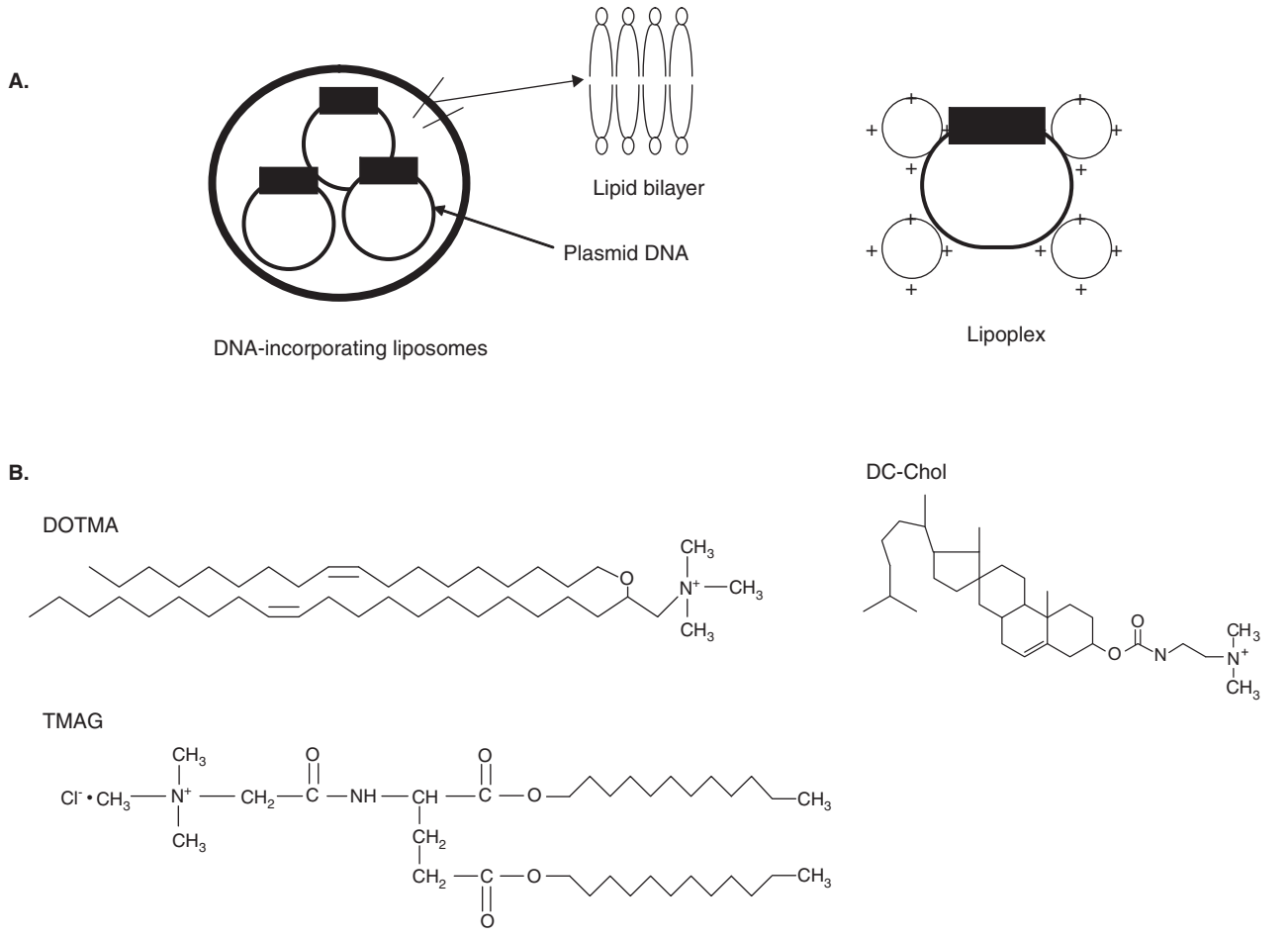

Figure 1. A. In classical liposomes, therapeutic molecules are incorporated into vesicles that have a lipid bilayer (liposomes). However, in a lipoplex composed of cationic lipids, the complex is formed by electric charges between negatively charged DNA and positively charged empty liposomes. **B.** Representative cationic lipids used for lipofection.

particles are believed to reach tumor cells by passing through the endothelial cells of the vessels (**Figure 2**). However, most colloidal particles, such as liposomes and polymeric micelles, are trapped by reticuloendothelial cells in the liver, spleen and lung. To avoid such nonspecific uptake and prolong the retention of particles in the circulation, polyethylene glycol (PEG)-conjugated (PEGylated) vectors have been developed [9-11]. In passive targeting, the accumulation of long-circulating small particles in tumor tissue is needed. This concept has been applied to other polymer-based delivery systems described below, as well as liposomes. In the liposome field, PEGylated liposomes are used for drug delivery to tumor tissues [24]. PEGylated liposomes containing doxorubicin (Doxil®; Johnson & Johnson , New Brunswick, NJ) are being used to treat cancer patients [25]. However, PEGylation decreases the efficiency of drug delivery into cells by reducing the association of liposomes with the cell membrane. One direction in liposome development is to neglect PEGylation. When non-PEGylated liposomes containing doxorubicin (Myocet®; Cephalon Inc., Frazer, PA) were intravenously administered to advanced breast cancer patients in combination with gemcitabine [26], antitumor effects were achieved without cardiac toxicity. Sphingomyelin/cholesterol liposomes containing vinblastine sulfate (Marqibo®; Hana Biosciences, San Francisco, CA) have been used to treat refractory non-Hodgkin's lymphoma [27]. The main adverse effect was neutropenia, which occurred in 9 of 119 patients. An approximately twofold dose of vincristine sulfate was tolerable when using the liposomes for drug delivery [27].

Another approach to enhance drug delivery is to bind tissue-selective molecules on DDS [9-11]. These molecules enhance the interaction of DDS with target cells and enable tissue-specific drug delivery. To target cancer cells, tumor-recognizing monoclonal antibodies or tumor-specific ligands have been examined. However, it is very hard to identify completely tumor-selective surface markers. Transferrin liposomes were developed because proliferating cells, especially cancer cells, express many more transferrin receptors than

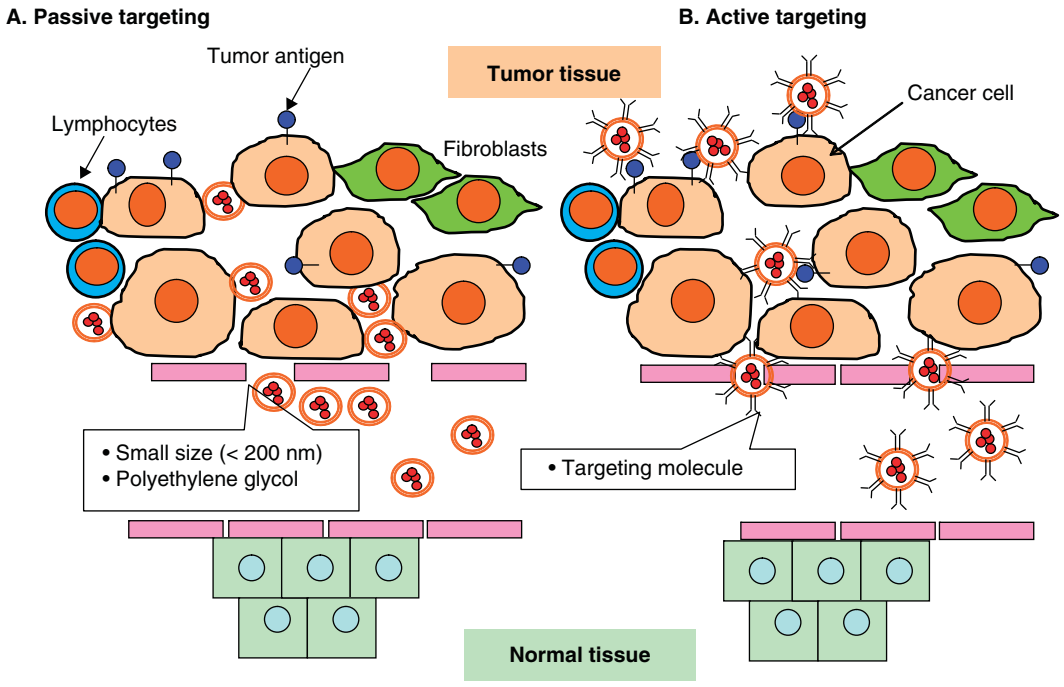

Figure 2. There are two approaches for tumor-targeting. A. DDS has been developed based on the characteristic structure of tumor vasculature, in which endothelial cells are not tightly linked with each other. Accordingly, small particles (< 200 nm in diameter) can pass through the leaky vasculature to reach tumor cells. Long-term circulation is required for the accumulation of the particles in tumor tissue. For this reason, small and PEGylated particles have been developed to achieve passive targeting based on enhanced permeabilization and retention. **B.** Active targeting DDS has been developed using tumor-recognizing molecules such as folate and transferrin.

other cells [28]. The folate receptor is also abundant in tumor tissues. When folate is conjugated with PEGylated lipid–DNA complexes, the lung accumulation of DNA is reduced and the amount of tumor-targeted DNA increases [29]. In the future, it will be absolutely necessary to identify cancer-targeting molecules. The concept of cancer stem cells is very attractive for the development of cancer therapies and the study of tumorigenesis. Although several molecules have been reported as markers for cancer stem cells in glioma and mammary carcinoma, the specificity and stability of those molecules in cancer stem cells are still controversial [30]. When molecules that recognize cancer stem cells are identified, tumor-targeting DDS should be equipped with such molecules.

3. Polymer-based delivery system

3.1 Polymeric micelles

Block-copolymers, which consist of two distinct linear polymers, are conjugated to therapeutic molecules by an electrostatic interaction, hydrophobic interaction or metal complex formation [31]. Those therapeutic molecule-interacting block-copolymers encapsulate the therapeutic molecule when the polymeric micelle forms in water (**Figure 3**). Based on this principle, appropriate block-copolymers should be selected for each therapeutic molecule. Paclitaxel and docetaxel were successfully encapsulated into PEG750-*b*-oligo (ε-caprolactone)$_5$ micelles, whereas adriamycin and doxorubicin were encapsulated into PEG-*b*-polyaspartic acid copolymer. Doxorubicin-encapsulating polymeric micelles provided from a venture company were studied in a Phase II clinical trial at the National Cancer Center Hospital in Japan [32]. The micelles are 20 – 100 nm in diameter, and the accumulation of the particles in tumor tissue is feasible by lowering the nonspecific uptake in the reticuloendothelial system. For DNA encapsulation, PEG-*b*-polyaspartamide with a 1,2-diaminoethane side chain is used as a block-copolymer. This micelle for DNA encapsulation enhanced the penetration into tumor tissue and enabled gene expression in the hypoxic state [33]. Such penetration into tumor tissue was not achieved by PEG liposomes. Bae *et al.* developed pH-sensitive micelles to enhance drug delivery from the endosome to the cytoplasm using an acid-labile hydrazone bond [34] and to release DNA gradually in the cytoplasm using a calcium phosphate/DNA complex. The amount of doxorubicin in tumors by pH-sensitive micelles was approximately fourfold higher than that by pH-insensitive micelles, and more efficient tumor regression was

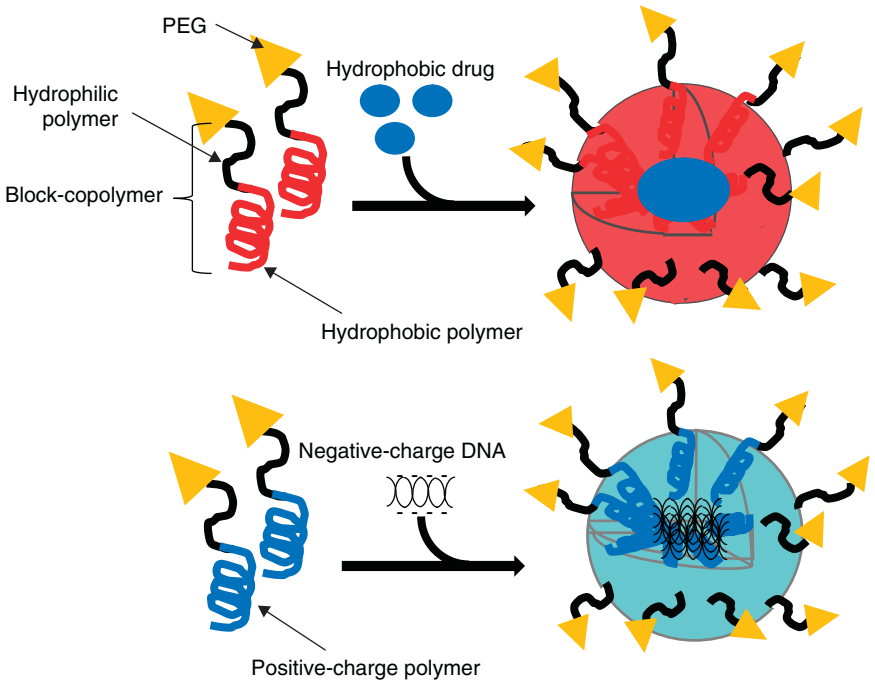

Figure 3. Polymeric micelles are constructed using therapeutic molecule-interacting block-copolymers. For the incorporation of hydrophobic molecules, block-copolymers containing hydrophobic and hydrophilic polymer chains are used. For encapsulating DNA, block-copolymers with a positive charge are used.

achieved by pH-sensitive micelles than by conventional micelles [35].

3.2 Atelocollagen

A trimer of type I collagen from bovine dermis forms a helix that is 300 nm long and 1.5 nm wide. Telopeptide at both the amino terminus and the carboxy terminus of type I collagen was digested with pepsin to form atelocollagen, which is much less antigenic than collagen and has been used clinically. This biomaterial becomes a gel at low temperatures (2 – 10°C) and can be mixed with nucleic acid solution. The atelocollagen has a positive charge and can be a carrier of DNA and siRNA [36]. When the siRNA/atelocollagen complex was intravenously injected into tumor-bearing mice, the siRNA existed in tumor tissue for > 1 week. SiRNA against Enhancer of Zeste homologue 2 or p110α of phosphatidyl-inositol-3-kinase, which are upregulated in prostate carcinoma, was complexed with atelocollagen and intravenously administered into mice with bone metastasis of human prostate cancer [37]. An *in vivo* imaging analysis revealed that both types of siRNA markedly suppressed metastatic tumors.

3.3 Cationized gelatin

Cationized gelatin is prepared by chemically introducing the amine residues to the carboxyl groups of gelatin. Cationized gelatin is crosslinked by various concentrations of glutaraldehyde to obtain cationized gelatin hydrogels with different *in vivo* degradabilities as the release carrier of DNA. The cationized gelatin hydrogels incorporating plasmid DNA prolonged the duration of gene expression [38]. The plasmid DNA release is driven only by the degradation of the release carrier, and the controlled release of plasmid DNA-cationized gelatin complexes enhances the concentration of plasmid DNA around cells, resulting in an increased gene transfection efficacy. The controlled release technology promoted the biological activity of an antitumor plasmid DNA of NK4, which is a protein composed of the NH_2-terminal hairpin and the subsequent four-kringle domains of hepatocyte growth factor (HGF) [39]. NK4 is an HGF antagonist that inhibits the ability of HGF to promote tumor metastasis and angiogenesis. The subcutaneous injection of hydrogel microspheres incorporating the NK4 plasmid DNA into nude mice with ascitic AsPC-1 tumor cells resulted in a significantly prolonged survival as compared with mice that received NK4 plasmid DNA in solution. Thus, the controlled release approach is a promising method to enhance the *in vivo* biological effects of plasmid DNA.

3.4 Chitosan

Chitosan is a linear polysaccharide consisting of one to four linked *N*-acetyl-D-glucosamine and D-glucosamine subunits. Chitosan is prepared from chitin by deacetylation. Chitosan has a positive charge and interacts strongly with negatively

charged DNA [40]. Numerous chitosan derivatives have been developed, including galactosylated, trimethylated and *N*-dodecylated chitosan [10].

Mannosylated chitosan was used as a carrier of IL-12 expression plasmid, and the complex suppressed colon carcinoma in mice by regional injection [41]. Chitosan is also used for the intravenous delivery of siRNA for cancer therapy [42].

3.5 Polyethylenimine

A linear form of polyethylenimine (PEI) is frequently used for DNA delivery to cultured cells. The cationic charge of PEI enhances its interaction with DNA [43]. PEI reagent is now commercially available. As PEI has a buffering action due to non-protonated amines with different pK_a, PEI enhances the escape of DNA from the endosome to the cytoplasm by the proton sponge effect [44]. Therefore, the transfection efficiency of PEI is generally higher than that of cationic liposomes. However, the number of reports on therapeutic experiments *in vivo* using PEI is still lower than that using liposomes. Nanoparticles based on PEI modified with stearic acid were developed to deliver siRNA against signal transducer and activator of transcription 3 (STAT3) to suppress mouse melanoma *in vivo* [45]. The PEI-stearic acid/siRNA complex was more effective for tumor suppression *in vivo* than the PEI/siRNA complex.

4. Physical methods

Gene expression by the direct injection of naked DNA was first reported for skeletal muscle [46] and subsequently reported for other tissues such as the thyroid, cardiac muscle, skin and liver [47]. The simplicity of naked DNA injection is advantageous. Although the gene expression level is varied, especially in large animals [48], clinical trials of this method are increasing [49]. Physical methods to push the DNA into cells have been developed to enhance gene delivery based on naked DNA injection.

4.1 Electroporation

In electroporation, an electric field increases the permeability of the cell membrane to facilitate the introduction of plasmid DNA into cells. Electroporation increases the expression of a gene of interest on naked DNA 10- to 100-fold [50]. Electroporation is a popular method for *in vitro* transfection. For *in vivo* transfection, skin and muscle have been the main target tissues. There are two types of electrode, the plate type and the needle type. The plate type provides more stable results, but the needle type is more useful for transfection in various tissues. Electroporation with high electric currents causes tissue damage. To solve this problem, electroporation at a lower voltage with similar transfection efficiency has been developed [51]. Electroporation is frequently used in animal models of cancer treatments for the intramuscular delivery of *IL-12* and *IL-27* genes [52]. Electroporation is also used for the regional tumor delivery of various therapeutic molecules, including microRNA against mutant K-ras [53]. For the clinical application of electroporation to the treatment of cancer, human dendritic cells were transfected with tumor mRNA using electroporation and then injected into melanoma patients in a Phase I/II study [54]. A Phase I trial of the direct injection of the *IL-12* gene by electroporation was performed with melanoma patients, and the safety of the method was shown [55].

4.2 Ultrasound

Ultrasound is also available for the delivery of therapeutic molecules to tissue [56]. This transfection method depends on cavitation activity, one of the biological actions of ultrasound. DNA is mixed with contrast reagents such as Optison and Levovist, which consist of gas-filled particles coated with lipids or albumin. The cavitation activity of ultrasound results in the formation and rupture of microbubbles (1 – 100 μm in diameter). Two mechanisms have been considered for the ultrasound-mediated delivery of foreign molecules into cells (**Figure 4**) [57]. When the bubbles are ruptured by the energy of ultrasound, the membrane permeability can be enhanced (**Figure 4A**). Alternatively, a high-speed jet flow (> 600 km/h) is generated by the cavitation activity to increase the membrane permeability (**Figure 4B**). With these activities, DNA is incorporated into target cells and most of the membranes are recovered in 60 s. The transfection efficiency is increased by increases in the mechanical index, frequency and exposure time. As these parameters are also related to cell damage, it is essential to determine the optimum conditions for each case. Immunogenes such as granulocyte macrophage-colony stimulating factor (GM-CSF) and B7-1 have been delivered to mouse tumors using sonoporation. The delivery efficiency is comparable to that of the electroporation, but the tissue damage caused by sonoporation seems to be less than the damage caused by electroporation [58]. Regional intratumoral delivery of toxic compounds, such as bleomycin and the cytolethal toxin gene, by sonoporation was successful in mice [59]. Sonoporation is also used for enhancing the liposomal delivery of the *IFN-β* gene to tumors [60]. Bubble liposomes containing perfluoropropane also enabled the successful delivery of the *IL-12* gene to tumors when combined with ultrasound [61].

4.3 Hydrodynamic gene delivery

Intravascular injection of naked DNA had been considered ineffective for gene expression. However, a rapid systemic injection of a large volume of solution containing DNA and siRNA enabled gene delivery to some organs, mainly the liver, in rodents [62]. Thus, this method appeared to be impractical for treating human disease. However, the rapid injection of a large volume of naked DNA through an arterial route with both blood inflow and outflow blocked achieved intramuscular gene expression [63], and this method has been clinically used for the treatment of Duchenne muscular dystrophy [64]. The mechanism of gene delivery into cells by the

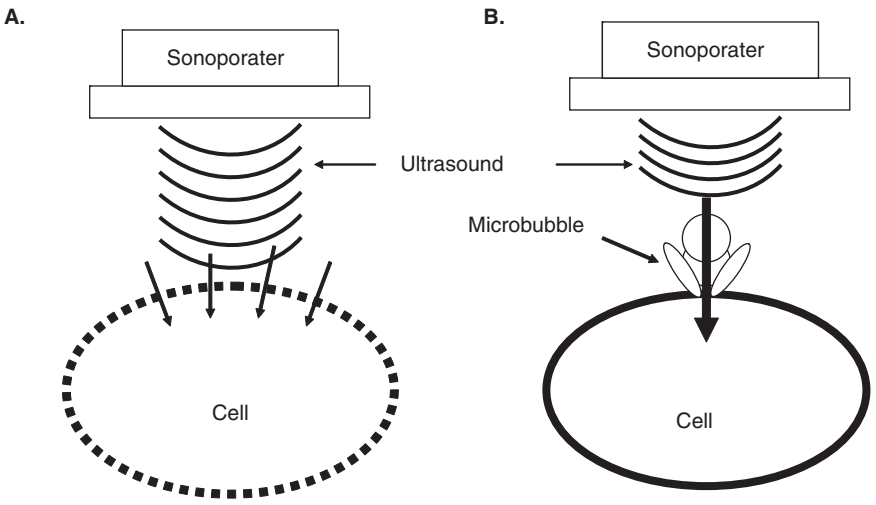

Figure 4. Two mechanisms for gene delivery to cells using ultrasound have been proposed. A. Reversible pores may be transiently formed in the plasma membrane by ultrasound, allowing DNA to enter the cytoplasm. **B.** When microbubbles are ruptured by ultrasound, the micro jet stream formed may accelerate the entry of DNA into the cytoplasm.

hydrodynamic method is being elucidated. The most important factor is considered to be the outflow obstruction of the DNA solution from a target organ for raising the vascular pressure in the organ [65]. As gene delivery to the cells of the organ is achieved by the pressure, the gene transfer efficiency is dependent on the elasticity of the target organ. Hydrodynamic gene delivery has been used for cancer therapy in animal models. A soluble form of the *fetal liver kinase-1* gene, a receptor for vascular endothelial growth factor (VEGF), delivered by hydrodynamic injection suppressed VEGF-driven angiogenesis and inhibited the growth of renal cell carcinoma and lung carcinoma in mice [66]. To develop a clinically relevant method, the intravascular hydrodynamic limb vein delivery of the human *gp100* melanoma-associated antigen gene to skeletal muscle was used for melanoma vaccination [67].

4.4 Other physical methods

Particle bombardment is the injection of plasmid DNA coated with gold particle (~ 1 μm in diameter) by compressed gas. The device for bombardment is called a gene gun. Gene-gun-mediated gene transfer has been successful mainly in skin or skin tumors [68]. Although it is available for gene transfer to various cells, the disadvantage is limited to the surface cells. Therefore, this method is suitable for DNA vaccination. Similarly, a simple tattoo device was also used for intradermal DNA delivery. Gene vaccination by this method activated both cellular and humoral immune response [69].

5. Virosomes

To avoid the degradation of the molecules before they reach the cytoplasm, fusion-mediated delivery systems have been desired. Despite several trials of synthetic DDS, it is difficult to realize the efficient membrane fusion that occurs with enveloped viruses. One approach is the development of virosomes consisting of viral envelope components or inactivated viral particles as a vehicle for therapeutic molecules [70]. Although virosomes have disadvantages such as immunogenicity and instability in the circulation, virosomes do have unique characters that seem to make them suitable for cancer therapy.

5.1 Synthetic DDS with viral components

Viral components are attractive tools for the delivery of therapeutic molecules. Viral envelope proteins with membrane fusion ability have been utilized to increase the efficiency of drug delivery to cells. A fusogenic viral liposome with a fusogenic envelope derived from the hemagglutinating virus of Japan (HVJ; Sendai virus) was constructed [71]. HVJ, a mouse parainfluenza virus, is not a human pathogen. The virus induces fusion with cell membranes at a neutral pH by means of the hemagglutinating (HN) protein and fusion (F) protein on the envelope [72]. For fusion-mediated gene transfer, DNA-loaded liposomes were fused with UV-inactivated HVJ to form the fusogenic viral liposome, HVJ liposome, which is 400 – 500 nm in diameter. The gene delivery efficiency of HVJ liposomes was not significantly inhibited when cells were incubated with wortmannin, an inhibitor of endocytosis. A study that used a fluorescence resonance energy transfer system revealed that a much greater number of intact oligonucleotides could be delivered to the nucleus by HVJ liposomes than by lipofection [73].

Viral fusion proteins have been used to enhance the gene transfer efficiency of receptor-mediated gene delivery systems by combining fusion peptide derived from influenza virus hemagglutinin (HA). The transferrin/poly-L-lysine/DNA

complex bound with the HA-2 peptide increases the gene transfer efficiency in cultured cancer cells > 1000-fold when compared with the efficiency in the absence of the peptide [74].

For cancer treatment, HVJ liposomes have been widely used in animal models. Melanoma-associated antigen gene or RNA injected into skeletal muscle or spleen successfully evoked tumor immunity to prevent melanoma growth [75]. Radio-inducible herpes simplex virus *thymidine kinase* gene driven by the early growth response-1 promoter enhanced the effects of cancer radiotherapy on hepatocellular carcinoma when delivered by HVJ liposomes [76].

5.2 Pseudovirion

5.2.1 HVJ envelope vector

The inactivated HVJ particle itself without liposomes was turned into a DDS for the delivery of DNA, siRNA and anticancer reagent [77,78]. Fusion between HVJ-E vector envelope and cell membrane occurs within only 3 – 5 s of the attachment of the plasmid-containing HVJ-E vector to the cell surface (**Figure 5A**). Although the receptor of HVJ is glycoprotein on erythrocytes, on many other cells it is a ganglioside having an α2-3 linked sialic acid at the terminal galactose, such as GD1a and sialylparagloboside. Those gangliosides distribute on the surface of various cells, but are particularly rich in cancer cells such as human prostate cancers [79], neuroblastoma, melanoma and glioblastoma (unpublished data by the author). Therefore, HVJ-E has high affinity to some human cancer cells. The application of HVJ-E and the development of tissue-targeting HVJ-E are described in a review article [73]. With pseudovirion it is generally hard to establish a system to produce homogeneous materials. However, the production system for HVJ-E vector has been established and GMP-grade HVJ-E (freeze-drying material) is available for clinical use.

5.2.2 Reconstituted pseudovirion

Reconstituted pseudovirions have also been developed. Influenza virion was completely lysed with detergent, and the lysates were mixed with siRNA or DNA solution. Reconstituted influenza particles containing therapeutic molecules were constructed by detergent removal after solubilization [80]. The particles having fusion protein HA on their surface deliver incorporated therapeutic molecules to the cytoplasm by endosomal fusion after endocytosis (**Figure 5B**).

A similar procedure is available for other envelope-type viruses [70].

5.2.3 Human hepatitis B virus nanoparticles

Overexpression of viral proteins in cultured cells can produce pseudovirions without a genome, although the efficiency is much less than that of native virus infection. Human hepatitis B virus (HBV) pseudoparticles were constructed by producing HBV L antigen in yeast (**Figure 6**) [81]. The particle without the viral genome is 210 nm in diameter and specifically targets human hepatocytes. Therapeutic DNA can be introduced into the particle by electroporation. The L nanoparticles containing the herpes simplex virus *thymidine kinase* gene were systemically administered to rats with human hepatocellular carcinoma. In conjunction with ganciclovir pro-drug administration, the growth of hepatoma in L particle-injected rats was significantly suppressed [82].

6. Conclusion

As described above, various non-viral DDSs have been developed to achieve highly efficient delivery with minimal invasiveness. The summary of each DDS is listed in **Table 1** from the view of administration, characteristic properties and clinical use for cancer. Each delivery system seems to have its suitable targets and applications. However, a conventional approach is not sufficient to eradicate cancers in patients. More antitumor activities will be necessary for cancer treatment in the future.

7. Expert opinion

Despite the progress that has been achieved in DDS, it is impossible to eradicate completely cancers in patients by a single treatment using either gene transfer or drug delivery. For example, *p53* has the activities of growth arrest and apoptosis, and the lack of *p53* is one of the main triggers for carcinogenesis. Although *p53* gene therapy has been performed in many cancer patients, the anticancer effects remain to be improved [83]. *p53* gene therapy must be combined with radiation or anticancer drugs [84]. Even with combination therapy, not all cancers are eliminated – probably because the tumor tissue is heterogeneous and contains various types of cancer cell – and tumors frequently recur. However, the human body is equipped with an immune system. If antitumor immunity were activated, the host immune system could eliminate residual cancer cells. This is a very optimistic view of cancer immunotherapy, which is quite different from immunotherapy against infectious diseases [85]. Numerous failures of cancer immunotherapy have indicated the difficulty of achieving anticancer immunity [86]. Cancer tissues produce factors that induce immunotolerance against cancers in tumor-bearing individuals. For more effective immunotherapy, both the activation of effector lymphocytes against cancers and the suppression of immunosuppressive factors are necessary [87]. At the tumor initiation stage, cancer cells can be eliminated by the host immune system. When the activities of the immune system are attenuated by factors such as senescence, stress and immunosuppressive drugs, cancers can escape from immune surveillance. Finally, a large tumor mass can overwhelm the immune system [88]. At that time, immunotherapy is too late to eliminate cancer cells; therefore, immunotherapy should be started at an early stage of cancer. Although it is difficult to conclude when immunotherapy should be started, postoperative immunotherapy might be beneficial

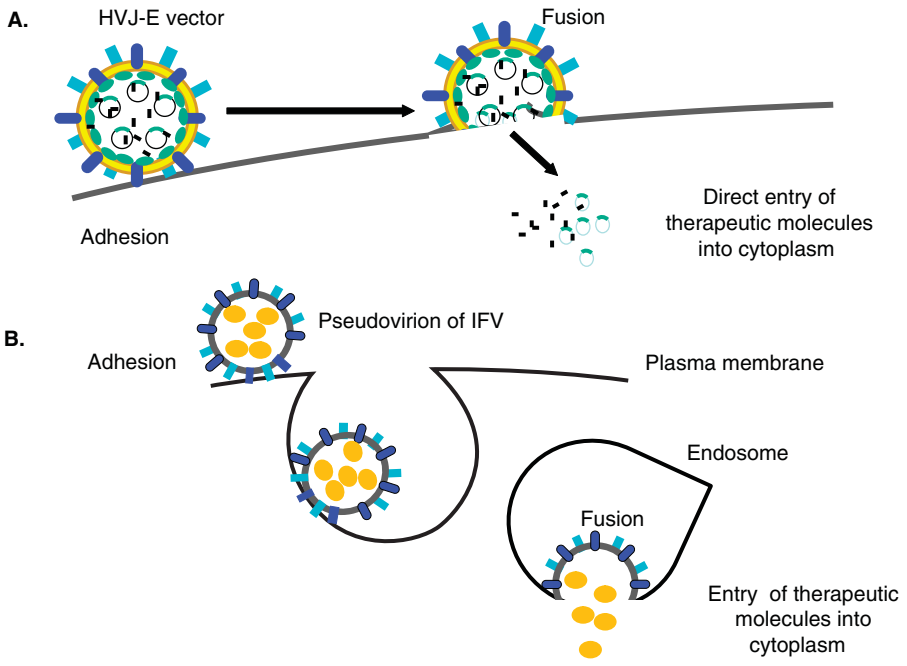

Figure 5. A. When HVJ-E vector attaches to the cell membrane via hemagglutinating protein, fusion between the plasma membrane and fusion protein on the envelope occurs very rapidly to introduce DNA directly into the cytoplasm. **B.** When IFV pseudovirion attaches to the cell membrane via neuraminidase protein, the virion is incorporated by receptor-mediated endocytosis. In the acidic endosome, fusion occurs by hemagglutinin protein to introduce therapeutic molecules into the cytoplasm.

HVJ-E: HVJ envelope; IFV: Influenza virus.

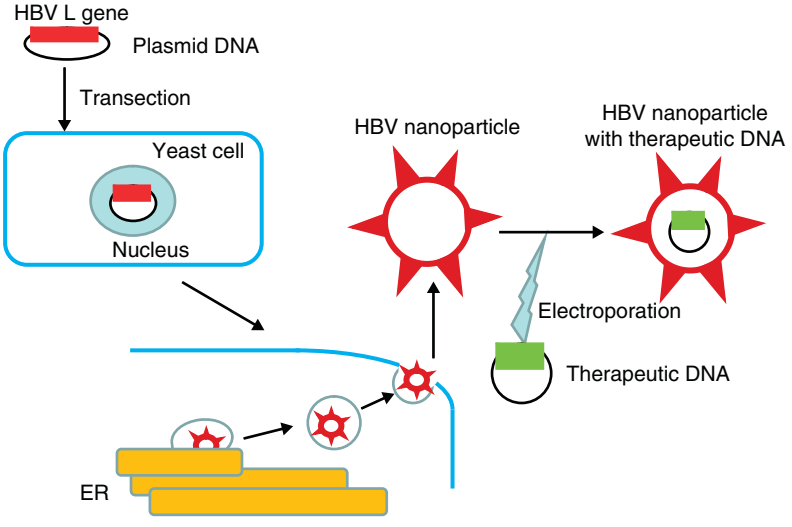

Figure 6. HBV pseudoparticles were constructed by introducing the HBV L antigen gene into yeast. The particle without the viral genome is produced from the yeast. Therapeutic DNA can be introduced into the particle by electroporation. The HBV pseudoparticle targets human hepatocytes and hepatoma cells.

HBV: Human hepatitis B virus.

Table 1. Comparison of each delivery system.

DDS	Administration	Characteristic properties	Clinical use for cancer
Liposomes	Mainly systemic	1. Delivery efficiency affected by lipid components	Gene and drug delivery to many cancers such as melanoma, glioma, etc.
Polymer-based			
1. Micelle	Mainly systemic	1. Smaller than liposome and efficient accumulation in tumor	1. Drug delivery to some cancers such as colon, gastric, pancreas, etc.; dependent on drug
2. Atelocollagen		2. *In vivo* use only	2. – 5. Not clinically tested
3. Gelatin	Mainly topical	3. Slow release of therapeutic molecules	
4. Chitosan	Mainly systemic	4. 5. More suitable for gene and siRNA delivery	
5. PEI	Topical		
Physical			
1. Electroporation	Topical	1. High gene expression	1. Gene transfer to melanoma
2. Sonoporation		2. Less invasive than electroporation	2. Not clinically tested
3. Hydrodynamic		3. Limited use for gene delivery	3. Vaccination (melanoma)
4. Gene-gun		4. Gene transfer to tissue surface	4. Tumor cell vaccine (melanoma, sarcoma)
Virosome			
1. HVJ-E	Mainly topical	1. Fusion-mediated delivery Tumor-specific killing, activation of tumor-specific immunity	1. Melanoma treatment using empty vector*
2. HBV	Systemic	2. Hepatocyte-specific delivery	2. Not clinically tested

*The trial was approved by the ethical committee of Graduate School of Medicine, Osaka University, on 28 July 2009.

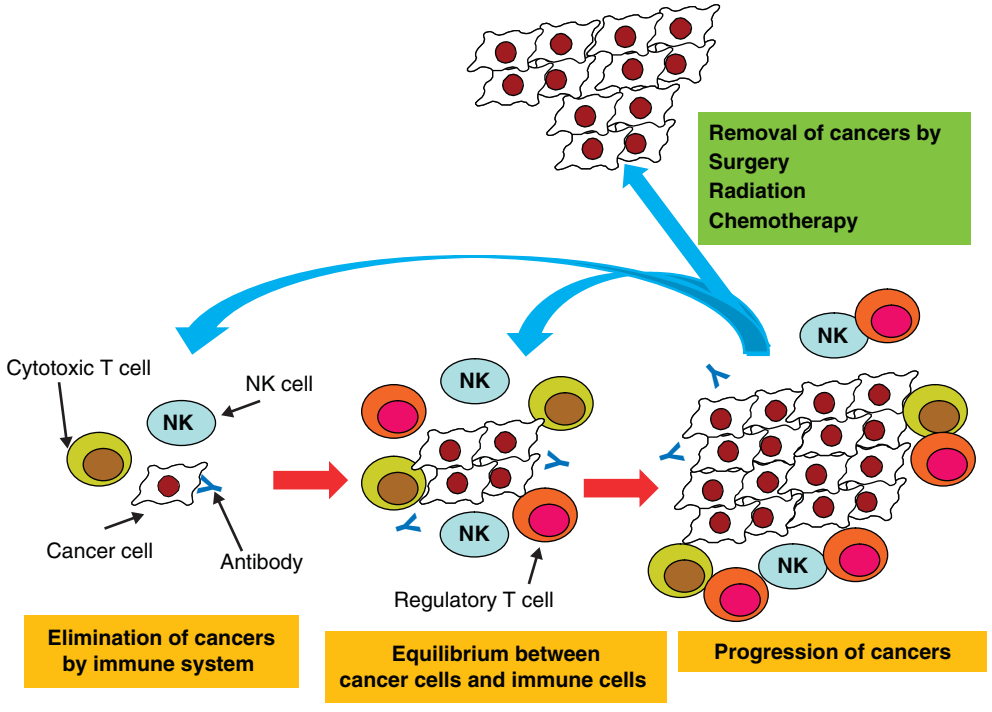

Figure 7. At the cancer initiation stage, cancer cells are easily eliminated by the host immune system. Cancer starts growing by escaping from the host immune system, probably owing to the attenuation of the immune system. Beyond the equilibrium stage between cancer cells and immune cells, tumors grow rapidly in the host by suppressing the host immune system. Unless tumor tissues are removed as much as possible by surgery, chemotherapy and radiation, immunotherapy is no longer effective for the suppression of tumor growth. Therefore, a combination of the removal of tumor tissues and immunotherapy is necessary for cancer treatment.

for prolonging survival rate [89]. This suggests that immunotherapy should be initiated after as much of the tumor mass has been removed as possible (**Figure 7**).

Multilateral therapeutic tools against cancer should be intensively developed. In the development of DDS, safer methods have been appreciated. The basic concept is correct; but, only for cancer treatment, DDS should have antitumor activities *per se.* The delivery of therapeutic molecules to cancer cells using DDS with antitumor activities would be an ideal cancer treatment.

Oncolytic viruses used for cancer therapy include natural mutants, such as E1B-55K deletion adenovirus and the Edmonston strain of the measles virus, and artificial mutants developed by viral gene engineering, such as telomerase-controlled adenovirus, ICP6/γ34.5-deleted herpes virus and thymidine kinase-deleted vaccinia virus (JX-594) [90]. These viruses are live viruses that are expected to replicate selectively in cancer cells. Recently, armed-type oncolytic viruses carrying therapeutic genes have been produced to augment the antitumor effects. Cytosine deaminase/thymidine kinase gene-loaded E1B-55K deletion adenovirus and GM-CSF gene-loaded JX-594 are representative [91,92]. These viral vectors will be promising therapeutic tools in the future. However, as long as the viral genome is intact, it is inevitable that live viruses will remain in the normal tissues of patients. Tumor-killing activity by those oncolytic viruses may be attenuated by the development of anti-viral immunity that can limit viral replication in cancer cells. Several issues remain to be solved. A similar anticancer strategy may be applicable to non-viral DDS. A DDS with function beyond delivery is being developed for cancer therapy.

7.1 DDS for inducing antitumor immunity

The enhancement of general immunity by several DDSs has been described [93]. As nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation by the CpG motif was the main factor for inducing inflammation, severe inflammation occurred in lung by systemic administration of DNA-loaded cationic liposomes [94]. The influenza virosome is also immunogenic and antigens delivered by influenza virosomes were presented by major histocompatibility complex-class I molecules and induced cytotoxic T cells that recognized the antigen [95]. Liposomes are also useful for cancer vaccine owing to the immunogenicity [96]. Thus, the liposome and virosome can enhance cancer immunotherapy [97].

Recently, it has been discovered that the HVJ-E vector itself induces antitumor immunity, including T-cell-mediated and non-T-cell-mediated immunity, through multiple pathways [98,99]. Furthermore, HVJ-E could suppress the function of $Foxp3^+CD4^+CD25^+$ regulatory T cells. Consequently, effector T cells against cancer were actively maintained in tumor-bearing mice. However, even in cancer treatment, an immunogenic DDS is a double-edged sword. An excess amount of vectors can induce fatal cytokine storm in patients, as reported in the administration of overdose adenovirus vector [100]. Generally, a less immunogenic DDS is recommended for the treatment of diseases including cancers. As an exception, DDS for inducing antitumor immunity may be a choice for cancer treatment.

7.2 DDS with tumor-killing activity

Oncolytic viruses are becoming more popular in cancer treatment. Paramyxovirus such as Newcastle disease virus, measles virus (Edmonston strain) and mumps virus have been used for cancer treatment [90,91]. However, these are live viruses and oncolysis is achieved by tumor-selective proliferation of the viruses. It was once thought that inactivated viruses lose their cancer-killing activity. However, a recent report indicates that hormone-refractory human prostate cancer cells can be killed by HVJ-E in a dose-dependent manner [79]. Moreover, HVJ receptor gangliosides were produced abundantly in those cancer cells as compared with hormone-sensitive human prostate cancer cells and normal prostate epithelium. It was revealed that cancer-specific apoptosis was induced by HVJ-E. Intracellular machinery for recognizing viral RNA fragments seems to be involved in the tumor-selective killing. The precise mechanism is now being elucidated.

7.3 Multilateral therapeutic strategies using DDS with anticancer effects

When the tumor-killing activity of DDS with anticancer effects is insufficient for tumor eradication, therapeutic molecules incorporated into the DDS can assist in tumor suppression. For example, although HVJ-E vector has antitumor activities [79,98,99], cell-cycle arrest is not induced by the vector. When HVJ-E containing bleomycin (BLM) (HVJ-E/BLM) was injected into intradermal murine colon cancer, tumor regression was more effective by HVJ-E/BLM than by either HVJ-E alone or BLM alone [101]. Antitumor immunity was enhanced by HVJ-E/BLM but not by BLM alone. Both direct tumor-killing activity and antitumor immunity were highly augmented by HVJ-E/BLM as compared with other treatments. Thus, to achieve multilateral therapeutic strategies, therapeutic molecules to compensate the anticancer actions derived from the vectors should be chosen.

Declaration of interest

The author states no conflict of interest and has received no payment in preparation of this manuscript.

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