Review Article

Pharmacokinetic considerations regarding non-viral cancer gene therapy

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Cancer gene therapy, in which pharmacologically active compounds are administered to cancer patients in a genetic form, has been examined not only in animals but also in cancer patients. Viral vector-induced severe side effects in patients have greatly underscored the importance of non-viral gene transfer methods. Even though the importance of pharmacokinetics is undoubtedly understood in the development of anticancer therapies, its importance has been less well recognized in non-viral cancer gene therapy. When transgene products express their activity within transduced cells, such as herpes simplex virus type 1 thymidine kinase and short hairpin RNA, the pharmacokinetics of the vectors and the expression profiles of the transgenes will determine the efficacy of gene transfer. The percentage of cells transduced is highly important if few by-stander effects are expected. If transgene products are secreted from cells into the blood circulation, such as interferons and interleukins, the pharmacokinetics of transgenes becomes a matter of significant importance. Then, any approach to increasing the level and duration of transgene expression will increase the therapeutic effects of cancer gene therapy. Here we review the pharmacokinetics of both non-viral vectors and transgene products, and discuss what should be done to achieve safer and more effective non-viral cancer gene therapy. (*Cancer Sci* 2008; 99: 856–862)

Since the first cancer gene therapy clinical trial was carried out in the early 1990s, many cancer patients have received gene transfer in an attempt to treat their cancer or to obtain clinical data. Even though a number of achievements in such clinical trials have been reported, gene therapy is still in its infancy phase especially when compared with other anticancer treatments. There are a number of major drawbacks, depending on the type of vector, the type of cancer, and the type of therapeutic compound (protein) used, and these variations make it very difficult to develop rational and universal cancer gene therapy protocols. In general, viral vectors are believed to be more hazardous than non-viral vectors, and the death of a patient receiving adenoviral vectors tragically proved this.[1] However, non-viral gene transfer is considered ineffective due mainly to the low and transient nature of transgene expression.

Continued progress has been made in improving viral and non-viral vectors in the last 15 years. As for non-viral methods, the hydrodynamic injection of naked plasmid DNA[2] has had a great impact on the subsequent development of non-viral methods. The level of transgene expression achieved by this method is as high as that obtained using adenoviral vectors, one of the most efficient viral vectors.[2,3] Complex formation of plasmids with cationic compounds has decreased as vectors for *in vivo* use because their levels of transgene expression are very low compared with those obtained by hydrodynamic injection or viral vectors.[3,4] Furthermore, extensive studies on the innate immune response against vectors have proven that non-viral vectors are not always safe, especially when cationic liposomes are used for DNA delivery. Large amounts of inflammatory cytokines are produced when plasmid DNA complexed with cationic liposomes are recognized by antigen-presenting cells.[3,5,6] Recognition of unmethylated CpG dinucleotides, or CpG motifs, by Toll-like receptor-9 is involved in such responses.[7,8] In addition, DAI, an intracellular DNA-recognizing protein, has recently been identified,[9] and this molecule may respond to DNA irrespective of the presence of CpG motifs.

Once it became known that high levels of transgene expression could be achieved by non-viral methods, including hydrodynamic injection, the reality of non-viral cancer gene therapy increased greatly. However, the therapeutic efficacy is determined not only by the level of expression, but also by other characteristics of gene transfer and transgene expression.[10,11] When transgene products distribute within transduced cells, such as herpes simplex virus type 1 thymidine kinase, both the pharmacokinetics of the vectors and the expression profile of the transgene determine the therapeutic efficacy. Ectopic expression could induce adverse effects, so that controlling the tissue distribution of vectors is the most important issue in the development of cancer gene therapy. An important class of intracellular therapeutic compounds is RNA, and plasmid vectors expressing small interfering RNA (siRNA) or short hairpin RNA (shRNA) have been investigated as a therapeutic tool to treat cancer and other genetic disorders. Silencing oncogenes or other genes contributing to tumor growth will provide a cancer-specific therapy with minimal side effects. RNA interference has realized the silencing of target mRNA expression in a sequence-specific manner. RNA interference, the event of mRNA degradation by siRNA or shRNA, takes place only in cells reached by these molecules. Therefore, the tissue distribution of vectors is highly important in the development of RNA interference-based cancer gene therapy.[12] However, when transgene products are secreted from transduced cells, the pharmacokinetics of vectors is of little importance. Then, the pharmacokinetics of the transgenes is the major factor determining the therapeutic efficacy of cancer gene therapy.[11]

Here we review first the pharmacokinetics of naked plasmid DNA, the most frequently used non-viral vector. Because of its polyanionic nature, it exhibits unique but common tissue distribution characteristics irrespective of the encoding gene, sequence, or size. Complex formation with cationic compounds, a standard method for increasing the level of transgene expression in cultured cells, is also reviewed. Then, the pharmacokinetics

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Table 1. Non-viral methods for *in vivo* gene transfer

Vector	Route of injection	Stimulus or additional treatment	Characteristics	References
Naked DNA	Extracellular space	None	Localized transgene expression High applicability to various targets	Muscle[15,17], liver[16,17], spleen,[17], kidney[17], brain[18], tumor[19–22]
Naked DNA	Extracellular or intravascular space	Electric pulse	Localized transgene expression High applicability to various targets Possible tissue damage caused by electric pulses	Muscle[17,26], liver[17,27], spleen[17], kidney[17], tumor[25]
Naked DNA	Extracellular or intravascular space	Ultrasound	Localized transgene expression High applicability to various targets	Tumor[29], carotid artery[30], femoral artery[31]
Naked DNA	Intravascular space	Massage	Localized transgene expression Applicability to other organs not reported	Liver[32,33]
Naked DNA	Intravascular space	Occlusion of blood flow	Localized transgene expression Surgery required for the occlusion	Liver[35,37], diaphragm[38]
Naked DNA	Intravascular space	Pre-injection of cationic liposomes	Lung-selective transgene expression Reduced immunostimulatory response compared with DNA–cationic liposome complexes	Lung endothelial cells[36]
Naked DNA	Intravascular space	Large volume injection at high speed	Extremely high transgene expression Possible tissue damage	Whole body[2,4,39]
DNA–cationic liposome complex	Extracellular or intravascular space	None	Low-level transgene expression *in vivo* High induction of inflammatory cytokines upon administration	Lung endothelial cells[46]
DNA–cationic polymer complex	Extracellular or intravascular space	None	Low-level transgene expression *in vivo* Concerns about cytotoxicity of cationic polymers	Lung endothelial cells[45]

of transgene products is discussed using our recent data on cancer gene therapy in which interferon (IFN)-expressing plasmids were injected into tumor-bearing mice.[13,14]

Non-viral gene transfer methods

So far, various non-viral gene transfer methods have been developed and their characteristics for gene transfer and transgene expression have been examined after administration *in vivo*. Table 1 summarizes the major non-viral methods for *in vivo* gene transfer.

Tissue injection of naked DNA. The usefulness of naked plasmid DNA as an *in vivo* gene transfer vector was realized as early as 1990, when Wolff *et al*. reported that transgene expression was obtained in skeletal muscle by a simple intramuscular injection of naked plasmid DNA.[15] This injection-mediated gene transfer had been discussed as a skeletal muscle-specific event, but the development of sensitive detection systems for expression has revealed that any organ or tissue examined can express transgenes at detectable levels.[16–18] The mechanism of gene transfer by tissue injection of naked plasmid DNA has been debated, but injection-induced cellular damage and increased pressure is involved in the entry of DNA directly into the cytoplasm and transgene expression follows. Tumor tissues are no exception, and direct injection of naked plasmid DNA into solid tumors results in detectable transgene expression.[19–22] Therefore, in spite of it being the simplest, most unsophisticated system, direct tissue injection of naked plasmid DNA is a useful method for *in vivo* gene transfer. The major drawbacks of this approach are the relatively low level of transgene expression and the limited distribution of the cells transduced.[23]

Physical method. Various physical forces have been used to increase the level of transgene expression by applying them to the target site for gene transfer after topical or systemic administration of non-viral vectors. Physical methods with proven positive effects include electric pulses (electroporation), ultrasound (sonoporation), and physical pressure (massaging), all of which are believed to increase the amount of DNA delivered to cells.

Electroporation-mediated gene transfer is believed to involve high-voltage pulse-mediated pore formation and electrophoretic delivery of charged molecules through the pores.[24] Because of its universality and flexibility, *in vivo* electroporation has been applied to increase transgene expression in various organs, including tumors.[25–27]

Another physical method used frequently to increase transgene expression is the application of ultrasound. Cavitation is considered a major mechanism for an ultrasound-induced increase in membrane permeability.[28] Several reports have demonstrated that transgene expression by plasmid DNA is greatly increased by application of ultrasound.[29,30] In addition, the expression can be further increased by using microbubble echo contrast agents, which enhance ultrasound-induced acoustic cavitation. Recently, Suzuki *et al*. developed bubble liposomes, a liposomal formulation of an ultrasound imaging gas perfluoropropane, and applied them to ultrasound-mediated gene transfer.[31]

A unique and simple gene transfer method was reported by Liu and Huang,[32] who manually pressed or 'massaged' mouse liver after systemic injection of naked plasmid DNA. The authors suggested the involvement of pressure-mediated effects in this mode of gene transfer.[33]

Intravascular and hydrodynamic injection of naked DNA. Administration methods and techniques are most critical when naked plasmid DNA is injected into the blood circulation. Because of the presence of high nuclease activity in serum, naked plasmids injected into the tail vein of mice are degraded rapidly,[34] leading to no detectable transgene expression in any organ. Several techniques have been developed to alter the ineffectiveness of naked plasmid DNA. Budker *et al*. injected naked plasmid DNA dissolved in hypertonic solutions into the portal vein of mice whose hepatic veins were transiently occluded.[35] Song *et al*. injected naked plasmid DNA into the tail vein of mice who had received an intravenous injection of cationic liposomes.[36] This sequential injection greatly prolonged the exposure time of plasmid DNA to the lung endothelial cells, the target cells, and increased the transgene expression. Increasing the retention time of DNA in target organs was simply achieved by occluding blood vessels, and significantly high transgene expression was obtained in the liver,[37] and in the diaphragm.[38]

Regarding gene transfer methods using intravascularly injected naked plasmid DNA, the most important finding was the hydrodynamic injection of naked plasmid DNA, which was first reported independently by Liu's group[2] and Wolff's group[39] in 1999. A simple injection of naked plasmid DNA solution into the tail vein of mice produces a significantly high level of transgene expression in internal organs, with highest expression in the liver. The key points of this delivery are the volume of solution and the injection speed.[2] Since these first reports, this method has been applied to deliver not only plasmids but also other compounds, such as siRNA.[4] Direct cytoplasmic delivery of the injected DNA is involved in the very high transgene expression obtained by this method.[40–43]

Cationic DNA complex. Since Felgner *et al*. proposed the concept of 'lipofection' in 1987,[44] lots of cationic lipids have been developed as transfection reagents. The report of an efficient transfection using polyethyleneimine[45] led to a rush of researchers searching for natural and synthetic polymers that possess gene transfer activity. Mixed formulations of cationic lipids and cationic polymers have also been developed.[46,47] In most cases, these DNA complexes are generally designed to have a net positive charge because binding to target cells, the first step of gene transfer, depends on the electrostatic interaction between positively charged complexes and the negatively charged cell surface.

Although the cationic nature of these DNA complexes is effective for delivering genes to cells in culture, this process is sensitive to the presence of any other negatively charged compounds. Therefore, serum proteins interfere significantly with the cationic DNA complex-mediated transfection to cultured cells.[48] This clearly means that it will be difficult to achieve *in vivo* transfection using cationic DNA complexes, and many reports have confirmed this. Because of the non-specificity of the interaction of cationic DNA complexes with negatively charged molecules, cationic DNA complexes administered *in vivo* interact with various biological components.[49]

Requirements for effective cancer gene therapy

Delivering plasmid vectors to tumor tissues has been carried out using some non-viral gene delivery methods, including direct injection into tumor tissues. Other gene delivery methods by which transgenes are hardly expressed in tumor cells, including hydrodynamic injection, can be used to express anticancer proteins that are secreted into the blood circulation. Thus, the requirements for effective cancer gene therapy vary markedly depending on what types of therapeutic proteins are used. Recently, rapid progress has been made in the application of siRNA and shRNA to cancer gene therapy.[12] Because the site of action of these RNA molecules is the cytosol, their requirements would be the same as those for intracellular proteins.

In a previous review article,[11] we discussed the efficacy of *in vivo* gene transfer in connection with the following four

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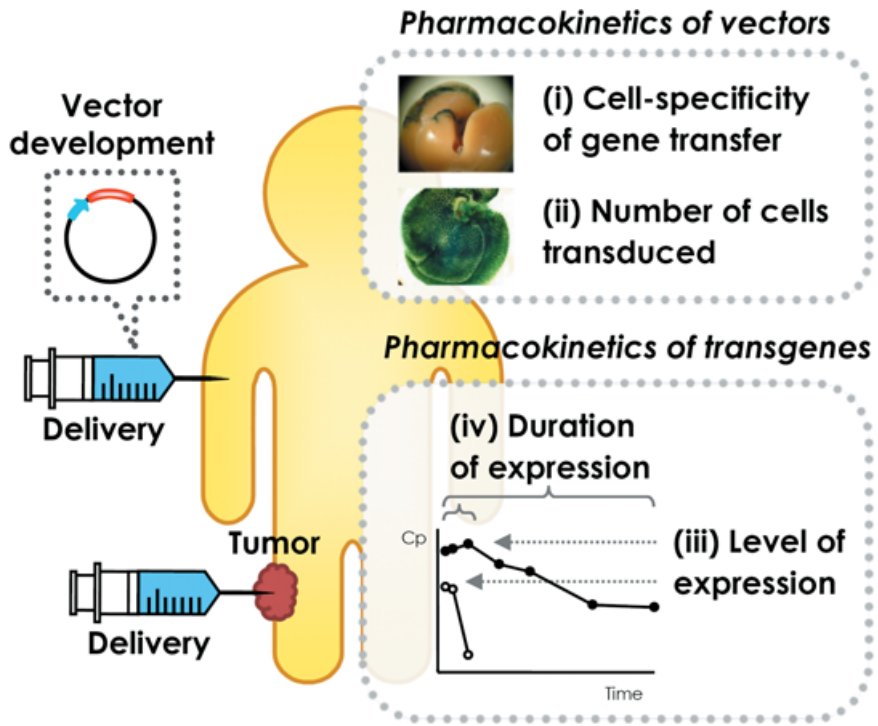

Fig. 1. Processes of non-viral gene transfer. Non-viral cancer gene therapy is achieved by delivering a proper vector directly to tumor tissue or to another part of the body. The therapeutic effects of gene transfer can be determined by the pharmacokinetics of both vectors and transgene products. Characteristics determining the effects are: (i) the target cell specificity of gene transfer; (ii) the number of cells transduced; (iii) the level of expression; and (iv) the duration of expression.

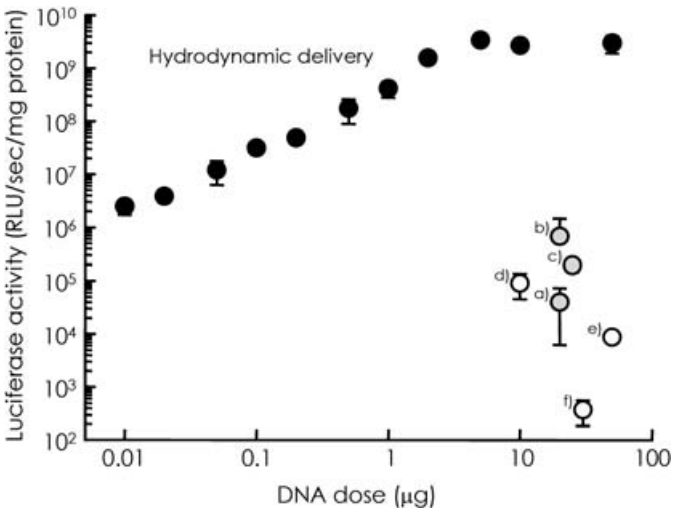

Fig. 2. Comparison of the level of transgene expression by several non-viral gene transfer methods. Firefly luciferase activity (relative light unit [RLU]/s/mg protein) in the liver after non-viral gene transfer to mice was plotted against the dose of plasmid vector expressing firefly luciferase (pCMV-Luc). Closed circles are the results of hydrodynamic delivery of pCMV-Luc injected at different doses.[41] (a) Direct injection of naked plasmid into the liver.[17] (b) Direct injection of naked plasmid into the liver followed by electroporation.[17] (c) Intravenous injection of naked plasmid followed by electroporation.[27] (d) Intravenous injection of plasmid DNA complexed with galactosylated poly ornithine-fusogenic peptide.[51] (e) Intraportal injection of plasmid DNA complexed with galactosylated polyethyleneimine.[54] (f) Intraportal injection of plasmid DNA complexed with cationic liposomes.[52]

characteristics: (i) target cell specificity of gene transfer; (ii) the number of cells transduced; (iii) the level of expression; and (iv) the duration of expression (Fig. 1). Even though the efficiency of non-viral gene transfer methods has improved significantly, the level and duration of expression are still major obstacles to achieving effective cancer gene therapy. Two other parameters, the specificity of gene transfer and the number of cells transduced, are important only when intracellular proteins or shRNA are used as therapeutic compounds. In these cases, all four parameters are important for better therapeutic effects, but the delivery of vectors to the majority of target cells in a cell-specific manner is often the bottle neck in the development of efficient gene therapy methods.

This problem relating to vector delivery is less important when secreted proteins, such as IFN, are used. The type of cells expressing transgenes can be chosen arbitrarily by researchers, depending on a number of factors, such as the accessibility for vector administration, the profile of transgene expression, the distribution of transgenes into the systemic circulation, and possible tissue damage. It is well known that the profile of transgene expression is highly dependent on the organ injected with naked plasmid DNA.[17] In addition, the level of transgene expression depends greatly on the type of non-viral gene transfer method. Figure 2 summarizes the luciferase activity in mouse liver after *in vivo* administration of a firefly luciferase-expressing plasmid vector using various methods. Plasmid DNA complexes with cationic liposomes or cationic polymers were much less effective in expressing the transgene, even though galactose, a hepatocyte-specific ligand, was incorporated into the complexes.[50–54] The expression level achieved by the hydrodynamic delivery of naked plasmid DNA is at least 1000 times greater than that of other methods.[41] Furthermore, hydrodynamic delivery of as little as 10 ng of naked plasmids (for an approximately 20-g mouse) reaches the highest expression levels achieved by other non-viral methods using more than 10 μg of the same plasmid (Fig. 2).

Pharmacokinetics of vectors

Intravascular injection. Unlike low molecular weight compounds, plasmid DNA and other macromolecular compounds are significantly limited in their distribution within the body. Plasmid DNA and its complexes with non-viral vectors can cross blood vessels only in organs with a discontinuous-type endothelium, such as the liver and spleen, or in solid tumors.[55]

Naked plasmid DNA is taken up rapidly by liver sinusoidal endothelial cells and Kupffer cells. This uptake reduces its concentration within the systemic circulation,[34,56,57] which results in little chance of DNA being delivered to tumor cells. When injected by the hydrodynamic delivery method, plasmid DNA shows different profiles for its tissue distribution, but the major organ involved in the tissue distribution is the liver.[56] Application of electric pulses to organs after intravenous injection of non-viral vectors has little effect on their tissue distribution.[17,27] Depletion of macrophages hardly changes the tissue distribution of naked plasmid DNA (unpublished data, Kako K, Nishikawa M, Yoshida H, Takakura Y, 2006). Thus, the tissue distribution of intravascularly administered naked plasmid DNA is hardly altered by any means, and a large fraction of the injected DNA is delivered to the liver.

Complex formation of plasmid DNA with cationic compounds is intended to increase the interaction with and, following uptake, by target cells. This inevitably increases the interaction of such DNA complexes with biological components.[50,58,59] In general, intravenously injected cationic DNA complexes are first trapped in capillaries in the lung, followed by accumulation in the liver.[60,61] Interactions of the DNA–cationic liposome complex with serum components results in disintegration of the complex, followed by the release and degradation of plasmid DNA.[58] Sakurai *et al*.[59] reported the involvement of erythrocytes in the interaction of cationic DNA complexes.

Use of receptor-mediated processes for cell-specific delivery of plasmid DNA complexes has been investigated extensively. Glycosylation (i.e. covalent conjugation of sugar moieties) of any compound greatly increases its affinity for cells expressing the corresponding sugar receptors. Precise control of the physicochemical properties of DNA complexes with galactosylated compounds increases their delivery to hepatocytes.[50] Some improvements in transgene expression were reported when DNA complexes were modified with transferrin,[62] folate,[63] or antibody.[64] However, the effects of these modifications on the tissue distribution of plasmid DNA complexes have not been investigated fully.

Blood flow to solid tumors is generally low compared with large organs, such as liver and kidney, so the delivery of any pharmaceutical compound to tumor tissues requires prolonged circulation in the blood. Several attempts have been made to deliver plasmid DNA to tumor tissues using DNA complexes.[65–67] However, the amounts of DNA delivered and the level of expression seem to be far below the thresholds required for effective cancer gene therapy.

Direct tissue injection. The pharmacokinetics of plasmid DNA after tissue injection is simple, because the large size of the DNA greatly restricts its distribution within and outside the site of injection. These characteristics are closely associated with the experimental finding that organs and tissues other than those injected show very little transgene expression.

As described above, tissue injection of naked plasmid DNA results in detectable transgene expression in almost all tissues and organs examined. However, cells surrounding the track of the needle injection only express transgenes.[16,23] Complex formation of DNA with cationic liposomes further limits the distribution of transgene-expressing cells.[20,21] It has been reported that the area of transgene expression is increased by physical methods, such as electroporation.[26,68]

Pharmacokinetics of transgene products. Because transgene products are responsible for therapeutic effects after gene transfer, the efficacy of any gene transfer application cannot be discussed without considering its pharmacokinetics. However, the pharmacokinetics of transgenes has not fully been discussed. This is largely because most non-viral methods are not efficient enough to express transgenes at therapeutic levels, and many reports have simply emphasized how expression is increased using a number of unpublished methods.

The importance of the pharmacokinetics of transgene products is easily appreciated when the therapeutic effects of chemically modified proteins are considered. Of all the technologies developed, conjugation of polyethylene glycol, or PEGylation, is the most successful for improving the pharmacological activities of biologically active proteins.[69–75] PEGylated derivatives show a much slower clearance from the systemic circulation than unmodified proteins, so that the area under the plasma concentration–time curve (AUC) and the mean residence time (MRT), two important pharmacokinetic parameters, are significantly increased for PEGylated derivatives.

Because the hydrodynamic delivery of naked plasmid DNA gives an enormously high level of transgene expression, its application to experimental therapeutic models has been reported.[4] Kobayashi *et al*. described how the hydrodynamic delivery of mouse IFN-β- or IFN-γ-expressing plasmid vector was effective in inhibiting metastatic growth of colon adenocarcinoma cells in mouse liver.[13] However, as demonstrated in this previous report the transient nature of transgene expression from a conventional plasmid DNA requires multiple injections. Therefore, increasing the duration of IFN transgene expression would be needed for better cancer treatments.

Various methods to increase the duration of transgene expression from plasmid vectors have been reported. For example, a controlled-release formulation of plasmid DNA greatly extended transgene expression.[76] Various plasmid vectors that promise sustained transgene expression have also been developed,[77–80] and any of these vectors would be useful for obtaining prolonged expression of therapeutic proteins. In a previous study, we constructed plasmid vectors with reduced numbers of CpG motifs.[14] Compared with conventional CpG-replete plasmids (pCMV-Muβ and pCMV-Muγ), the CpG-reduced plasmid vectors pGZB-Muβ and pGZB-Muγ resulted in sustained expression of mouse IFN-β and IFN-γ, respectively, after their hydrodynamic delivery to mice. Significant increases in the pharmacokinetic parameters of the transgenes were obtained. The AUC and MRT of IFN-γ after injection of pGZB-Muγ were approximately 60- and 4-fold, respectively, greater than those of pCMV-Muγ. The survival time of the pGZB-Muγ-treated mice was significantly longer than other groups, clearly demonstrating that long-term expression of IFN enhances the therapeutic effects of IFN cancer gene therapy.

Conclusions and perspectives

Although the initial enthusiasm for gene therapy has been tempered, continuous progress in both viral and non-viral vector development and administration devices and methods has greatly increased the reality of cancer gene therapy. Because of the difficulty of controlling the pharmacokinetic characteristics of plasmid DNA, irrespective of the naked or complexed form, gene delivery of proteins that are released from transduced cells, such as IFN and interleukins, can be considered to be much easier than that of intracellular proteins. Although skeletal muscle has been considered a suitable target for gene transfer, because of its accessibility, size, and the unique characteristics of sustained transgene expression compared with other organs, its usefulness as a platform for producing anticancer proteins into the systemic circulation remains to be verified. The liver seems to be another promising 'factory' producing secreted proteins as demonstrated, but less-invasive and safer methods than hydrodynamic delivery for gene transfer would be required for future clinical applications.

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