Review

Development of Novel Technology of DDS for Gene Therapy

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Summary: In the near future, not only "systemic pharmacokinetics" but also "intracellular pharmacokinetics" seems to be important in Drug Delivery System (DDS) research for gene therapy. Beyond the basic philosophy of DDS of "delivering the optimal amounts of drugs to a target site", it is now necessary to "express the gene (as a drug) efficiently in a target cell for a required period" in gene therapy. To achieve these objectives, vectors for introducing the gene into the target cell are being improved, and techniques to efficiently express the transgene and to regulate the transgene expression are being developed. DDS is expected to play a large part in achieving this goal. Here, we review a novel DDS technology to satisfy these criteria.

Key words: fusogenic liposome; gene therapy; DDS; cytoplasmic gene expression system; tetracycline-regulatable gene expression system

Introduction

Drug Delivery System (DDS) techniques are under active investigation to optimize therapeutic effects by controlling the systemic pharmacokinetics. In fact, many DDS pharmaceuticals using macromolecules, liposomes, and microcapsules, etc. have been developed. In this respect, DDS technology allowing control over the kinetics of drugs in the body is considered as an important basis for support of pharmacotherapy and basic research.

Today, a great deal of attention is being paid to next-generation therapies utilizing biological macromolecules such as plasmid DNA, siRNA or antisense nucleotides, and there is a need for new DDS techniques that maximize therapeutic effects by taking into account the mechanisms of action of these next-generation drugs. The action of these next generation drugs doesn't appear, as long as it isn't introduced into the cell. For example, siRNA and antisense oligonucleotides need to reach cytoplasmic mRNA, while plasmid DNA needs to be delivered into the nucleus. However, since these drugs are biological macromolecules, they cannot easily pass through cells and nuclear membranes.[1–3] Furthermore, these drugs are not stable, so the action dosen't continue. Therefore, it is essential to develop new technologies to introduce these drugs into the cytoplasm and to deliver the target organella (nucleus), or to keep

the optimal concentrations in cytoplasm in gene therapy.

On the other hand, stringent control of gene expression in gene therapy strategies is important for both therapeutic and safety reasons. However, in most gene therapy, a transgene is under the control of a promoter that allows constitutive expression. Due to recent advances in gene transfer vectors, a gene can be delivered directly into tissue cells *in vivo*. One cancer gene therapy approach involves direct injection of cytokine genes into the tumor cells. The rationale behind this strategy is that high intratumoral cytokine levels can be maintained to effectively and optimally activate the antitumor immune response without the toxicity frequently associated with systemic cytokine administration.[4–7] As the control of kinetics of gene products is virtually nonexistent, production of proteins that cause strong adverse reactions, such as cytokines, by gene therapy may lead to severe adverse reactions due to the excessive expression of a therapeutic gene.[8,9] To date, the main focus of research on gene transfer vectors has been to establish a high gene expression system. In order to develop safe and effective gene therapy, a system must be developed in which the *in vivo* production of a protein (or the expression of a transfected gene) can be regulated.

The aim of DDS research in gene therapy is to deliver optimal amounts of a drug (plasmid DNA, siRNA, or antisense nucleic acid, etc.) to the cytoplasm or nuclei in
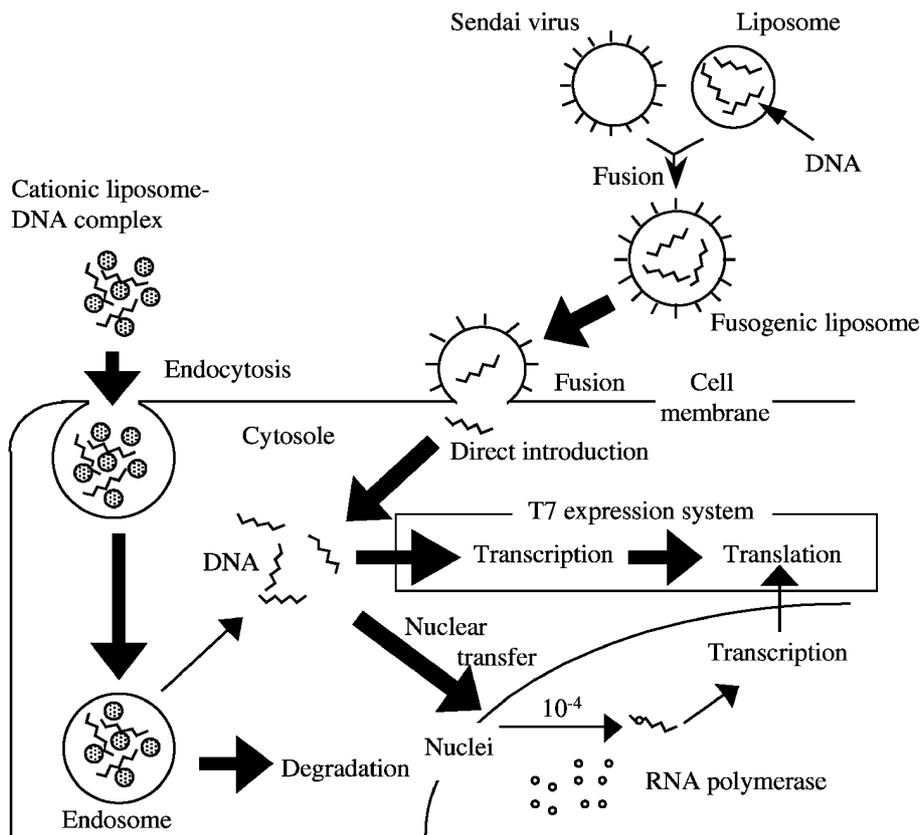
Fig. 1. Gene delivery by cationic liposomes and fusogenic liposomes.
Cationic liposomes do not directly fuse with the cell membrane. Cationic liposomes and plasmid DNAs complex are taken into the cells by endocytosis. Therefore, most plasmid DNAs are degraded in endosome, resulting in loss of their efficiency. On the other hand, fusogenic liposomes, which are prepared by fusing conventional liposomes with the Sendai virus, bind to the cell surface and fuse with cell membrane, and then directly deliver encapsulated plasmid DNAs into the cytoplasm.

a target cell, and regulate the function of these drugs. In this article, we describe some of the research we are undertaking to achieve this goal, and describe the DDS technology for gene therapy. As an example of the potential utility of this technology, we highlight a novel hybrid vector, fusogenic liposome, to introduce the macromolecules into the cells, a novel cytoplasmic expression system in order to obtain the high gene expression efficiency, and tetracycline-regulatable gene expression system to regulate the transgene.

Direct introduction of macromolecules into cells

Many viral and nonviral vectors for gene transfer have been developed, and they have both advantages and disadvantages. Viral systems using such as retrovirus, adenovirus, and adeno-associated virus have a high gene transfer and gene expression efficiency and some of them can be used for gene transfer into tissue cells *in vivo*. However, the structure and stability of transferred genes are restricted by the character of the virus genome. Furthermore, viral vectors can introduce only genes, not other macromolecules such as siRNA or antisense nucleotides. In contrast, nonviral vectors such as cationic liposomes are safe and doesn't restrict the character of the genes. Liposomes can encapsulate various drugs and macromolecules, and are useful as carriers for their delivery to cells. However, conventional liposomes don't directly fuse with the cell membrane. Even when they are actively bound to the cell surface by adding an antibody or ligand on the surface, they are taken into the cell by endocytosis. Most substances encapsulated in liposomes are degraded by lysosomal enzymes, which significantly reduced the introducing efficiency. Therefore, delivery by direct fusion of the liposome membrane with cell membrane is essential for efficient introduction of macromolecules into the cytoplasm.

We have been developed fusogenic liposomes which have two envelope glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F) protein, from Sendai virus on the surface of liposome membrane. Fusogenic liposomes are prepared by fusing conventional liposomes with Sendai virus which belongs to the paramyxovirus family. The HN protein is responsible

for viral attachment to the cellular receptors containing sialic acids and also plays an undefined role in subsequent fusion, while the F protein plays a major role in fusion of the envelope with the cell membrane. These two envelope glycoproteins on the fusogenic liposomes have critical roles in the process of membrane fusion. Fusogenic liposomes can fuse with the cell membrane like the native virus particle and deliver encapsulated contents directly and efficiently into the cytoplasm (**Fig. 1**). Although fusogenic liposomes are composed of Sendai virus, the RNA gene of the Sendai virus is chemically modified or fragmented by β-propyl lactone or ultraviolet irradiation. Also, as the Sendai virus is not pathogenic to humans, it is safer than other virus vectors. Fusogenic liposomes have the advantages of efficient gene introduction and high gene expression in comparison with cationic liposomes, a common non-viral gene transfer vector.[10-12] For example, fusogenic liposomes transferred genes efficiently into cultured cells within 10 min when they were incubated at 37°C, while cationic liposomes-gene complex required at least 30 min to reach the same expression level. Fusogenic liposomes are also less cytotoxic than cationic liposomes and showed more than 1,600 times higher gene expression than cationic liposomes *in vivo*. Fusogenic liposomes can introduce substances with 100% efficiency into the most of mammalian cells. Furthermore, virus vectors such as retrovirus and adenovirus have a limitation in the size of the genes that can be introduced. However, the fusogenic liposomes can introduce any kind of macromolecules such as plasmid DNAs,[10-12] antigenic proteins,[13,14] toxin proteins,[15,16] oligonucleotides,[17] phages and nano-particles as long as they can be encapsulated in a liposomes. Due to these advantages, it can be thought that fusogenic liposomes have a wide range of applications, possess the functions of a gene-transfer vector, and also can be used as an antigen protein (vaccine) carrier, as a vector to introduce macromolecules into cells.
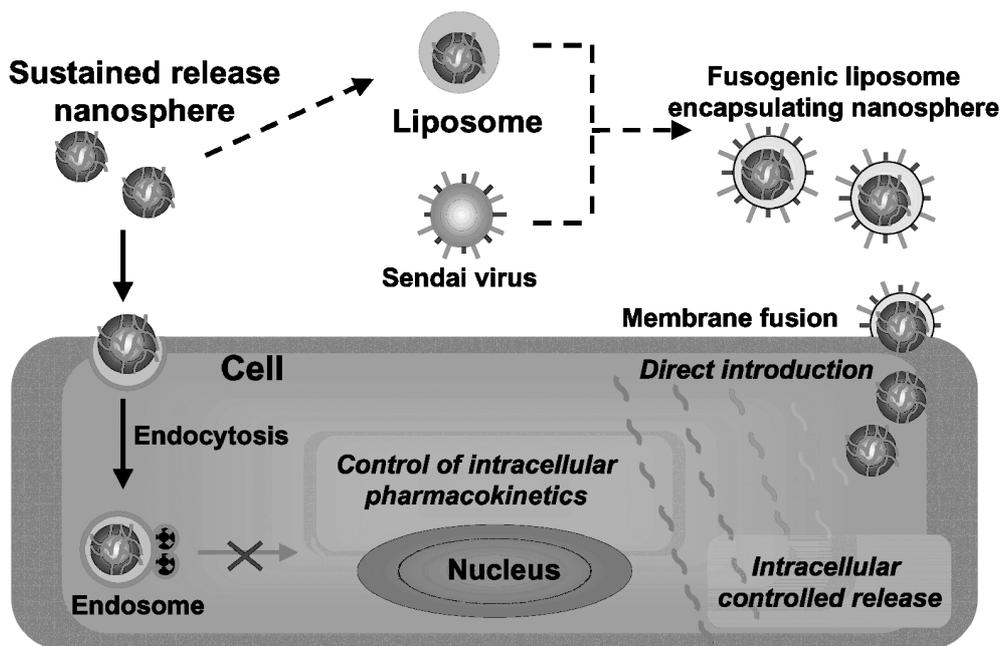
Intracellular kinetics

At the present time, the adenovirus vector is superior in terms of transgene expression efficiency. Adenovirus vector is incorporated into the cell by the endocytosis pathway through a receptor. However, as it has an outstanding capability of escaping from endosome to cytoplasm, it reaches cytoplasm efficiently without the gene being decomposed by the lysosome enzyme.[18] Moreover, since it also has the nuclear transfer function required for the gene to be expressed, a high gene expression can be obtained. Fusogenic liposomes can efficiently introduce encapsulated plasmid DNAs into cytoplasm by direct fusion with cell membrane, resulting in obtaining the high gene expression in comparison with cationic liposomes. However, plasmid DNAs

introduced into cells by fusogenic liposomes don't have the function to be delivered into the nucleus, so it is generally difficult to obtain a gene expression efficiency equivalent to that of adenovirus vector. The nuclear membrane restricts the transport of the plasmid DNA, and the efficiency of DNA transfer from the cytoplasm to the nucleus has been estimated to be about 10^{-4}. Therefore, in order to obtain a high gene expression efficiency, the genes introduced into the cell must be reduced to a compact size which can pass through nuclear pores, and they must also be given an ability to target the nucleus. As regards intracellular targeting, specific aminoacid sequences (signal peptides) of virus proteins and intracellular proteins can control transfer of substances into organella, and much information has been accumulated. Such information will be important in the future when controlling intracellular pharmacokinetics, and will be very useful in the realization of intracellular targeting.

On the other hand, there is an another method to express transgenes in the cytoplasm. Gene expression in the cytoplasm can be expected to achieve higher efficiency, because it does not require transport of genes into the nucleus, which is one of the most significant limiting steps in gene transfer using non-viral vectors. We have tried to develop a T7 expression system which efficiently expresses genes in the cytoplasm[19] (**Fig. 1**). This system realizes gene expression by introducing into cytoplasm both T7 RNA polymerase and plasmid DNA with a T7 promoter, which are not present in eucaryocytes. With this expression system, we can expect gene expression in a completely independent manner from the usual gene expression in the nucleus, without the problem of whether the cells are mitotic or non-mitotic, and regardless of differences in promoter activity according to cell type. In this approach, T7 RNA polymerase or mRNA[20] which codes for T7 RNA polymerase must be introduced into the cytoplasm together with the target gene. Only fusogenic liposomes which can directly introduce any kind of substance (genes, proteins and particles such as nanospheres) into cytoplasm, can solve the difficult problem.

Intracellular controlled release

Antisense oligonucleotides, ribozymes and siRNA, etc. have been shown to be very efficient in the selective inhibition of gene expression. However, the therapeutic application of these macromolecules is currently limited by their physiological properties such as low cellular uptake and instability. These macromolecules are incorporated into the cell by an endocytosis pathway, and most will be decomposed in the endosome, so there is a very low efficiency of reaching the cytoplasm. Even if they can be delivered to the cytoplasm, as they have poor stability, it is very difficult to expect continuity of

Fig. 2.   Novel hybrid vector, fusogenic liposome, for the delivery of sustained release nanosphere.
In order to control the intracellular pharmacokinetics, technology for delivering sustained release particles such as nanospheres into cytoplasm is indispensable. However the nanoparticles generally enter cells via endocytosis. In other words, even when these nanoparticles enter cells after encapsulation by an endosome membrane, they still can not reach the cytoplasm. However, fusogenic liposomes can directly introduce nanoparticles into the cytoplasm of living cells at one time without serious cytotoxicity.

activity. Therefore, in addition to control of the systemic pharmacokinetics which have already been exploited, an efficient technology must be developed for delivering them into cytoplasm, and new DDS technology must be established which can control the intracellular pharmacokinetics such as the regulation of the drug concentrations in cytoplasm using sustained release system.

Various techniques have already been developed for sustained release of drugs, one of which is "nanospheres". Nanospheres, which comprise a polymer matrix that can enclose a large amount of drugs, can control the release of drugs by the matrix component, and may also be able to suppress the decomposition of unstable drugs. We have succeeded in directly introducing these nanospheres into cytoplasm by using fusogenic liposomes (**Fig. 2**). By introducing a nanosphere which has adsorbed an oligonucleotide with a fluorescent label into the cell using fusogenic liposomes and observing with a confocal laser scanning microscope, it was found that the fluorescence of the oligonucleotide diffused through the whole cell in a time-dependent way. In the future, however, evaluation of "intracellular pharmacokinetics" rather then "systemic pharmacokinetics" will be required, and a technique to introduce nanoparticles into the cytoplasm will become necessary. Our technique is further expected to contribute greatly to the development of nanoscience and life science of the next generation as a method

indispensable for evaluation of intracellular activities of functional nanoparticles, which will be created by the extensive use of nanoscience and nanotechnology.

Regulation of the expression of exogenous genes

To date, heat shock,[21] hormones such as glucocorticoids, estrogens, progesterones, and androgens[22–25] and heavy metal ions[26–28] have been used to control gene expression. However, for various reasons, including pleiotropic effects caused by the inducers or interference of transgene expression by endogenous inducers, these systems are not well suited for gene therapy. The ideal system for gene therapy requires a non endogenous inducer that specifically regulates transcription of the transgene. The inducer must not affect other endogenous genes.

The Cre-loxP system of P2 phages has been used in recent years to regulate the expression of exogenous genes. In this system, an exogenous gene is incorporated between loxPs, and Cre protein is then used to release the exogenous gene.[29,30] Cre protein specifically recognizes the 34 bp loxP sequence and performs a series of reactions on its own that are necessary for homologous recombination (cutting and binding).[31] However, a drawback of this system is that it is not reversible; once the segment of DNA incorporated between two loxP sequences is excised by Cre recombinase, gene expression cannot be restored to the original state. As a result,
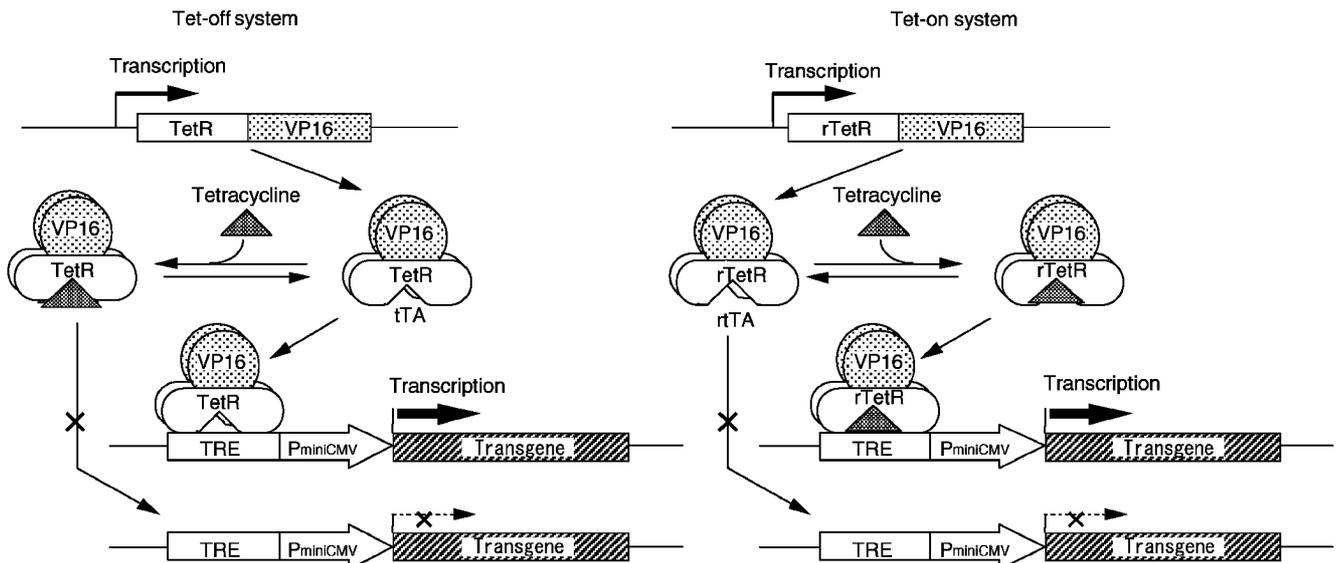
Fig. 3. Transgene regulation by tet-off and tet-on system.
Tet-off system: The tTA can induce enhanced transgene transcription only from the tetracycline response element (TRE) promoter in the absence of tetracycline. In the presence of tetracycline, a conformational change in the transactivator abolishes its ability to drive transcription.
Tet-on system: The rtTA can not bind to TRE without tetracycline derivatives like doxycycline. In the presence of tetracycline, rtTA binds to the TRE and triggers transcription.

gene expression can be regulated only once. Thus, an important aspect of the Cre-loxP system is to determine when and how Cre protein is expressed.

As first described by Gossen and Bujard,[32] gene expression regulatory systems that involve the use of tetracycline-responsive promoters have led to the establishment of highly efficient regulatory systems in mammalian cells. Because the method involves a regulatory circuit not used by eukaryotic cells, transgene expression can be stringently controlled without affecting the expression of endogenous genes. The present gene expression system is based on two regulatory elements derived from the tetracycline-resistance operon of the *E. coli* Tn10 transposon[33]: Tet repressor protein (TetR) and the Tet operator DNA sequence (tetO) to which TetR binds. Tetracycline-responsive promoters are a compound promoter consisting of the tet-responsive element (TRE), which contains seven copies of tetO, and the minimal immediate early promoter of cytomegalovirus. The tetracycline-controlled transcriptional activator (tTA) is a complex consisting of the wild-type TetR and the VP16 activation domain of herpes simplex virus. tTA binds to TRE and activates transcription. However, tTA does not bind to TRE in the presence of tetracycline and transcription is inhibited as a result (**Fig. 3**). Thus, when a vector that is capable of expressing a target gene under the control of a tetracycline-responsive promoter and a vector that is capable of expressing tTA are simultaneously transfected into a cell, the expression of the target gene can be regulated via the addition of tetracycline (a Tet-Off

system). Conversely, if a fusion protein (reverse tTA, rtTA) of reverse TetR (rTetR) and VP16 is used, it can combine with TRE only in the presence of tetracycline, and this is a Tet-On system which can activate transfer.[34] Also *in vivo*, we have reported that optimization of cancer gene therapy is possible by regulating the expression of the transgene (interleukin-12; IL-12) by applying a Tet-Off system to an adenovirus vector in a tetracycline concentration range which does not show any toxicity.[35] It is thought that as the promoter and inducer do not exist in mammalian cells, this system can permit the expression of the target gene alone to be strictly controlled without affecting expression of the host genome genes in any way, and will become a useful tool in the formulation of a safe and effective gene therapy technique.

Conclusion

In the pharmacotherapy carried out to date, optimal medication was possible by implementing the concept of DDSs, i.e., delivering the optimal amount of a drug to the target site. Thus, for example, if a sustained release pharmaceutical which controls the release speed and amount of the drug can be administered to the target site, pharmacotherapy can be optimized, and such a DDS pharmaceutical has in fact already been developed. Regarding gene therapy, the function of the vector is of great importance. Also, in addition to the function of the vector, the expression efficiency of the gene depends on the characteristics of the therapeutic gene including expression-regulating sites such as those

of the promoter. Therefore, in the case of gene therapy, it is important to regulate the expression period, expression intensity and expression site of the gene depending on the disease by formulating various combinations of vectors and the therapeutic genes including expression-regulating sites.

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