Provisional Translation*

Points to Consider in the Development of *in vivo* Gene Therapy Products with Target Specificity

– Including *in vivo* CAR-T Development

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Subcommittee on Cell and Gene Therapy Products Produced in vivo

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1. General Remarks

1.1. Background

In recent years, the development of gene therapy products has rapidly progressed, and the number of approved products has also been increasing (1). Gene therapy can be roughly divided into two categories: *in vivo* gene therapy, where viral or non-viral vectors are directly administered to patients, and *ex vivo* gene therapy, where tissues or cells are taken out of the body for gene transfer or modification before being administered to patients. Examples of *in vivo* gene therapies with significant benefits include adeno-associated virus (AAV) vector treatments for Leber congenital amaurosis (LCA) and spinal muscular atrophy (SMA). For *ex vivo* gene therapy, chimeric antigen receptor (CAR) transduced T-cell (CAR-T) therapy has shown remarkable efficacy against malignant B cell tumors. However, CAR-T therapy problematically requires substantial resources (facilities, goods, and personnel) for production; accordingly, attempts are being made to produce CAR-T *in vivo*. In the future, this approach will expand further, and there will be a push to switch from *ex vivo* gene therapy to *in vivo* gene therapy (2).

To realize efficacy and safety that are similar to or better than *ex vivo* gene therapy by direct administration of a vector, high target specificity is required to deliver and express therapeutic genes and gene-modifying tools restricted to the target cell or tissue. It would be meaningful for developers and regulators to share basic ideas in evaluating such targeting approaches, particularly regarding safety. Therefore, the Science Board of the Pharmaceuticals and Medical Devices Agency has decided to establish an expert committee to discuss this issue. The readers of this report include developers (companies) of gene therapy products, researchers involved in vector development (both from companies and academia), and regulatory authority reviewers.

1.2. Scope of the report

This report discusses the development of *in vivo* gene therapy products that require enhanced target specificity, transitioning from *ex vivo* gene therapy to *in vivo* gene therapy. On the other hand, for technologies that are already established as *in vivo* gene therapies or their extensions (such as those targeting tumors and parenchymal organs by local administration or oncolytic viruses that rely on tumor-specific infection and growth mechanisms), current regulations may provide guidelines and literature as necessary.

In addition to the above, there are two main safety concerns that this expert committee emphasizes. The first concern is the risk of permanent changes in the host genome caused by genome-integrating vectors or editing procedures. This is particularly important when genetically modified cells are germ cells, special consideration is required because their effects extend across generations. The second concern is the occurrence of serious systemic reactions. Thorough examinations are mandated to ensure safety when a large amount of an integrating viral vector (retrovirus/lentivirus) is systemically administered, since this kind of vector has not been systemically administered to humans.

1.3. Target specificity

Determining “target specificity” is roughly divided into three phases: The first phase is based on the affinity of the vector for cell surface molecules and can be referred to as “(cell/tissue) tropism.” Viral vectors have

been studied for their interaction of envelopes and capsids with cell surface receptors, but their tissue specificity is not necessarily high. For mRNA and plasmid DNA, which are non-viral modalities, drug delivery systems (DDS) with nanocarriers, such as lipid nanoparticles (LNP), determine their tropism. The second phase is the host factor-dependent intracellular trafficking of the vector and the life cycle of viruses, but the knowledge about the actual mechanism is limited. The third phase involves the modulation of expression levels, including transcriptional regulation with tissue-specific promoters/enhancers and tissue-specific post-transcriptional regulation or RNA interference. This report will primarily discuss cell/tissue tropism for each vector/modality, in combination with addressing expression control where necessary.

1.4. Definition of terms

Vectors: In this report, gene transfer/modification tools are collectively referred to as gene therapy vectors. Viral vectors are derived from viruses such as retroviruses (RV), lentiviruses (LV), adeno-associated viruses (AAV), and adenoviruses (AdV). Non-viral vectors include plasmids, mRNA, and nanocarriers containing LNPs, as defined below.

Nanocarriers: Collectively refer to submicron-sized carriers that form complexes with drugs (including DNA, RNA, proteins, and low-molecular-weight compounds) for delivery to the target tissue. These include polymer nanoparticles, lipid nanoparticles, inorganic nanoparticles, liposomes, nanotubes, nanocomplexes, and niosomes.

LNP (lipid nanoparticle): A type of nanocarrier, typically 10-1000 nm in diameter, with lipids as their main component. The LNPs currently used for mRNA delivery are composed of pH-sensitive lipids (ionized lipids), polyethylene glycol-modified lipids, and helper lipids (phospholipids, cholesterol).

2. Strategies for Conferring Target Specificity to Gene Therapy Vectors/Modalities

This section describes the characteristics and target specificity conferring strategies for LV vectors, AAV vectors, AdV vectors, mRNA, and DNA, which are primarily studied as target-specific *in vivo* gene therapy vectors.

2.1. Lentivirus (LV) vector

Vectors derived from LV, belonging to a family of retroviruses, allow for the stable integration of the transgene into the genome in target cells. Therefore, LV vectors are suitable for applications such as stem cell gene therapy, where the functions of the therapeutic gene can persist for a lifetime. LV particles are covered with a lipid bilayer envelope embedding several proteins. Their infection tropism can be altered through pseudotyping, which involves replacing the envelope protein with that of other viruses, or by modifying the envelope protein (3). In general, target-specific LV vectors are developed by incorporating a specific ligand, such as single-chain variable fragments (scFv) from antibodies that bind to a targeted molecule, and designed ankyrin repeat proteins (DARPins) (3,6,7), as well as pseudotyped with an envelope protein from Sindbis virus (4), measles virus (5), and others. The hematopoietic stem cell (HSC) marker CD133 (8), the HSC/vascular endothelial cell marker CD105 (9, 10), and the T cell markers CD8 (11) and CD4 (12) have

been reported as surface antigens recognized by scFv.

In addition, LV gene expression can be controlled by a tissue-specific promoter that activates only in target cells, and the target sequence of microRNAs (miRNAs) that suppress gene expression in non-target cells (13).

2.2. Adeno-associated virus (AAV) vector

AAV is a non-enveloped particle that packages a single-stranded DNA viral genome in an icosahedral capsid. After infection, the vector genome does not integrate into the host genome and exists as an episome in the nucleus. The cell/tissue tropism of AAV vectors is determined by the affinity of the capsid to the host receptor, and the tropism depends on the amino acid sequence of the capsid. Although there are more than 100 AAV serotypes in nature, it is difficult to achieve highly specific tropism with these different capsids alone. Therefore, AAV vectors with high target specificity have been developed by adding arbitrary peptide sequences to the surface of the capsid or inserting scFvs or DARPins. Recently, in silico capsid engineering using bioinformatics and computational tools has attracted much attention (14).

Meanwhile, tissue-specific promoters/enhancers have been used to control transgene expression, but optimal vector construction remains a challenge due to limited genome size of AAV vectors.

2.3. Adenovirus (AdV) vector

AdV is a non-enveloped virus with a double-stranded DNA genome. There are more than 100 serotypes, such as serotype 5 (Ad5), that differ in the amino acid sequence of their capsid. Infection tropism is primarily determined by the affinity between the fiber knob protruding from the viral particle and cell surface receptor. Exploration of natural isolates and modification of the fiber knob have been continued to manipulate the tropism. For example, the fiber knob sequence of serotype 35 (Ad35), which uses CD46 highly expressed on HSC as a receptor, or serotype 3 (Ad3), whose receptor is desmoglein 2 (DSG2), has allowed gene transfer to HSC (15-19). More recently, several modifications have led to the development of an AdV vector that further enhances tropism to HSCs (20). Other attempts include the use of a B-cell specific promoter in combination with the insertion of an integrin-bound RGD motif into the fiber (21), as well as adapters that bridge the Ad5 knob to molecules on the T cell (CD3, CD28, or interleukin [IL]-2 receptor) to achieve selectivity (22).

AdV is more immunogenic than AAV and, therefore, has been considered unsuitable for massive systemic administration due to post-treatment deaths from cytokine storms (23). However, if target specificity improves, it is expected that therapeutic effects can be achieved with reduced doses. The AdV vector also has the advantage of accommodating large genes and easily combining with a tissue-specific promoter/enhancer.

2.4. mRNA and DNA

mRNA is a new modality for gene transfer, which recently gained particular attention due to the success of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine. Compared to DNA, which requires delivery to the cell nucleus, mRNA is advantageous because proteins can be produced only by its introduction into the cytoplasm. This eliminates the need for delivery to the nucleus, resulting in high

expression, especially in non-dividing cells. Additionally, there is no risk of random integration into the genome. Whereas, mRNA is easily degraded in the body, resulting in a short duration of protein production, and is chemically unstable, leading to low storage stability. Thus, DDS for mRNA is crucial by twofolds: mRNA protection and target cell/tissue targeting. LNP has been used in nearly all cases for the mRNA vaccine (24). Since lipids are the main components of the cell membrane, LNPs not only promote the uptake of mRNA into cells, thereby exerting high transfection efficiency, but also act as adjuvants that induce an immune response. Other DDS (e.g., synthetic polymer-based nanocarriers) not using lipids have also been developed. In particular, they are expected to be applied to therapeutic mRNA drugs.

For providing cell/tissue targetability, there are various candidates, from small to large molecules, including approved chemical products or DDSs for nucleic acid delivery (25): For examples, the use of scFv and DARPins like viral vectors (26-28), modification of lipid components in LNPs to target the lung, spleen, or liver (29, 30), and nanocarriers screened by a high-throughput analysis of biodistribution (31).

For providing target specificity by regulating the gene expression from delivered DNA or mRNA, DNA can modulate the expression in the transcription level using tissue-specific promoters/enhancers as well as the control in the translation level via miRNAs existing in the cytoplasm. In contrast, although regulatable at the translation level, mRNA would have greater potentials in its efficiency and versatility for cell/tissue-specific targeting. Various methods have been reported for cell-selective translation control by the delivered mRNAs: (i) The use of cell-specifically miRNA activity (32-34); (ii) complementary strand formation between cell-specifically transcribed RNA and administered mRNA (35, 36); (iii) the use of interactions between administered mRNA and the proteins expressing in a cell-specific manner (37-39); and (iv) the use of cell-specific proteolytic mechanisms (40).

3. Development Trend of Noteworthy Cases

3.1. CAR-T

Numerous studies have reported generating CAR-T in the body through direct administration of gene therapy vectors. In nonclinical stages, active CAR-T have been successfully manufactured in the body (26, 41). With much experience in *ex vivo* gene therapy, most *in vivo* CAR-T research has been focused on LV-based CD19-CAR-T (42-47). Recently, nonclinical results from the pharmaceutical industry have been reported not only in small animals but also in primates (48, 49). Furthermore, nonclinical studies of *in vivo* CAR-T have also been performed using AAV vectors (47, 50), DNA (27), or mRNA (51, 52, 53). Of note, in addition to hematologic malignancies, which have been the primary indications to date, cardiac disease and other conditions have also been considered as therapeutic targets (53). (Table 1)

Table 1. *In vivo* CAR-T nonclinical studies (excerpted and modified from [26])

Platform	Target cell binding	Target cell receptors	References
LV vector	Antibody, DARPins	CD3, CD4, CD7, CD8	42 - 49
AAV vector	DARPins	CD4, CD8	47, 50

DNA/Nanocarrier mRNA/Nanocarrier	Antibody	CD3, CD8	27, 51, 52
mRNA/LNP	Antibody	CD5	53

3.2. Hematopoietic stem cell (HSC) gene therapy

HSCs are responsible for the lifelong production of all lineages of blood cells. If their genes can be repaired, a long-term therapeutic effect could be achieved with a single treatment for various genetic diseases. To date, successful trials in HSC gene therapy are based on *ex vivo* methods (54), but it requires resources similar to or greater than those for *ex vivo* CAR-T therapy.

Main delivery tools for *in vivo* HSC gene therapy include viral vectors and mRNA/LNP. Viral vectors are more reliable for efficient and stable gene delivery, compared with LNPs; however, viral vectors are more immunogenic due to virus-derived proteins. The development of gene delivery methods using AdV vectors is the most advanced. *In vivo* prime-editing treatment of CD46-targeted AdV vectors (Ad35, described in Section 2.3) with drug selection was reported in a mouse model of sickle cell disease (19). In addition, an AdV-vector gene transfer to HSCs has been reported at higher efficiency (20). However, AdV vectors are highly immunogenic and is generally expected to be administered once. The LV vector has not been extensively investigated *in vivo* (55), and there are limited data on its immunogenicity.

The non-viral delivery tools such as mRNA/LNP are relatively less immunogenic and able to be administered multiple times. HSC-specific antibodies (or scFv) are commonly used for targeted gene delivery with LNPs. *In vivo* genome editing of HSCs at the LNP-based gene delivery has been reported (56, 57).

3.3. Anti-malignant tumor drug (other than CAR-T)

Other than CAR-T, oncolytic virus (OV) is one of the drugs being developed as an *in vivo* gene therapy product for malignant tumors. AdV, herpes simplex virus, vaccinia virus, and reovirus are often used as the OV platforms (58). OV is divided into two types: (i) Attenuated viruses derived from spontaneously isolated ones, and (ii) genetically modified viruses using tumor-specific promoters, etc., so that they replicate only in tumors and do not grow in normal cells. In other words, OV has been designed from the beginning to ensure safety and efficacy, considering the possibility of infection to non-target cells to some extent. This aligns with the opinions from the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), and the following considerations have been issued such as “Oncolytic Viruses” (59; hereinafter referred to as “ICH Consideration: Oncolytic Viruses”) and “General Principles to Address the Risk of Inadvertent Germline Integration of Gene Therapy Vectors” (60; hereinafter referred to as “ICH Consideration: Risk of Germline Integration”). The major route of administration for OV is intratumoral, but intravenous administration also accounts for nearly a quarter of cases. AdV and vaccinia virus are primarily administered intratumorally, although protocols for intravenous administration are occasionally used. Reovirus and Newcastle disease virus are mainly used for intravenous injection (58). In the case of intravenous administration, the natural infection by parent viruses and the neutralizing antibodies induced by previous OV administration are problematic, in addition to the various physical barriers that need

to be overcome to reach the tumor (61).

Some of the modifications on the vector side that further enhance OV target specificity include changes in cell tropism associated with capsid modification, as described in Sections 2.2 to 2.3, and an increase in tumor-specific proliferative capacity by directed evolution (62). Meanwhile, administration methods to promote the delivery of systemically administered OV to tumor targets include normalization of blood vessel construction (63), adjustment of tumor perfusion pressure (64), and the use of physical devices such as ultrasound and magnetic fields, as well as the combination of carrier cells, etc. (65). The Committee considers that the development of OV, including these improvements, can be addressed for the time being with the existing guidelines, such as the aforementioned ICH considerations.

3.4. Regenerative medicine

Regenerative medicine has long attracted attention as one of the promising applications of gene therapy. Tissue regeneration is essentially a local phenomenon, and spatially regulated activity of the administered gene is essential. There are few reports using viral or non-viral gene vectors by systemic administration for regenerative medicine. Instead, local administration to target sites and tissues has been the main route. Local administration of viral vectors aiming at sustained release of cytokines and growth factors has been repeatedly investigated (66). However, even the local administration of viral vectors may induce immune response, which may provide adverse effects on tissue regeneration not only at the injection site but also at distant sites (67). Thus, the regenerative medicine using viral vectors is still challenging.

Whereas, mRNA is superior in safety because mRNA has the negligible risk of insertional mutagenesis in the genome. Currently, there are some trials for regenerative medicine by local mRNA administration. A most-progressed clinical trial is the myocardial injection of vascular endothelial growth factor (VEGF) mRNA for treating ischemic heart disease, where a significant enhancement of vascular regeneration was observed at the injection site (68). Preclinical stages include bone regeneration using mRNAs encoding bone morphogenetic protein (BMP-2) (69), and neuroprotective therapy for cerebral ischemic diseases using mRNA encoding brain-derived neurotrophic factor (BDNF) (70). Furthermore, because mRNA can express factors such as transcription factors that function inside the cells, mRNA is used for promoting tissue regeneration by controlling signals in the target cells, such as direct reprogramming (71). A preclinical study is ongoing in Japan for the treatment of osteoarthritis with an mRNA encoding a chondrogenic transcription factor (72). In the field of regenerative medicine, mRNAs are administered not by LNPs, which is commonly used for mRNA vaccines, but by other methods such as collagen- or polymer-based DDSs, or even by the naked mRNA form. This is likely because the immune response induced by LNPs at the injected site would impair tissue regeneration.

3.5. Genome editing (other than the above)

Genome editing tools broadly include two systems: One is a sequence-specific artificial endonuclease, such as zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN), where protein performs sequence recognition; the other is a clustered regularly interspaced short palindromic repeats

(CRISPR)/CRISPR-associated (Cas) endonuclease (CRISPR/Cas) system, where RNA performs sequence recognition. In the case of ZFNs and TALENs, DNA or mRNA encoding these proteins is delivered to each target tissue. To date, AAV vectors with appropriate tropism for their targets have often been used. However, there is an increasing trend towards delivering mRNA using LNPs with tissue specificity. For tools derived from CRISPR/Cas, the DNA-cutting protein (Cas) or the gene encoding Cas and a guide RNA (gRNA) that recruits Cas to the target DNA sequence are delivered as separate molecules. The gene size may exceed the packaging limit of AAV for Cas and Cas-derivatives, such as base editor and prime editor. Therefore, the genes need to be split into different AAV vectors, and then reconstructed in the transduced cells. When LNPs are used, Cas mRNA and gRNA are simultaneously encapsulated within them. In addition, attempts have been made to deliver the complex of Cas protein and gRNA, rather than AAV or mRNA, using lentivirus-based virus-like particles (73, 74).

As an example of clinical application, the results of a phase I study of *in vivo* genome editing therapy using LNPs with high liver tropism have been reported (75). This LNP has been approved as a DDS for oligonucleotide therapeutics (25). Although the LNP does not contain cell-specific ligands, it effectively bound to apolipoprotein E in the blood when administered intravenously. It was then taken up by the liver through endocytosis via low-density lipoprotein (LDL) receptors, which are highly expressed on the surface of hepatic parenchymal cells. The Cas mRNA and gRNA encapsulated in the LNP disrupted the transthyretin gene in the liver, effectively reducing amyloid deposition. Similarly, in an *in vivo* base editing approach targeting the liver, an interim report of a phase I study of proprotein convertase subtilisin kexin 9 (PCSK9) gene modification in patients with hypercholesterolemia showed dose-dependent decreases in LDL-cholesterol after the administration of LNPs containing mRNA encoding the adenine base editing tool and gRNA targeting the PCSK9 gene (76). For base editing of PCSK9, another clinical study has been initiated using LNPs with *N*-acetylgalactosamine on their surface to improve targeting, specifically incorporating them into hepatic parenchymal cells via the asialoglycoprotein receptor.

4. Points to Consider at the Start of Clinical Studies

4.1. Characterization and quality control

As a general quality principle, understanding the critical quality attributes (CQAs) of the gene therapy product to be developed is important for initiating a clinical study. To justify the evaluation of efficacy and safety in both nonclinical and clinical studies, characterization information should be provided to ensure the quality consistency throughout the products used in nonclinical and clinical studies, and the marketed gene therapy products. Furthermore, for *in vivo* gene therapy products requiring high target specificity, it is necessary to collect information and identify the CQAs related to target specificity (target-specific CQAs) from the early stages of development. Although quality control for general gene therapy products is described in the “Guideline on Ensuring Quality and Safety of Gene Therapy Products” (hereinafter referred to as “Guidelines for Gene Therapy Products”) (77), issues of quality control associated with achieving high target specificity, which are highlighted in this report, are discussed in this section.

With high target specificity, the products will be developed with the aim of reducing or optimizing the

dose required to achieve efficacy and minimizing safety hazards associated with dose reduction by delivering or introducing them to the objective cells or tissues. Developers should be able to explain the scientific mechanism of action for accomplishing high target specificity and the significance of this accomplishment. Because target specificity is directly linked to efficacy and safety, quality attributes associated with cell/tissue specificity are likely to be target-specific CQAs. As mentioned above, since the consistency of CQAs is considered to be critical for the evaluation of nonclinical and clinical studies, the control of target-specific CQAs should also be rigorously performed from the early phase of development, in addition to the quality control required for conventional gene therapy products.

For example, modifications that confer target specificity to an active substance (such as the binding of a target cell-specific antibody or a ligand binding to a target cell receptor) should be evaluated and controlled as target-specific CQAs. These include the rate of modification and the binding capacity to the target molecule with such modification. If LNP or a similar kind of nanoparticle contains active substances, the type and percentage of lipids comprising the LNP, the amount of antibodies bound to the surface of the LNP, and the rate of inclusion of active substances (including nucleic acids) in the particle may constitute target-specific CQAs. In addition, *in vitro* tests may be performed to confirm cell-type specific gene expression, inhibition of gene transfer to non-target cells, or the absence of unexpected gene mutation. These may also be critical characteristics related to safety.

Before initiating a clinical study, it is necessary to identify the quality attributes, including all known CQAs at that time, and to examine the acceptable range of the target-specific CQA in the quality control. In other words, it is necessary to expect the hazards caused by the reduction of target specificity (such as reduction of efficacy, effects of gene expression in non-target cells, germline integration, etc.), to evaluate the final risk, and to determine an acceptable range, considering factors such as the type of vector, route of administration, and expected administration dose. For evaluation, the results of nonclinical studies will help as reference data. The specification range of the final product for commercialization will be determined based on the results of clinical studies.

When model animals and/or other experimental means are available, it is desirable to confirm in nonclinical studies that the expected target specificity effectively in the body. However, the evaluation of target specificity may vary between species, and the assessment of effects in humans by nonclinical studies is often limited. In such cases, target specificity needs to be analyzed *in vitro* using human cells and tissues. For example, if specificity to a particular human organ is expected, it may be possible to evaluate the localization to the target organ or the absence in non-target organs using a cell panel that covers many cell types.

In addition, analytical methods for target specificity in quality characterization include: (i) Detection of vector transgenes by quantitative PCR/reverse transcription-quantitative PCR (qPCR/RT-qPCR), and (ii) detection of gene expression products from vectors by antigen-antibody reactions (e.g., immunostaining and ELISA). Furthermore, (iii) it may be possible to use a vector including the transgene of fluorescent proteins or similar markers to evaluate expression. Analysis by qPCR/RT-qPCR seems to have high sensitivity; however, this sensitivity may be reduced for modalities like mRNA, which has a short

intracellular half-life. Analysis with control probes such as cell-specific miRNAs described in Section 2.4 (32-34), cell-specific transcripts, and proteins expressed specifically in cells (37-39), may be helpful.

4.2. Nonclinical studies

When conducting clinical studies of *in vivo* gene therapy products with target specificity, it is important to perform the following nonclinical evaluations for both efficacy and safety, in addition to analyzing the quality data obtained thus far.

4.2.1. Biodistribution evaluation

Biodistribution studies for *in vivo* gene therapy products allow sponsors to evaluate distribution not only to target organs, tissues, and cells but also to others. Therefore, these studies provide valuable information for evaluating the efficacy and safety of *in vivo* gene therapy products with target specificity.

In the development of *in vivo* gene therapy products with target specificity, genome-integrating viral vectors such as RV or LV vectors, which have not been previously used in *in vivo* gene therapy, may also be employed. When systemic exposure to gene therapy products with genome-integrating ability is planned, the risk of unintended gene integration in germ cells and gene modification should be carefully evaluated in accordance with the ICH Consideration: Risk of Germline Integration (60). A similar risk assessment is required for the development of genome-editing products for systemic administration.

When planning a biodistribution study for an *in vivo* gene therapy product with target specificity, the Guidelines for Gene Therapy Products (77) and the "Nonclinical Biodistribution Considerations for Gene Therapy Products" (hereinafter referred to as "ICH-S12") (78) will serve as useful references, as with conventional *in vivo* gene therapy products. However, *in vivo* gene therapy products with target specificity may bind only to human target cells. In such cases, tissue cross-reactivity studies using human tissue panels or cells expressing human proteins may be used. If nonclinical pharmacology or safety studies are conducted using model animals, it may be useful to evaluate the biodistribution using a product with the homologous gene of the test animal species for interpreting the study results. These studies do not necessarily need to be conducted independently; efficacy and safety can be evaluated in nonclinical pharmacology and toxicity studies, respectively.

4.2.2. Nonclinical pharmacology (primary pharmacodynamics)

Nonclinical pharmacology studies of an *in vivo* gene therapy product with target specificity should be conducted to assess the appropriateness of conducting clinical studies of the product from the viewpoint of efficacy, with reference to the Guidelines for Gene Therapy Products (77). When selecting an animal species for *in vivo* nonclinical pharmacology studies, it is important that the animal has target specificity as similar to that in humans as possible, and that the expression product of the gene therapy product induces biological activity similar to that in humans, as in the nonclinical safety studies described below. If it is difficult to meet these conditions, it is recommended to select the animal species by prioritizing target specificity and to perform the study using a product with a homologous gene of the test animal species. Secondary

pharmacodynamics information (i.e., pharmacodynamic effects of a test substance not related to the desired therapeutic target) may also be useful in assessing the target specificity of an *in vivo* gene therapy product.

4.2.3. Nonclinical safety evaluation

Because it is difficult to expect complete target specificity even for *in vivo* gene therapy products with target specificity, it is necessary to evaluate not only target-specific expression but also the "toxicity attributable to the expression product" expressed in non-target cells/tissues. Furthermore, as with conventional *in vivo* gene therapy products, evaluation of "toxicity not attributable to the expression product" is also required.

4.2.3.1. Toxicity caused by expression products

For assessing toxicity caused by the expression products of *in vivo* gene therapy products, it is necessary to use at least one animal species expected to mimic the pharmacological action in humans, referring to the Guidelines for Gene Therapy Products (77). In addition, for *in vivo* gene therapy products with target specificity, it is desirable to use an animal model in which the transgene is expressed in the cells or tissues targeted in humans, and where the expression products exert biological activity. However, given the species differences between humans and animals regarding target specificity, selecting an appropriate animal species may be difficult. In such a case, based on various information including the biological properties of the target cell or expression product, it is recommended to evaluate the toxicity resulting from the target-specific expression product, by using a method that estimates the risk to humans (a weight-of-evidence approach). After implementing such an approach, if additional nonclinical safety studies are needed to estimate the human risk, studies using homologous genes from the test animal species or genetically modified animals (e.g., transgenic animals) may be performed. However, the need for such studies should be agreed upon through consultation with the appropriate regulatory authorities.

4.2.3.2. Toxicity not caused by expression product

The toxicological evaluation of an *in vivo* gene therapy product, which has specific targeting and whose toxicity is not caused by the expression product, depends on whether the product includes a chemical with unknown safety in humans. When a chemical substance (e.g., a novel lipid) whose safety in humans has not been confirmed is contained in the product (e.g., LNP), it is necessary to consider nonclinical safety studies (e.g., general toxicity study, genotoxicity study, reproductive and developmental toxicity study, using two animal species) in accordance with the "Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals" (hereinafter referred to as "ICH-M3") (79) in order to confirm the safety of the chemical substance. However, toxicity not caused by the expression product of a gene therapy product without such an ingredient may be evaluated along with toxicity attributable to the expression product in a general toxicity study using any one animal species.

4.2.3.3. Gene integration evaluation

In the development of conventional *in vivo* gene therapy products, intravenous administration (associated with systemic exposure) of an integrating viral vector to humans has been avoided because of concerns about the risks of cancer development and next-generation effects due to integration in the genome. However, in the future, systemic administration of an integrating viral vector may be considered, provided that target specificity is enhanced to reduce unintended effects on non-target cells/tissues (e.g., germ cells) as much as possible. Therefore, in the development of such products, it is necessary to perform a more detailed evaluation by referring to ICH Consideration: Risk of Germline Integration (60), particularly to assess the effects on germ cells, in addition to the Guidelines for Gene Therapy Products (77) and the "White Paper for Quality and Safety for Gene Therapy Products Using Gene Editing Technology" (80). This evaluation should be conducted in the same manner as the concept applied to conventional *in vivo* gene therapy products and *in vivo* genome editing products. In this regard, it is important to estimate the risk of germline integration, based on biodistribution evaluation or estimated blood exposure in humans. Accordingly, if there is a concern about the risk of integration in human germ cells, the risk/benefit should be carefully evaluated. If a clinical study is conducted, measures such as management procedures, informed consent, and appropriate precautions should be taken to reduce the risk to patients.

4.3. Matters to be considered when planning a clinical study

4.3.1. Vector toxicity

A considerable amount of clinical experience with AAV vectors has been accumulated as gene therapy vectors administered systemically, and serious adverse events (SAEs) including deaths from liver, kidney, heart, and lung disorders have been reported (81-84). Liver disorders are the most common SAEs, with 4 deaths reported in patients with SMA and 4 deaths in patients with X-linked myotubular myopathy (81-84). Hepatotoxicity may be caused by multiple factors, including liver stress and immune response due to a high vector load, because the liver is the primary sink for AAV vectors administered systemically (84, 85). When the dose is high, preventive measures against hepatotoxicity, such as corticosteroid administration, will be necessary (86). Thrombotic microangiopathy (TMA) was also observed as a SAE, resulting in the death of a patient with SMA (87), and similar events have also been reported in various diseases such as Duchenne muscular dystrophy (DMD), Fabry disease, Danon disease, and methylmalonic acidemia. TMA is a disease that causes microangiopathic hemolytic anemia, thrombocytopenia, renal disorder, and microthrombosis due to endothelial cell damage. The activation of the complement system is a cause of its aggravation. For this reason, anti-complement therapy (e.g., C5 inhibitors such as eculizumab and ravulizumab) and hemodialysis are effective treatment strategies. Cardiotoxicity has been observed in patients with DMD who received mini/micro-dystrophin gene therapy, of which cause has been suggested to be a cellular immune response generated by recognition of the therapeutic gene product as non-self (88). The cytokine storm observed after high-dose systemic administration of AdV vectors may also occur with other viral vectors and should be noted (23). While no serious neurotoxicity in humans has been known, there have been reports of inflammatory mononuclear cell infiltration and neuronal necrosis/loss in the spinal dorsal root ganglia after

intrathecal administration of AAV9 vectors in nonclinical studies with cynomolgus monkeys (89). Therefore, it is necessary to consider that risks may occur according to dose increase of vectors that can easily pass through the blood-brain barrier.

There is little human experience with systemic administration of RV/LV vectors, which have been mainly used for *ex vivo* gene therapy. If these vectors are used for *in viv*o gene therapy, a careful approach should be taken, including dose adjustment and post-treatment monitoring.

Meanwhile, mRNA/LNP have been administered intramuscularly in billions of doses cumulatively worldwide as COVID-19 vaccines. The major adverse reactions were post-vaccination inflammatory reactions (such as pain/swelling at the injection site, fever/chills, fatigue, muscle pain/joint pain, nausea/vomiting, and diarrhea) (90), probably mainly due to reactions to the lipid component of LNP (91). Less frequent but potentially serious events include myocarditis/pericarditis (92) and anaphylactic reactions. Clinical studies of systemic administration of mRNA/LNP have been conducted for genome editing and hereditary refractory diseases, providing valuable information (75, 76, 93). Intravenous drip infusion of LNP is used as a method of administering oligonucleotide therapeutics. Significant adverse reactions include infusion-related reactions and atrioventricular block (94). Other adverse reactions may include general and gastrointestinal disorders that are observed after administration of the mRNA/LNP vaccine. Hepatotoxicity and pulmonary disorders were also reported in nonclinical studies, and thus attention should be paid to these events in clinical studies (95, 96). As some ingredients overlap with those of LNP, the “Guideline for Development of Liposome Drug Products” (97) has been issued. This document can be used as a reference along with points to consider in quality and nonclinical studies.

4.3.2. Risk of malignant transformation

The risk of malignant transformation by genomic insertion of the vector (insertional carcinogenesis) has been widely recognized in vectors derived from RV/LV, which are naturally integrating, and a significant attention revisited. Recently, the United States Food and Drug Administration (FDA) has reexamined the incidence of secondary malignant tumors following CAR-T therapy (98). Regarding CAR-T treatment, the incidence of secondary cancer is fairly low, and the nature of the target disease and preceding treatments may contribute to it. Despite this, a warning about the risk of developing T-cell malignancy is again required to be included in the package insert.

AAV vectors are essentially non-integrating, but the possibility of malignant transformation caused by genome insertion of AAV vectors has been suggested since hepatocellular carcinoma developed following the administration of AAV vectors to neonatal mice (99-103). Genomic insertion of AAV has been found in humans, but no malignancy has been reported to date (104, 105). However, a study has reported that if genomic DNA is damaged by double-strand breaks during genome editing, AAV vectors are frequently integrated at the cleavage sites (106). There is a concern that genome editing using AAV vectors could increase the risk of insertional mutagenesis. Although long-term follow-up has not been required for AAV to date, long-term follow-up comparable to that of integrating vectors is required for a non-integrating vector when administered *in vivo* for treatments with persistent effects, such as genome editing.

4.3.3. Risk of germline integration

Even in *in vivo* gene therapies using vectors with high targeting specificity, nonclinical biodistribution studies should be conducted, in principle, to evaluate the distribution not only to the target cells or tissues but also to unintended sites. If the results show distribution to the reproductive organs, the risk of germline integration must be evaluated with reference to the ICH Consideration: Risk of Germline Integration (60). Additionally, measures such as using physical contraception should be taken, depending on the risk, to prevent genetic modifications from spreading to offspring.

4.3.4. Immunogenicity

Measures are required to prevent or mitigate SAEs due to an acquired immune response to capsid, genome, or transgene expression products (83, 104, 107), in addition to the innate immune response described in Section 4.3.1. Vector toxicity. Prior to the initial dosing, patient background factors, such as antibodies against the capsid, T cell response, human leukocyte antigen (HLA) type, and screening for complement-related gene mutations, should be taken into consideration. Post-treatment options include anti-CD20 monoclonal antibody targeting acquired immune B cells/antibodies and mTOR inhibitors targeting T cells, in addition to systemic corticosteroid therapy, which is standard in current AAV gene therapy protocols.

4.3.5. Potential for non-target cell modifications and safety evaluation if modified

As an example of transduction/modification of non-target cells and the resulting expression of the therapeutic gene, a case of CD19-CAR transduction in B cell lineage tumor has been reported (108). With reference to nonclinical biodistribution evaluations, in principle, assessments should determine which non-target cells/tissues can be modified and whether these cells/tissues express the therapeutic gene. Furthermore, potential adverse reactions should be considered according to the characteristics of the product.

4.3.6. Excessive pharmacological effects

The typical adverse events due to the excessive pharmacological effects of the intended product include cytokine release syndrome (CRS) and central nervous system toxicity with CAR-T administration. CRS is a systemic reaction caused by the excessive inflammatory cytokines produced by CAR-T, macrophages, and other inflammatory cells upon recognition of B-cell tumors, which are the targets of CAR (109, 110). CRS can range from mild cases with fever and chills to severe cases leading to circulatory disorders, capillary leak syndrome, and multiple organ failure. The management, including drug therapy targeting upstream inflammatory cytokines such as IL-6 and IL-1, is recommended and generally effective. Central nervous system toxicity secondary to CAR-T treatment is called immune effector cell-associated neurotoxicity syndrome (ICANS) and includes cognitive dysfunction, attention disorder, delirium, convulsion, and brain edema (110-112). The early onset of symptoms after CAR-T treatment is considered to be associated with CRS, and many of them improve within 3 weeks with appropriate treatment. However, some events occur independently from CRS. Although the mechanism of onset is still unknown, the breakdown of the blood-

brain barrier and vascular endothelial damage have been suggested to be the cause in serious cases. Therefore, attention should be paid to updates on the safety information of CAR-T therapy.

5. Summary

Toward the spread and implementation of gene therapy, *in vivo* gene therapy has become more accurate, leading to a shift from *ex vivo* gene therapy to *in vivo* gene therapy. The greatest key to this increased accuracy is improved cell/tissue specificity, which continues to be refined for each vector. Quality control strategies to support and ensure improved target specificity are essential for the development of these vectors. It is appropriate to conduct nonclinical biodistribution studies based on the ICH-S12 guideline for the time being. Toxicity evaluations should be divided into two aspects: One due to the overexpression of therapeutic gene products, and the toxicity due to other than that. When starting clinical studies, dose selection and measures to prevent AEs should be determined by predicting the systemic administration toxicity for each vector, with reference to past clinical experience. The risks associated with the distribution of the vector to non-target cells/tissues, the immune response, and the expression in target cells/tissues need to be addressed separately, according to the characteristics of the vector and inserted genes.

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