Provisional Translation*

Report on Therapeutic Products Based on Extracellular Vesicles (EVs) Including Exosomes

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Subcommittee on Therapeutic Products Based on Extracellular Vesicles (EVs) Including Exosomes

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Table of contents

1. Introduction....3
   1.1 What are extracellular vesicles (EVs)?....3
   1.2 Composition of EVs....3
   1.3 Development of therapeutic products using EVs....4
   1.4 Problems in development....5
2. Manufacturing method development and quality characterization....6
   2.1 Establishment and characterization of cell banks....6
   2.2 Manufacturing methods of EV preparations....10
   2.3 EV-specific quality characterization....12
   2.4 Safety evaluation of infectious agents such as viruses contaminated in EVs – Measures taken by overviewing manufacturing processes....14
3. Nonclinical studies....17
   3.1 Pharmacokinetics....17
   3.2 Pharmacological studies....19
   3.3 Nonclinical safety studies....20
4. Clinical development....23
   4.1 PK/PD and efficacy assessment....23
   4.2 Undesirable immune responses such as allergy and rejection....24
   4.3 First-in-human study design....25

1. Introduction

1.1 What are extracellular vesicles (EVs)?

Cells contain and secrete functional molecules such as proteins and nucleic acids in vesicles with lipid bilayer membranes (1). In the living body, secreted vesicles are transported through body fluids to surrounding cells and cells in distant organs, and they deliver functional molecules to recipient cells. These extracellular vesicles (EVs) are an intrinsic delivery system of functional molecules in our body and have been shown to be involved in various life phenomena and diseases as intercellular communication tools (2). They have also been reported to be used not only as communication tools but also for discharging unwanted molecules from cells (3). Such EVs can be classified into three types depending on their formation and secretion route or size: Exosomes, which are derived from endosomes and whose size is approximately 100 nm; microvesicles, micro-sized vesicles derived from cell membrane; and apoptotic bodies, derived from dead cell membranes. However, confusion often occurred for the following reasons: The definitions of these EVs were vague, it was difficult to identify the formation and secretion pathway unless the moments of release are captured by live imaging technology, and the names were not generally standardized. Under such circumstances, the International Society for EVs (ISEV) was established in 2011, and ISEV has been defining EVs, standardizing the names, and establishing guidelines, etc. for research. ISEV recommends the use of " EVs " as the generic name of the above-mentioned EVs (4). In addition, in 2018, minimal information for studies of EVs es 2018 (MISEV2018) was published as a guideline in the Journal of EVs, published by ISEV, in which it is recommended to call medium/large EVs (m/l EVs) and small EVs (sEVs) according to the size and density of EVs (1). Because the size of sEVs is defined as ≤ 200 nm, exosomes are categorized as sEVs. However, because an individual vesicle cannot be always addressed by the size or density and accurate measurement of their size is difficult, the term, EVs, is used in this report to collectively refer to EVs. In this regard, ISEV is currently (September 1, 2022) preparing to issue MISEV2022, a revised version of MISEV2018, and thus the above matters may be changed.

1.2 Composition of EVs

EVs are a complex of molecules, and their composition include various proteins as well as nucleic acids such as microRNA (miRNA) and mRNA. Cells encapsulate these molecules in EVs and deliver functional molecules to other cells, and the recipient cells show phenotype changes, and gene expression changes in response to them. EVs exert various actions, and the compositions of EVs vary depending on the cell type and even in the same cell, these may vary depending on the surrounding environment. even in the same cell (5). In addition, EVs contain molecules called EV markers, such as CD9, CD63, CD81, annexins and heat shock proteins. Such EV markers may be used for identification or quality evaluation of EVs in some cases, but they may not always exist in all EVs (6). As described above, EVs are a complex of molecules, and thus an individual EV may not always hold the same components. Furthermore, it has also been reported that constituent molecular groups differ depending on the subtypes of EVs (7). Thus, although EVs are collectively categorized, the component molecules are heterogeneous and it is difficult to define the characteristics of EVs using specific marker molecules. Therefore, EVs are not a term for specific molecules but generally refer to cell-derived complexes consisting of a variety of molecules in lipid membranes, with sizes of several tens of nm to several μm in size.

1.3 Development of therapeutic products using EVs

Therapeutic products using EVs described here are preparations of cell-derived EVs administered into the body. They are roughly divided into two types. One includes preparations that utilize the therapeutic effect of EVs themselves, and they are used without modification after isolation from cell culture supernatant or other sources (natural EVs). The other employs engineered EVs, which include EV preparations surface-decorated with peptides or some molecules after purification and collection of EVs from specific cell line culture supernatant, body fluid, or fruit juice (engineered EVs). Similarly, EVs can be functionalized by genetic modification of the donor cells of EVs. These engineered EVs encapsulating specific therapeutic drugs can also be used as a carrier of a drug delivery system (DDS) The characteristics and examples of practical research are described below for each of these two types of forms.

1.3.1 Characteristics of natural EVs and examples of therapeutic products

EVs exert various effects on cells, tissues, and organs. Among these effects, such as, anti-inflammatory effect, immunoregulatory effect, and tissue repair effect, are expected to be used as therapeutic drugs for specific diseases. In particular, attention has been focused on EVs derived from mesenchymal stem cells (MSC). MSCs are known to have an ability to support the repair of tissues injured in a variety of ways and are now expected to be a cell source in regenerative medicine (8). Recent study results indicate that a large part of the therapeutic effect of MSCs is due to secretions of MSCs (secretomes), although in some cases the cells themselves differentiate into the tissue cells to repair the tissue. Secretome includes various EVs, and in fact, therapeutic effects in the use of EVs of MSCs have been frequently reported (9, 10). In recent years, therapeutic effects on humans have also been confirmed. Austrian and German research groups conducted a clinical study in which EVs of MSCs derived from umbilical cord were applied to the inner ear to attenuate inflammation as an adverse reaction due to cochlear implantation (11). The results have shown improved speech perception. Furthermore, acoustic trauma causing hearing loss has also been investigated in an experiment in mice, and it has been clarified that application (administration) of EVs of MSCs derived from umbilical cord to the inner ear improved hearing loss and protected hair cells from noise-induced trauma, demonstrating the therapeutic effects in EVs of MSC derived from umbilical cord (12).

Moreover, the involvement of EVs in immune control has been revealed in early EV studies, and it has been reported that EVs derived from B cells and dendritic cells (DC) were involved in antigen presentation and activation of CD4 positive T cells (13-15). Based on these reports, a method in which EVs are used in immunotherapy to activate immunity as a vaccine and prophylactic use were proposed. In 2002, a phase I study in melanoma patients in France (16) and a clinical study in patients with non-small cell lung cancer (NSCLC) in the US (17) were conducted. The studies involved administration of collected EVs subcutaneously, etc. after differentiation of DCs from patient-derived monocytes and antigen presentation with tumor peptides. In addition, a phase II study of EVs derived from DCs that have interferon-γ on the surface in patients with advanced NSCLC was conducted in France between 2012 and 2014 (18). Treatment outcomes appeared not as good as expected, but in some patients, natural killer cells tended to be activated and progression-free survival was prolonged. Currently, clinical studies by other groups are also being conducted, and the development is proceeding toward practical use.

1.3.2 Characteristics of engineered EVs and examples of therapeutic products

In contrast to natural EVs, engineered EVs refer to EVs whose contents are changed by genetic modification or transfection to the donor cells, EVs for which a peptide or antibody is conjugated to

the surface after secretion from the cells, or EVs in which a specific drug is encapsulated by application of physical force or other methods (19, 20). The primary purpose of use of engineered EVs is related to the use in DDS because EVs can deliver functional molecules to remote organs and specific tissues and cells. Nano-sized EVs such as exosomes are less immunogenic and less mutagenic than viral carriers and are biocompatible. Furthermore, nucleic acids in EVs have been reported to be resistant to degrading enzymes, such as nucleolytic enzymes in blood. Thus, a possibility as a stable DDS carrier in circulation has been increasingly expected. In addition, it has been suggested that EVs may have a specific tropism towards target cells, and the construction of a delivery system with high specificity in addition to stability and low toxicity is expected (21). Therefore, engineered EVs, encapsulating a therapeutic drug or equipping preferable functional features to EVs with the above characteristics, have been prepared and their therapeutic effects have been investigated. In 2011, researchers from Oxford University, U.K., showed the potential of EVs as a DDS carrier. In this report, DCs were transfected with an expression vector for a rabies virus glycoprotein-derived peptides (RVG peptides), and then EVs were isolated and loaded with siRNA using the electroporation method (22). This delivery system employs the RVG peptide for specific targeting of nerve cells and includes siRNA as a pharmaceutical drug. Starting with this report, research reports on the application of EVs to DDS have been increasing. Also from Japan, Tokyo Medical University reported in 2013 on the preparation of EVs displaying GE11 peptide, an artificial ligand of EGFR, loaded on the membrane, targeting tumors expressing EGFR (23). In addition, there is a report on the therapeutic application of EVs using engineered EVs, where a tumor-suppressor miRNA let-7a was transfected into the donor cells, and EVs containing a large quantity of let-7a were purified. As in the above examples, engineered EVs or DDS preparations are currently obtained by the genetic modification or transfection to the donor cells or by the loading of specific molecules or drugs after the purification of EVs (these two methods are combined in some cases). However, general approaches for practical application have yet to be established, with many of the problems being pointed out.

1.4 Problems in development

There have been many papers and reports from clinical studies on the possibility of EV therapeutic preparations. However, there has been scarce knowledge based on regulatory science for actual manufacturing of products (therapeutic drugs), and various problems have been pointed out. The problems and check items are listed below.

1.4.1 Selection of original materials

EVs are biologically active substances derived from cells. However, EVs and they are not unique or homogeneous but contain different molecules depending on the donor cells and cell conditions. Thus, in EV therapeutic preparations, quality control of the cells, producing EVs, is important from the perspectives of quality control of preparations and obtaining EVs with the intended activity. Establishment of cell culture methods and quality control of culture media are also required. In addition, it will be necessary to decide how to establish rules for the use of genetically modified cells. There are studies using not only cultured cells but also, for example, body fluid- and milk-derived EVs as well as vesicles obtained from fruit juice as the source of EVs for DDS (21), and it is necessary to examine how to deal with cases without culture such as milk EVs and fruit juice EVs. However, in this review, the main focus is human cell-derived EVs.

1.4.2 Safety

There are two safety issues. The first is the safety of EVs themselves. The second is related to contamination with, for example, mycoplasma, viruses, endotoxins, and medium components. For the first issue, EVs are involved in various physiological phenomena and diseases, and there are not only EVs that exhibit beneficial efficacy showing the therapeutic effect. For example, EVs derived from cancer cells are known to promote cancer malignancy (24) and may cause unexpected adverse reactions in some cases. Therefore, sufficient consideration is required for development and manufacturing. The second issue, which is also related to Chapter 2, includes adverse reactions, etc. caused by ingredients other than EVs. Because adverse reactions caused by contaminants such as mycoplasma, viruses, endotoxins, and medium components can be substantially prevented by quality control, it is important to establish appropriate control methods.

1.4.3 EV purification methods

There are many EV purification methods while the best applicable methods have not been established at this time, which has been an issue. In many reports and studies, ultracentrifugation methods have been used. However, in practical application, the use of ultracentrifugation is considered difficult because multiple samples and large quantities of samples cannot be processed with this method. Moreover, a large quantity of impurities other than EVs remain with some purification methods. Thus, when considering which method should be employed for purification, it is important to evaluate the method based on the purity of EVs so that adverse reactions are also reduced. Furthermore, if there is a difference in the active ingredients depending on the size of EVs or if no active component is contained in EVs not of a specific size, such EVs may also be regarded as impurities, and thus determination of the size may also be important. Based on the above, methods such as those that purify only EVs containing active ingredients are more preferable.

1.4.4 How to define the active ingredient and dose

As EVs are a complex of molecules, not all the molecules are active ingredient. It is desirable to list the components of the preparation, but it is unrealistic to list all components for every lot because of the heterogeneity. Therefore, it is desirable that the active ingredients and the mode of action have been clarified for the formulation, and it is further desirable to determine at least the amounts of these active ingredients. For this purpose, it is useful to establish and use *in vitro* evaluation systems for their efficacy. However, it is not easy to elucidate the mode of action in some cases. In such cases, it is necessary to conduct multifaceted evaluation using multiple *in vitro* evaluation systems, or secure the efficacy using *in vivo* evaluation systems as necessary. In addition, when administered to the body, the unit of EVs to be administered should be clarified. For example, the amount of EV protein, the number of EV particles, or the amount of active ingredient may be considered. Additionally, it is decided to clarify the conditions, such as which reagents and instruments were used to determine the protein concentration and particle count, and to fix the measurement conditions.

2. Manufacturing method development and quality characterization

2.1 Establishment and characterization of cell banks

In order to ensure the quality of EV preparations, isolation of cells for manufacturing or establishment of cell lines and their control is necessary, and it is important to maintain various characteristics that may affect the product quality in an appropriate range throughout the period of manufacturing culture. As shown in Table 1, human autologous cells, human allogeneic cells such as MSCs, iPS/ES cell-derived cells, and established cell lines are used for manufacturing of EV

preparations. Except when autologous cells are used, a cell bank should be established according to the ICH Q5D guideline (25) or other appropriate documents and qualified before use in manufacturing.

Table 1

Cell source		Establishment of cell bank for manufacturing	Establishment of cell bank from a single clone	Cell homogeneity*
Primary cell	Autologous	× (impossible – unnecessary)	× (impossible)	×
	Allogeneic	○	△ (difficult)	△
iPS/ES cell-derived cells		○	○	○
Established cell line		○	○	○

*When relative evaluation is performed based on an established cell line that has already been widely used in the manufacture of drugs

In addition to Table 1, genetically modified cells prepared by transfer of a specific gene, etc. may be used. Characteristics of each cell source, points to consider for establishment of cell lines for manufacturing, and items recommended for establishment, evaluation, and control of cell banks are shown below. For these items, the methods for establishment and control of cell banks used for biopharmaceuticals and cell therapy products can be referenced.

Characteristics of each cell source and points to consider for establishment of cell lines for manufacturing

To ensure consistency of cell characteristics throughout the manufacturing culture period, a homogeneous population of cells from cloned cells should be obtained, where possible, so that a cell line for manufacturing is established. If it is difficult to obtain a homogeneous cell population, it is desirable to identify cells producing EVs that are used as an active ingredient in a heterogeneous cell population to the extent possible, but the method has not been fully established. The history of steps for the isolation of cells and the establishment of cell lines used for the establishment of cell banks should be clearly recorded.

Human allogeneic cells:

It is difficult to obtain a homogenous cell population, and multiple types of cells may be included. Virus safety is an issue because cell banks are established for each donor. In addition, when a cell bank is renewed, assurance of the comparability of the quality of EVs as the final product before and after the renewal will be an issue.

iPS/ES cell-derived cells:

Cloned cells can be used to establish cell lines for manufacturing; however, the phenotype is not always stable, and thus established cell lines and cell banks derived from these cell lines are not necessarily homogeneous cell populations. The risk of tumorigenicity due to contamination of EV products with cell-derived DNA or EVs derived from tumorigenic cells that may exist as impurities in the cell bank may need to be considered.

Established cell lines:
Large scale manufacturing is possible, and the method established in biopharmaceuticals can be referenced for manufacturing method development. Because cell lines for manufacturing can be established from cloned cells, it is expected that cell banks will be established using homogeneous cell populations. However, it should be noted that there is no information on the stability of the phenotype during long-term repeated subculturing when a new cell line is established. The risk of tumorigenicity due to contamination of EV products with cell-derived DNA or inclusion of EVs derived from tumorigenic cells included as impurities in the cell bank may need to be considered.

For genetically modified cells, analysis of the gene expression construct and control of the cell bank should be performed with reference to the ICH Q5B guideline (26) or other appropriate documents in addition to the above points to consider.

Establishment of cell banks

If human allogeneic cells are used, a donor cell bank (DCB) shall be established using cells prepared from raw materials collected from a qualified donor. For iPS/ES cell-derived cells, established cell lines, and genetically modified cells, a master cell bank (MCB) shall be constructed from established cells, and a working cell bank (WCB) shall be constructed using a part of MCB. The routine manufacturing shall be performed using a WCB. If the WCB needs to be replenished, the cells from the MCB shall be prepared again for renewal of the WCB.

The established cell banks should be qualified through characterization, purity, and storage stability tests as shown in Table 2. In addition to these, evaluation of the gene expression construct is required when genetically modified cells are used. Furthermore, to evaluate the stability throughout the manufacturing culture period, the same tests shall be conducted also for the cells at the limit of *in vitro* cell age (LIVCA) for manufacturing of EVs.

Table 2

	Characterization test	Purity test	Storage stability test	Gene expression construct*
DCB	○	○	△	-
MCB	○	○	○	○
WCB	-	-	○	-
LIVCA	○	○	-	○

*For genetically modified cells

Characterization test of cell banks

In the characterization test, the characteristics of cells and the characteristics of EVs produced shall be evaluated. Regarding the characteristics of cells, to confirm that the cells are the intended ones, phenotypes such as genotype, isozyme, and expression of cell surface marker molecules shall be analyzed. Regarding the characteristics of EVs produced, analysis shall be performed on items appropriate for evaluation of cells for manufacturing among the quality characteristics whose variations may affect the efficacy/safety (i.e., critical quality attributes) with reference to "EV-specific quality characterization" section of this report to confirm that EVs with intended characteristics are produced. For engineered EVs, confirm that the intended molecule is included in the EVs after modification.

Footnote: Because MSCs are known to have different characteristics depending on the tissue of origin, necessary characterization, including the confirmation of appropriate cell surface markers, should be performed.

Purity test of cell banks (evaluation of contaminants such as viruses)

Because EV preparations have similar physicochemical properties to those of viruses such as the particle size and charge, removal and inactivation of viruses in the purification process are assumed to be difficult. Thus, for cells used for manufacturing, sufficient virus safety analysis should be performed with reference to the ICH Q5A guideline and various guidelines of the Ministry of Health, Labour and Welfare regarding the quality and safety of Cell therapy products (27). Cells in which a type of endogenous virus known to be an obvious hazard or such virus-like particles are detected by tests such as a retrovirus test and *in vitro* test should not be used for the manufacturing of EV preparations. It is also necessary to confirm that contamination with microorganisms such as fungi and mycoplasma does not exist.

(For prevention of contamination with adventitious viruses, etc. during the manufacturing process and removal/inactivation of viruses during the purification process, see Section 2.4 of this report.)

Analysis of gene expression construct

Analysis of the gene expression construct is required when genetically modified cells are used. The nucleic acid sequence of the vector used for gene transfer shall be determined with reference to the ICH Q5B guideline or other appropriate documents, and the entire nucleic acid sequence of the target gene introduced in the established cell and the copy number of transferred genes shall be analyzed. It should be confirmed that there is no insertion or deletion in the transferred target genes.

Analysis of cells at the limit of *in vitro* cell age (LIVCA)

In addition to the analysis of the MCB, WCB, or DCB, main characteristics of the cells at an *in vitro* cell age reached at the end of the longest culture period (or longer) for EV manufacturing shall be determined focusing on the characteristics of the EVs produced in characterization to confirm that the required cell characteristics are maintained throughout the manufacturing culture period. When genetically modified cells are used, it is necessary to confirm the sequence and copy number of the transferred genes, etc. Testing for endogenous and adventitious viruses should also be conducted.

Storage stability test of cell banks

Because cell banks are stored frozen, stability should be checked periodically. The endpoints of the storage stability test should follow those of the characterization tests conducted at the establishment of cell banks.

Renewal of cell banks

The MCB and WCB are permanently stored throughout the product life cycle, but they need to be renewed depending on the number of vials used for manufacturing. If renewal is expected, the method and criteria for renewal should be specified in advance. Because the DCB is repetitively prepared from a new donor, it should be prepared in accordance with the predetermined preparation method of the DCB and the conformity to the criteria should be confirmed. In all cases, the criteria for renewal should

be set based on the results of characterization and purity tests performed at the establishment of the cell bank. When a cell bank is renewed, evaluation of virus safety is essential.

2.2 Manufacturing methods of EV preparations

Basically, cell culture methods for the manufacturing of EV preparations shall use cell sources as stated in Section 2.1. Because the components of EVs secreted may change depending on the culture conditions as well, it is important to use a consistent culture method and prevent contamination with factors from external environments such as viruses. It is a prerequisite for EV therapeutic drugs that EVs themselves that are secreted from cells under such conditions exert the intended pharmacological action. This means that, for preparations made from the culture supernatant without the EV purification step, substances other than EVs, for example, cytokines not derived from EVs, may also be the pharmacologically-active body, but such preparations are not categorized as EV preparations. Thus, it is desirable to purify EVs only if it is possible, but it is difficult to completely separate EVs from other substances with the current technology (28). On the basis of this matter, points to consider in the process of purification/recovery and formulation of EVs as the active ingredient from culture medium are shown below.

2.2.1 Culture methods

When EVs are used as a therapeutic product, the quality characteristics of the EVs produced are considered to be affected by the conditions and status of the cultured cells and the culture conditions. Thus, sufficient characterization of the cells, establishment of the manufacturing process to produce EVs with consistency, and process control in manufacturing are very important. Furthermore, in consideration of the above, precautions specific to EVs are described below.

Because of the characteristics of EVs, secretion of EVs from cells are affected by the medium components, culture conditions, and the conditions of cells themselves which are changed because of these (5), and thus process regulate to control the quality characteristics required for the target EVs is also important. For example, apoptotic vesicles are known as a type of EVs, secreted from apoptotic cells, and have different functions and roles from other EVs (exosomes and microvesicles) (2); thus, they may become impurities in EV preparations. Therefore, it may be useful to monitor the rate of apoptotic cells in culture, etc. Moreover, as described above, it is assumed that the components of EVs may vary depending on the conditions and characteristics of the cells. It is important to control the composition of culture media containing additives such as fetal bovine serum (FBS) and cytokines, culture temperature, oxygen concentration, and CO_2 concentration. In particular, evaluation of the safety of raw materials such as factors added to culture media and their residual in the final product is required. When raw materials of biological origin such as FBS containing animal-derived components are used, evaluation including the risk of infectious factors is required. It is also necessary to evaluate whether these components are sufficiently removed/reduced in the purification process. In addition, because cell density also affects the amount of EVs secreted and components, it is important to evaluate the equivalence before and after the change in manufacturing scale-up, etc. Thus, it is recommended to establish several checkpoints from culture to EV purification. This means that it is advisable to confirm before purification whether certain criteria are satisfied, such as the conditions of cells after seeding (morphology, apoptotic cell rate, and survival rate), amount of EVs present in the culture medium before EV purification, and the amount of active ingredient contained in the EVs. In addition, the details of the points to consider concerning inclusion of impurities from the external environment, especially infectious factors such as viruses, are described in Section 2.4.

2.2.2 EV purification methods

Although various EV purification methods have been proposed, it is necessary to develop methods in consideration from the purification process at the pilot scale in the early stage of development to the commercial manufacturing after approval. At the early stage of development, the characteristics of the active ingredient may not be fully understood, and the purification method may not have been established. However, it is desirable to have the purification method established before producing lots to be used in nonclinical studies. This means that, even if the active component as EVs cannot be sufficiently isolated, it is required to clarify the ingredient (active ingredient) necessary for its biological activity (efficacy) and establish a purification method of EVs containing the active ingredient. In addition, it is necessary to establish purification processes that can reduce inclusion of unintended EVs and culture medium components. Furthermore, because commercial preparations are expected to be administered to a large number of patients, it is necessary to produce them at a scale applicable to commercial preparations. Thus, it is desirable to use column chromatography or other methods that can be scaled up as the culture scale is increased. For example, purification of EVs by using the size exclusion chromatography method, which sieves molecules on the basis of size, and the anion exchange chromatography method, which separates molecules on the basis of physicochemical properties such as electric charge, and the separation results have already been reported (29, 30). However, it is not necessary to limit the method to column chromatography, and the purification method shall be selected according to the characteristics of EVs with the intended biological activity. Examples of purification methods using individual characteristics and points to consider are described below. In addition, in any purification method, the application of ultra-concentration, etc. should be considered for bulk harvest recovered at the end of the culture process, as necessary. Furthermore, in any purification step, it is necessary to establish a manufacturing method with indices such as removal of process-derived impurities (e.g., ligands and column carriers derived from culture process or used in purification process) and unintended related substances, and in some cases, it may also be considered that the indices shall be the extent of the removal of impurities by comparing before and after purification.

Purification by the ultracentrifugation method is often performed in research stages and in laboratories, but the application to commercial preparations is difficult as described above, and concentration of bulk harvest is required as a pretreatment. Similar to ultracentrifugation, there are methods to precipitate and concentrate EVs by low-speed centrifugation using commercially available polymers such as PEG as EV purification reagents in research stages and in laboratories, but there is a concern that the reagents may remain when these methods are used. Thus, it is essential to establish a method to determine the residual amounts of impurities after purification.

In cases where the active component or active ingredient is identified and the molecules a protein on EV membranes, etc., affinity purification using antibodies, etc. is a useful method for purification by using those as indices. Furthermore, affinity purification of CD63 and CD9, which are referred to as EV markers, is also possible. However, as mentioned in Section 1.2, these markers do not necessarily exist in all EVs (6), and it is necessary to determine whether or not the purified CD63 or CD9-positive EVs have the intended biological activity also from the viewpoint of the heterogeneity of molecules encapsulated in EVs. In addition, the safety of reagents such as buffers used to remove EVs from the antibodies and for purification should also be confirmed in consideration of potential inclusion of impurities. If necessary, solvents shall be replaced further by another method.

Purification based on size is also useful when EVs with the intended biological activity are defined with their size, for example, in cases where small particles with a size of ≤ 200 nm (called sEVs)

strongly exhibit the intended biological activity, or conversely, large particles (m/l EVs) ≥ 200 nm have the activity. The examples include separation by size exclusion chromatography and filtration using ultrafiltration membrane. However, in these methods, when proteins aggregate or other materials of the same size are present, the amounts of remaining impurities may increase, and thus it is necessary to establish a method to determine the residual amounts of impurities.

2.3 EV-specific quality characterization

Because EV preparations are considered to be a type of biopharmaceutical produced from cells, the points to consider described in "Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products" (ICH Q6B) are useful for the quality characterization.

In addition, the quality characterization needs to take into account the characteristics specific to EVs. After full consideration of the development of manufacturing methods in which EVs with the intended biological activity and active ingredients are purified as much as possible or to a certain level (see Section 2.2), the characteristics of the EVs obtained by the purification method shall be clarified by EV-specific characterization (see Section 2.3.1). However, because the separation/purification methods of EVs have not yet been fully established, it is necessary to pay sufficient attention to inclusion of components other than intended EVs that remain after purification. Consideration should also be given to the stability of EVs (potential degradation and transformation of products) during the manufacturing process and storage period. Qualitative and quantitative detection, establishment of a contamination acceptance limit, and safety evaluation are required for potential impurities, degradants, etc. (see Section 2.3.2).

2.3.1 EV-specific characterization

EV-specific characterization includes analyses of EV composition, physicochemical properties, and biological activity. Because EVs consist of heterogeneous particle populations, evaluation of the distribution and variability of characteristic values for each particle is considered important in addition to the evaluation of the overall mean characteristic values of particles. An approach to evaluate the heterogeneity of EVs at single particle-level resolution is considered useful for understanding the quality characteristics of EV preparations, and thus it is desirable that such analytical methods are incorporated in test items. In the field of EV research, development of new purification and analytical technologies and improvement of existing technologies are being carried out every day, and thus they should be incorporated in a timely manner.

2.3.1.1 Composition analysis

Analyses related to molecular composition, positive rate of EV marker molecules, and surface molecular profile are included. Determination of the presence or absence and quantitative analysis of EV-related molecules such as proteins, lipids, sugar chains, RNA, and metabolites present on the surface and inside of EVs are performed. Moreover, when the target active ingredient has been identified or when engineered EVs are used, qualitative and quantitative analysis of the target ingredients and the molecules transferred by modification is necessary. Tetraspanin (e.g., CD9, CD63, and CD81) and late endosome-associated proteins (e.g., Tsg101 and Alix) are commonly known as EV marker molecules. Other examples include the use of ganglioside GM1 as a marker for endosome-derived EVs and the use of major histocompatibility complex (MHC) class I as a criterion for dosage of DC-derived EVs. In any case, it is important to analyze with multiple combinations of EV marker molecules that are suitable for the purpose, keeping in mind that there are currently no EV marker molecules for which a consensus is reached. In fact, the ISEV guideline (31) stated that at least three

types of proteins specific to EV fractions or other EV-associated molecules should be analyzed semi-quantitatively. In addition, MISEV2018 also stated the need to evaluate the topology of the particles, which is to confirm whether the constituents of EVs are present in the interior of particles or on the surface of particles, considering that EVs may be decomposed/changed in the manufacturing /storage steps (1). On the other hand, there is a possibility that components not related to EVs (or components that are considered less relevant) may be included in EV preparations as impurities. The examples include factors related to intracellular organelles such as mitochondria and Golgi bodies, culture medium- derived components such as serum proteins, and virus particles and their fragments. For impurities that may be included, their detection methods and assay methods should also be investigated. For EV composition analysis, bulk analytical methods such as Western blotting and mass spectrometry are commonly used. Meanwhile, the flow cytometry method has been developed intensively in recent years, which is expected to enable various molecular composition analyses at a single particle level.

2.3.1.2 Analysis of physicochemical properties

The examples include the analysis of particle size, particle number, and surface charge (zeta potential). High-resolution imaging using microscopy such as electron microscopy and atomic force microscopy can measure particle size by direct observation, but it is practically difficult to obtain a statistically sufficient number of data. Dynamic light scattering (DLS) is an established method widely used for evaluation of particle size distribution of microparticles in a sub-micron region, but it should be noted that the accuracy of measurement may be deteriorated for polydisperse particles. Single particle analysis methods are considered to be preferred for the analysis of the size and number of heterogeneous microparticles, and nanoparticle tracking analysis (NTA), resistant pulse sensing technique, fluorescence correlation spectroscopy (FCS), etc. are often used. It should be noted that the measured values of particle size distribution are readily affected by the measurement method (principle) and equipment (model). For the determination of zeta potential, the electrophoretic light scattering (ELS) method, a combination of the light scattering and electrophoresis methods, has been frequently used. Recently, single particle-level analytical instruments for zeta potential measurement are being developed by improving NTA and the resistance pulse sensing technique. In any analysis, it is required that an individual particle is in a dispersed state, not aggregated.

2.3.1.3 Evaluation of biological activity and related molecules

The biological properties of EVs shall be clarified by the potency assay (32). In general, the potency assay shall use test methods that reflect the clinical mode of action of the drug. However, because of their characteristics, it is often difficult to fully elucidate the mode of action of EVs. Thus, it is desirable to investigate the mode of action of EVs as much as possible, and then to set up multiple tests based on the pharmacological action of the EVs to clarify the biological activity characteristics multidimensionally. The potency assay is performed using test systems such as cells, tissues, and individual animals in addition to *in vitro* biochemical tests. For example, *in vitro* assays include quantitative measurement of EV active ingredients (e.g., miRNA, mRNA, and proteins) and enzyme activity assays. Furthermore, cell assays include evaluation of cell activity in terms of cell proliferation, migration, toxicity, activation/suppression of immune cells, gene expression regulation, signal transduction, etc. It is considered that the obtained activity values may be normalized by particle number or protein amount depending on the purpose to evaluate the specific activity. Biological activity evaluation of EVs is important not only for clarifying EV properties but also for quality control and securing consistent characteristics of EVs.

2.3.2 Impurities in EV preparations

EV preparations are expected to contain not only EVs with the intended active ingredient (intended EVs, i.e., active component) but also EV-related substances such as EVs without the active ingredient (unintended EVs) and EVs changed or decomposed during the manufacturing /storage steps (EV variants). It is desirable to eliminate unintended EVs and variants as much as possible by chromatography or purification processes focusing on characteristics such as surface molecules and surface charge. In addition, in the culture process, purification concentration process, formulation process, and other processes in the manufacturing of EV preparations, it is considered that impurities such as other EVs, viruses/virus-like particles/microorganisms/mycoplasma, and medium components/reagents may be included via cell substrates, cell culture media, equipment and reagents used for purification and concentration, etc. Furthermore, there is a sufficient possibility for inclusion of airborne particles in the work environment and artificial microparticles derived from labware, etc. such as tubes. It should be noted that separation of particles with a similar size to that of EVs is generally difficult once they are included, whether they are of biological origin or artificial substances. Thus, it is extremely important to design and control appropriate manufacturing processes to minimize the inclusion of components other than the intended EVs. In addition, it is important to establish qualitative and quantitative test methods for impurities that may be included and to examine the acceptable limit of impurities from the viewpoint of efficacy and safety. Indicators for evaluation of inclusion of components other than the intended EVs include the evaluation of the proportion of target EV particles by vesicle profile analysis, potency measurement of EV preparations by biological tests, and comparison based on the potency standardized by the number of particles or other quantities.

In the manufacturing of EV preparations, it is desirable to avoid the use of animal-derived raw materials (including humans) to the extent possible. For additives of human or animal origin, viral contamination may occur, and if FBS is used, EVs of bovine serum origin may be included, leading to unexpected biological activities. Generally, use of reagents and recombinant proteins free from animal-derived components is recommended.

2.3.3 Immunogenicity of EVs

The immunogenicity of EVs is classified into the immunogenicity of EVs themselves and that of components other than EVs. For the former, because the immune response induced is different depending on the surface antigen of EVs, attention should be paid to the source cells of the EVs (autologous, allogeneic, cell type) and the surface antigen expression pattern (See Section 4.2.1). The latter is mainly due to impurities contained in the EV preparation (see Section 2.3.2).

2.4 Safety evaluation of infectious agents such as viruses contaminated in EVs – Measures taken by overviewing manufacturing processes

The following is known regarding the virus safety of EVs: because EVs and viruses are similar in size and EVs are heterogeneous constructs encapsulating a variety of molecules, 1. It is difficult to apply viral clearance processes in manufacturing and 2. EVs are highly likely to show the same behavior as that of viruses in processes such as purification because of the characteristics (33, 34). Thus, with regard to virus safety, it is most important to use cells not contaminated with viruses as the manufacturing substrate of EVs, and thus it is important to confirm the eligibility by performing virus screening for donors of cells used for manufacturing of EVs. The collected cells are examined for multi-dimensional infectious factors including viruses. A cell bank (see Section 2.1) is established by separation and proliferation of the target cells from the collected cells/tissues. Meanwhile, in a case

where the patient and the donor are the same person (so called autologous product), the donor screening is not required on the prerequisite that adequate measures are taken against cross-contamination in the manufacturing process.

The established cell bank is the starting material for EV manufacturing and is the starting point of manufacturing process in accordance with GMP. Thus, established cell banks should be tested in accordance with virus safety evaluation for cell bank in ICH Q5A. In particular, it is necessary to demonstrate the absence of viral contamination by performing tests such as an endogenous retrovirus test, *in vitro* virus test, and *in vivo* virus test.

A WCB for manufacturing may be prepared from the MCB. However, because human-derived cells are raw materials, it may be difficult to create a large bank that uses a continuous cell line (CCL) in some cases. In such a case, a DCB from a different donor may be newly established. In this case, virus tests as stated above should be performed for every bank renewal.

In the manufacturing of EVs, for example, when raw materials of biological origin are used for cultivation of manufacturing cells, it is important to use materials not contaminated with viruses, etc. In addition, it is important to take measures against contamination with infectious factors such as viruses due to improper handling in manufacturing.

Adventitious virus testing should be performed for bulk harvest produced in a culture process. Drug substance and drug products of EVs are prepared from the bulk harvest, and the application of viral clearance process is often assumed to be difficult in EV purification processes; thus, virus testing after harvest or subsequent processes is important as process control. Particularly in cases where a process of EVs concentration is performed, because if there is viral contamination, the viruses are also likely to be concentrated. Thus, in some cases, it may be more reasonable to perform a sterility test or virus test on in-process products after concentration.

It has been assumed that among viruses that may be included in EVs, enveloped viruses, which have lipid membranes, are likely to behave similarly because of the similarities in characteristics. Meanwhile, some non-enveloped viruses have been reported to have an envelope when released from cells, and thus careful consideration should be given to these points in testing of bulk harvest, etc. (35).

[Attachment] Manufacturing process of manufacturing cells to manufacturing of EVs
– Manufacturing of EVs using allogeneic cell banks and safety measures against viruses and other infectious agents at each step

1. Donor qualification
   As donor screening, it is necessary to conduct interviews and tests with reference to screening performed on donors for blood products and cell therapy products in principle.

   - Virus test: Serology test and nucleic acid amplification test (NAT) for HBV, HCV, HIV, HTLV-I/II, etc. should be performed and measures in consideration of the window period should also be considered.

   - Blood transfusion, organ transplantation, treatment with regenerative medicines, etc.
   - Dura mater transplantation and treatment with pituitary-derived human growth hormone
   - Medical history including history of infections such as hepatitis
   - Tests in consideration of the medical history of infections other than the above such as syphilis and tuberculosis, and if the cell donor`s history of travel and live abroad, epidemiologic conditions on the onset of infectious diseases in each region should be considered.

   Regarding confirmation of items to be tested and medical history by interview or other methods, it is necessary to refer to "Medical examination based on Article 25 of the Act on Securing a Stable Supply of Safe Blood Products and the Standards for Biological Materials 2 (1) and medical interview specified in 2 (1)," "Standards for Biological Ingredients," "Basic Concepts for Handling and Use of Drugs and Devices Utilizing Cells or Tissues (revised version)" and other relevant documents (36-38).
2. Test of collected cells
   Tests of collected/isolated cells/tissues: Tests for viruses, etc. in accordance with the type of cells/tissues collected should be conducted in addition to tests for viruses, etc. at screening (Even if a blood sample is negative for Parvovirus B19, bone marrow cells can be positive. Moreover, many people are latently infected with human herpes virus type 6). In addition, sterility test, mycoplasma negative test, etc. are required to be performed.
3. Tests for viruses as the characterization of cell banking
   Banked cells established by isolation and expansion culture of target cells from collected cells/tissues and DCB: Extensive virus testing performed in accordance with the tests on cell bank in ICH Q5A.
   - Electron microscopy observation
   - Reverse transcriptase activity
   - Retrovirus infectivity test
   - *In vitro* virus test
   - *In vivo* virus test
   - NAT test
     For application of NAT testing, attention should be paid to viruses derived from humans, which are the donors of manufacturing cells, and to viruses, etc. that have strong infectivity risk, in consideration of the administration site and route.

4. Safety measures for infectious agents in manufacturing

In cases where EVs are produced by expansion culture of a WCB (or MCB), the evaluation from safety perspectives should be performed when raw materials of biological origin are used for media used for culture. Measures to prevent contamination with infectious factors such as viruses by inappropriate handling during manufacturing are also required.

5. Tests on produced bulk harvest

Implementation of adventitious virus test for bulk harvest containing EVs produced, concentrated intermediate process products, and other products.

- *In vitro* virus test
- NAT testing, etc.

6. Tests on drug substances and preparations

A sterility test and mycoplasma test should be conducted on drug substances and preparations. Generally, the sensitivity is not higher in biopharmaceutical drug substances and preparations than in the bulk harvest, and thus virus testing is not required for them. However, while virus inactivation/removal processes are performed in the purification process for biopharmaceuticals, those processes may not be incorporated for EVs. An EV concentration process is likely to be performed particularly in manufacturing of EVs, and thus viruses may be concentrated. In such cases, bacteria and viruses may be concentrated, and thus sterility and virus tests of the drug substance and drug products may also be very useful.

3. Nonclinical studies

3.1 Pharmacokinetics

In usual drug discovery, absorption, distribution, metabolism, and excretion studies are conducted to clarify the pharmacokinetics, but for EV drugs, it is considered difficult to perform any evaluations such as the determination of blood concentrations, absorption or excretion rate for reasons that prepared EVs are not homogeneous, and evaluation methods for separating and analyzing specific EVs containing the bioactive substances have not been established at present. Thus, there are technical limitations in the evaluation of pharmacokinetics of EV drugs. In addition, naturally occurring EV drugs derived from human cells are considered to consist of human-derived components, and thus it may be supposed that there is little need to evaluate the metabolism and excretion. On the other hand, for artificial engineered EV drugs that contain chemical substances, quantitative evaluation of absorption, distribution, metabolism, and excretion according to the level of exposure to the relevant substances is considered important (see Section 3.3.2-2)-(1)-[1]-ii). It is considered that biodistribution of EV drugs can be evaluated by the following methods according to past reports, and it may become important information in elucidating the pharmacological and toxicological target organs, duration of action, and mode of action of the relevant EV drugs. It should be noted, however, that artificially modified EV drugs may not reflect physiological dynamics by any visualization method.

3.1.1 Differences in biodistribution by types of EV drugs

EVs are broadly divided into exosomes (sEVs) derived from multivesicular late endosomes and microvesicles (lEVs) derived from cell membranes (plasma membranes). Mouse PK studies have been reported that exosomes (sEVs) are distributed to the systemic organs including kidney, liver, lung, and spleen compared with dominant accumulation of microvesicles (IEVs) in the liver (39). In the investigation of the PK of EV drugs isolated by methods such as ultracentrifugation and gel filtration,

significant distribution to the liver in addition to the systemic organs such as the kidney, liver, lung, spleen, bladder, bone marrow, and heart have been reported, and distribution to the brain has been occasionally reported (40).

3.1.2 Evaluation methods of biodistribution

3.1.2.1 EV-labeling methods

Current EV-labeling methods used for biodistribution studies include the followings.

3.1.2.1.1 EV-labeling methods using cell membrane-labeling dyes

EV membrane labeling methods using fat-soluble dyes such as PKH dyes, Dil dyes, and near-infrared absorption dyes (NIR) including DiR and DiD are available (41).

These dyes are lipid-like molecular probes, which are inserted into the lipid bilayer membrane, and are used as in simple labeling methods in many PK studies. However, it should be noted that if lipophilic proteins such as lipoproteins coexist in prepared EV drugs, they will also be stained, leading to erroneous results. For example, the accumulation of large amounts of EVs in the liver, which has been observed in biodistribution studies of EV drugs using this labeling method, is considered to be one of the erroneous results. However, because this labeling method is the simplest, it is recommended for exploratory investigations at the organs/cell levels especially in the early stage of development.

3.1.2.1.2 Labeling methods using EV-dominant proteins

Labeling methods using the fusion proteins such as CD63 and Lamp2, which are concentrated in EVs, with reporter protein such as gLuc and GFP and lactadherin, which has an affinity for EVs, or fusion proteins of Moloney murine leukemia virus-derived protein (Gag), which binds to PI(4,5)P2 and is abundantly found in the inner leaflet of EV lipid bilayer) with gLuc or GFP (42, 43).

It is considered that these labeling methods are more sensitive and quantitative than the labeling methods shown in Section 3.1.2.1.1 and are appropriate for in vivo imaging. However, because the amounts of EV proteins used are not the same among in individual EV particle, the obtained results may be biased, and there is also concern that these fusion proteins prepared by genetic manipulation may affect on the biological characteristics of EVs. Thus, although these labeling methods are highly sensitive, it is recommended that they should be used in combination with the labeling methods in Section 3.1.2.1.1 to assess biodistribution at organ levels.

3.1.2.1.3 Labeling methods using radio isotopes

There are labeling methods using a fusion protein of lactadherin-streptavidin with radioisotopes such as biotinylated ^{125}I or ^{99m}Tc (44).

These labeling methods are the most sensitive reported to date and suitable for quantitative in vivo imaging, and the results are unlikely to be biased. However, these methods require operations in RI facilities where animal experiments can be conducted. Thus, these methods are recommended in investigation of biodistribution of EVs at low doses, but the methods in Section 3.1.2.1.1 or 3.1.2.1.2 should be used for investigation at medium or high doses.

3.1.2.1.4 Fluorescent staining methods of EV lumen

There are fluorescence staining methods for EV lumen using SytoRNASelect, CFSE, etc.

SytoRNASelect and CFSE, membrane permeable dyes, are the most suitable dyes to study unbiased biokinetics because they become fluorescent upon interaction with substances in EV lumen. However, sufficient sensitivity may not be obtained because of the weak fluorescence at present. Because these methods are mainly used in studies of microvesicles (lEVs), without protein markers, the methods in

Sections 3.1.2.1.1 to 3.1.2.1.3 should be used in PK investigations of exosomes (sEVs). They are recommended for tissue staining in studies of biodistribution.

3.1.2.2 EV detection methods (41-44)

In study at organ levels, IVIS is commonly used for methods in Sections 3.1.2.1.1, 3.1.2.1.2, and 2.1.2.1.3. BLI is used for 3.1.2.1.2.PET and SPECT/CT are used for 3.1.2.1.3. In many reports, histological staining of tissues and flow cytometric analysis of tissue-derived cells are performed in PK studies using fluorescent dyes in addition to studies at organ levels.

3.1.3 Scientific interpretation of uptake in organs and cells

Many studies reveal that the EVs appear to be distributed systemically regardless of the source of released cells or the animal species to be administered (39, 40). However, the observation of fluctuations in the distribution of administered EVs to target and non-target cells/organs by using appropriate control EVs is important for predicting safety in humans. For these evaluations, it is desirable to conduct PK studies at organ/cell levels using multiple labeling methods for the intended EVs and to include groups in the relevant studies to administer appropriate control EVs (e.g., if the target EVs are derived from MSCs, EVs derived from fibroblasts; if the target EVs are derived from $CD8^+$ T cells, EVs derived from $CD4^+$ T cells or natural killer [NK] cells; if the target EVs are derived from DCs, EVs derived from macrophages; and if the target EVs are artificially engineered EVs, EVs from original cells before modification). If safety is questionable, approaches such as Section 3.1.4 can be considered.

3.1.4 Countermeasures for distribution other than the target organs and cells

Countermeasures for reducing distribution in non-target organs and cells

- If the EV population of the EV drugs (e.g., exosomes [sEVs] or microvesicles [lEVs]) has been identified, the EV population should be isolated and purified as much as possible.
- For artificially engineered EV drug s, enhancement of specificity (affinity) to target cells would be effective.
- If the fraction of prepared EV drugs contains large amounts of unnecessary proteins, lipids, etc., the EVs should be prepared by methods to achieve high purity (for example, the affinity method and ion exchange method (30)).

3.2 Pharmacological studies

Pharmacological studies using EV preparations are basically the same as studies conducted for low-molecular drugs or protein preparations and studies that are suitable for individual diseases shall be conducted using cells, tissues, individual animals, etc. However, for the selection of the control group and dose unit of EVs, the following measures may be considered as EV-specific items. Furthermore, as most of the active ingredients of EVs are proteins and nucleic acids, attention should be paid in handling of differences in animal species in pharmacological studies.

3.2.1 Control groups

In functional studies of EVs, the basic objective is to evaluate the dose-response relationship. According to MISEV2018, in cases of high-recovery and low-specific EV isolation, quantitative comparison of functional activity between the three groups, EV-containing sample solution, sample solution after EV removal, and EVs alone, is required, and in cases of low-recovery and high-specific EV isolation, comparison between the EV fraction and other fractions is required. Moreover, as the control group, natural EV preparations collected from different biological fluids or tissues/cells may

be used for natural EVs, and EVs that do not contain functional molecules or those that contain replaced variants may be used for engineered EV preparations.

3.2.2 EV dose unit

The number of particles and the total amount of protein are widely used as the unit of EV dose, and these units may also be used in nonclinical studies. Meanwhile, these units are likely to be impacted by the purity and quality of EVs, leading to difficulty in obtaining stable test results in some cases. In such cases, if the active ingredient has been clarified, the content can be used as the unit of EV activity (e.g., equivalent to IL-2 XX mg and equivalent to miRNA XX copies). On the other hand, if the functional molecule is unknown, the biological potency defined in the representative pharmacological study may be used as the unit.

3.2.3 Selection of animal species

Because most of the functional molecules of EVs are proteins and nucleic acids and the effects of differences in animal species are significant, it is desirable to select animal species that exhibit the intended pharmacological action in humans when treated with the EV preparation. For biotechnological products, if the relevant animal species are not available, nonclinical evaluation can be performed using equivalent animal products, but for EV preparations, it is considered difficult to demonstrate the equivalence between clinical candidates and animal products. In such cases, the efficacy of the EV preparation should be explained on the basis of scientific evidence obtained from various published information.

3.3 Nonclinical safety studies

3.3.1. General principles

Nonclinical safety studies of an EV preparation should be planned on a case-by-case basis after fully understanding its characteristics and conducted in accordance with GLP in principle. Because scientific progress and accumulation of experience in the field of EVs are constantly advancing, if new strategies and testing methods to replace the nonclinical safety evaluation described below are developed, they can also be used in nonclinical safety evaluation.

3.3.2. Evaluation strategy

To conduct nonclinical safety assessment of EV preparations, it is imperative to evaluate the safety profile upon human administration, considering both on-target toxicity, which results from the intended pharmacological action and off-target toxicity, which is not directly related to the intended pharmacological action, as is done with other drugs.

1) On-target toxicity

The on-target toxicity of EV preparations is considered to be induced by proteins, oligonucleotides (mRNA, miRNA, and DNA), etc. contained as active ingredients, and it should be evaluated based on the mode of action. Therefore, for evaluation of on-target toxicity attributable to proteins, mRNA and DNA, and miRNA, "Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals (45)," "Ensuring the Quality and Safety of Gene Therapy Products (46)," and "Guideline for preclinical safety assessment of oligonucleotide therapeutics (47)" can be used as references, respectively.

2) Off-target toxicity

(1) Safety related to extracellular vesicles

[1] General toxicity

It is considered that EV preparations consist of various types of EVs, and proteins and nucleic acids in each EV have high target specificity. Thus, EV preparations are considered unlikely to cause off-target toxicity due to these molecules, and their species specificity is considered to be high. On the other hand, off-target toxicity of engineered EV preparations with chemical modifications (e.g., modification of nucleic acids and conjugation) could be a concern when administered to humans, and sponsors should evaluate it on a case-by-case basis using scientifically appropriate approaches.

i. Natural EV preparations

For EV preparations from cells with minimal concern about safety in humans, such as natural EV preparations that have already been utilized in cell therapy products, off-target toxicity concerns are considered to be low. Therefore, in the case of natural EV preparations, it is not required to conduct an independent study for off-target toxicity, as it is sufficient to evaluate it in nonclinical safety studies for on-target toxicity.

ii. Engineered EV preparations

If any chemical entities whose safety in humans has not been assured (e.g., chemical modification by bioconjugation) are to be used in a engineered EV preparation, nonclinical safety studies should be conducted with reference to the "Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals" (48) (ICH M3) or other appropriate safety documents related to chemical entities.

[2] Carcinogenicity

It has been reported that EVs derived from immortalized cells contain numerous RNA and DNA sequences of retrotransposon, which can transfer the malignant phenotype of immortalized cells to normal cells via retrotransposons (49). Furthermore, retrotransposons have been detected in FBS, which is a commonly used cell culture supplement (50-52). Due to the challenge of evaluating the risks associated with retrotransposons in nonclinical studies. it is advisable to prioritize the use of cells with minimal safety concerns, such as those intended for cell therapy products in the manufacturing of EV preparations, and FBS should be avoided as much as possible. However, if it is indispensable to use cells with a unknown, safety profile, at least, retrotransposon activity in the cell substrate should be measured to ensure low risk.

Rn addition, the regarding carcinogenicity of EV preparations, including both natural and engineered EV preparations using chemical entities without confirmed safety in humans, carcinogenicity studies of the chemical entities need not be conducted unless the preparations are continuously used for a prolonged period (6 months or longer) in the clinical use ("Guidelines on Testing for Carcinogenicity of Pharmaceuticals") (53).

(2) Impurities

It is important to reduce manufacturing process-derived impurities such as reagents used in the manufacturing of EV preparations as much as possible by using the information on manufacturing. On top of that, it is considered appropriate to grasp the amounts of impurities

remaining in the preparation as much as possible and evaluate the safety based on the nonclinical safety study results of the preparation, published data (e.g., toxicity profile, information on human endogenous substances, and experience of administration to humans), guidelines on impurities (54-58), and toxicological concepts (e.g., threshold of toxicological concern).

3.3.3. Design of nonclinical safety studies

1) Selection of animal species

(1) On-target toxicity

In assessing the on-target toxicity of EV preparations, it is generally appropriate to conduct nonclinical safety studies in one animal species that exhibits the intended pharmacological action in humans when treated with the EV preparation. In the case of biopharmaceuticals and cell therapy products, when no animal exhibits the intended pharmacological action in humans, alternative models using analogous animal products are considered. However, evaluating the similarities between an EV preparation with an animal equivalent is considered complicated. Thus, when no animal exhibits the pharmacological action of an EV preparation, hazards caused by the on-target toxicity should be evaluated based on scientific evidence obtained from various published information for risk management in humans.

(2) Off-target toxicity

For natural EV preparations derived from normal human cells, the concern for off-target toxicity when administered to humans is considered low, and it is supposed sufficient to evaluate in toxicity studies (e.g., single-dose toxicity studies and repeated-dose toxicity studies) in one animal species including rodents. On the other hand, when a chemical entity whose human safety has not been assured is used in a engineered EV preparation, two animal species (rodent and non-rodent) are generally required to evaluate off-target toxicity focusing on the chemical entities with reference to ICH M3.

2) Dose and administration

(1) Route and frequency of administration

In nonclinical safety studies of EV preparations, in principle, it is desirable to administer EVs by the intended therapeutic route at a frequency equal to or more than that in clinical use. If the therapeutic route is not feasible, the influence of the difference in the administration route on the safety evaluation should be explained. Furthermore, repeated administration is not necessarily required when accumulation in the body and exacerbation of toxicity findings are not expected after repeated administration of an EV preparation to humans and animals.

(2) Dose

Given that exosomes contain a wide variety of proteins and oligonucleotides and the biological activity of EV preparations is assumed to differ between animal species, addressing the quantitative human risk caused by the on-target toxicity of EV preparations is difficult, and it is necessary to identify the hazards. The dose levels of natural EV preparations are considered evaluable in at least two groups, the control and treatment groups. On the other hand, if a chemical substance whose safety in humans is not identified is used in an engineered EV preparation, it is desirable to set multiple dose groups as for chemically synthesized drugs so that the dose-response relationship can be evaluated. Moreover, for all EV preparations, it is considered that the maximum dose should be as high as possible while considering the maximum tolerated dose (MTD), maximum feasible dose (MFD), and animal welfare (3Rs).

3) Reversibility

If serious toxicological findings are observed in nonclinical safety studies and extrapolation in humans is concerned, the reversibility should be evaluated with reference to ICH M3.

4) Toxicokinetics

For usual drugs, toxicokinetic data (systemic exposure data in toxicity studies) are collected to assess the extrapolation of nonclinical safety study results to humans. However, EV preparations are not necessarily homogeneous, and there is no established evaluation method for analyzing specific EVs separately. Thus, it is difficult to evaluate the toxicokinetics of EV preparations at present. In addition, given that biological activity differs between animal species for many EV preparations, it is considered unnecessary to collect toxicokinetic data in nonclinical safety studies to evaluate the human safety. On the other hand, if any chemical entity whose human safety has not been identified is used in an engineered EV preparation and there is a particular concern about its safety, the necessity of collecting toxicokinetic data focusing on the relevant chemical substance should be considered.

4. Clinical development

4.1 PK/PD and efficacy assessment

PK/PD studies are important for the evaluation of maximization of therapeutic effects and minimization of adverse effects of drugs, but in light of the current situation where it is difficult to quantify the concentration of EV preparations in the body, it is not necessary to strictly determine the ADME/PK/PD, and it is realistic to analyze the relationship between the dosage and administration and effects/actions. Evaluation in clinical studies is desirable, but nonclinical studies or *in vitro* studies shall be conducted for items with difficulty. However, it should be noted that animal study data are not necessarily applicable to humans. The effects and actions to be evaluated are different depending on the disease or purpose as shown in the examples below. If there is a possibility that concomitant use of drugs with the same type of action or related action may enhance the main action, it is necessary to examine the interaction with the concomitant drug. In efficacy and safety evaluation in clinical development, immunogenicity and on-target/off-target toxicity should be considered as special properties of EVs and the development methods of low molecular weight compounds and RNA/protein drugs should be referred to. Regarding immunogenicity, if an EV preparation is administered repeatedly, attention should be paid because the effects/actions may be attenuated due to antibody manufacturing against molecules on EVs. Furthermore, for quantification methods of EVs, attention should be paid to whether the intended dose is administered accurately because values may differ depending on measurement conditions.

4.1.1 Tissue damage repair by MSC-derived EVs and their anti-inflammatory effect

MSC-derived EVs are expected to restore functions in various types of damaged tissues and anti-inflammatory/anti-fibrosis effects, and the following endpoints are assumed to be evaluated in combination depending on the target disease: Inflammatory/anti-inflammatory mediators in tissues (e.g., IL-1β, IL-5, IL-10, IL-12, chemokines, and TGF-β1), fibrosis markers (e.g., collagen I, fibronectin, αSMA, and hydroxyproline), infiltration of inflammatory cells (e.g., neutrophil count, eosinophil count, and lymphocyte count), tissue functions (e.g., forced vital capacity [FVC], SpO_2 on

exertion, and Saint George's Respiratory Questionnaire [SGRQ] total score, for the lung), and clinical findings (incidence of acute exacerbation).

4.1.2 Activation of immunity by DC-derived EVs

DC-derived EVs are expected to induce T cell activation and have an immunostimulatory effect against cancer or infection. When used as cancer vaccines, the following endpoints are assumed: Cytotoxic activity by killer T cells, activation of helper T cells (e.g., T cell proliferation and IL-2/IFN-γ production), increase of antigen-specific T cells, tumor growth inhibition, response rate, progression-free survival, overall survival, etc. When used as vaccines against infections, endpoints such as neutralizing antibody titer and prevention of onset/aggravation are additionally assumed. It is desirable to analyze the time of onset and duration of action as much as possible. For both types of vaccines, it is important to assess adverse effects such as allergic reactions (including anaphylactic shock) and induction of autoimmunity.

4.1.3 Control of disease-related protein expression by miRNA-containing EVs

Certain miRNAs contained in EVs are expected to have the effect of decreasing the expression of disease-related proteins, and the following endpoints are included: The expression level of disease-related proteins targeted by the miRNAs, the time of onset and duration of action, changes in the cellular activity and function by the miRNAs, effects thought to be related to clinical benefits (e.g., biomarkers and hematology parameters), and ultimate clinical benefits (e.g., response rate, severity rate, and survival time). Because miRNAs control the expression of multiple proteins, control of expression in non-disease related proteins may occur. Assessment of potential adverse effects caused by the expression control of off-target proteins is required.

4.2 Undesirable immune responses such as allergy and rejection

When EVs derived from allogeneic humans, non-human animals, or plants are administered to humans, risks due to undesirable immune responses such as allergy and rejection are assumed, but the significance of evaluating these risks in nonclinical studies in animals is limited. Thus, in clinical studies, it is necessary to advance clinical studies carefully after considering measures to reduce risks related to undesirable immune responses in humans from the following viewpoints:

4.2.1 Immune responses to EVs

The immunogenicity of an EV preparations differs depending on the origin of manufacturing cells.

If it is derived from autologous cells, there is no need to consider the immunogenicity of the EVs themselves. In the case of allogeneic human origin, immunogenicity resulting from the incompatibility of the histocompatibility complex expressed by EVs themselves, especially MHC class I and II of surface antigens, should be considered.

No immunosuppressants are used in treatment with cell therapy products or cell processing products that are expected to have a short-term effect, such as MSC, a therapeutic drug for acute graft-versus-host disease (59). In addition, no description requiring actions relating MHC (matching of MHC) is found at present in the investigation of EV-related clinical studies using public information such as the US clinical trial registration database (ClinicalTrials.gov). Furthermore, considering that transfusion of platelets expressing human leukocyte antigen (HLA)-A and HLA-B (so called random platelet transfusion) has been performed globally including Japan for a long time, serious immune responses via MHC are unlikely to occur in clinical application of EV preparations and safety concerns are considered to be low.

On the other hand, in treatment with allogeneic human cell-derived cell therapy products and cell processing products for which long-term engraftment is expected, immunosuppressants are used, and long-term engraftment requires control of rejection due to MHC mismatch (60). To reduce immune responses such as acute and chronic rejection, the same approach in hematopoietic cell transplantation, i.e., matching of MHC class I A, B, and C loci and class II DR locus in the direction from the recipient (patient) to the donor (EVs administered) (so-called the host versus graft [HVG] direction) as much as possible, might be useful.

It is important to consider the necessity of matching MHC class according to the purpose for which EV preparations are applied.

4.2.2 Immune responses to impurities other than EVs (e.g., antibiotics and medium components)

For impurities (e.g., antibiotics and medium components) contained in therapeutic preparations using EVs, risks such as allergy are considered, and treatment with an appropriate combination of drugs such as antihistamine, anti-inflammatory drug, and antipyretic analgesic before the use of EV preparations is also an option to avoid the relevant risks. At least, when the relevant preparation is administered to humans, appropriate clinical safety management will be necessary, for example, fully explaining and calling attention to subjects and investigators after reducing the relevant risks as much as possible (see Section 2.3.2).

4.2.3 EV-induced immune responses

The intended pharmacological action (on-target effect) may be caused by immune responses induced by EVs unlike the cases stated in Section 4.2.1 for responses caused by the immunogenicity of EVs. However, it should be noted that the response may exhibit excessively (on-target toxicity) (see Section 3.3). For example, there is a possibility for the onset of infection caused by excessive immunosuppression because the intended pharmacological effect of MSC-derived EVs is immunosuppression. Conversely, the intended pharmacological effect of DC-derived EVs is to activate the immune response (see Section 1.3), and thus prophylactic and therapeutic use of immunosuppressants, etc. for excessive immune response (e.g., cytokine storm) should be considered.

4.3 First-in-human study design

Ensuring safety in humans is an extremely important issue in clinical studies. In particular, in a clinical study in which a drug is administered to humans for the first time (FIH study), it is necessary to carefully determine the FIH study design including the initial dose, dosing interval, and risk management method based on all available information.

Because many EV preparations are expected to induce biological reactions via highly species-specific molecules such as proteins and nucleic acids (mRNA, miRNA, and DNA), there is a limitation in estimating the initial dose in the FIH study based on the results of nonclinical studies in animals. Thus, if the results of clinical studies of similar EV preparations have already been obtained, it is recommended to draw up an FIH study design including the initial dose by utilizing these information as much as possible. On the other hand, if no information on similar EV preparations is available, the initial dose in the FIH study should be determined from the perspective of both on-target and off-target toxicity using the nonclinical data of the EV preparation. From the standpoint of on-target toxicity of EV preparations, if a critical safety concern due to excessive pharmacological activity is expected when administered to humans, it is necessary to determine the initial dose by estimating the minimal anticipated biological effect level (MABEL) in clinical studies of the EV preparation with reference to the "Revision of the Guidance For Establishing Safety in First-in-Human Studies during Drug

Development" (61) (Guidance for FIH studies). Meanwhile, if there is no critical concern about on-target toxicity of the EV preparation, it is not necessarily required to calculate the MABEL. In addition, if a safety concern is expected from the viewpoint of off-target toxicity, the initial dose should be determined based on the no observed adverse effect level (NOAEL) calculated based on the general toxicity studies of the EV preparation. Based on the above, in the FIH study, it is appropriate to determine the initial dose by selecting a dose determined based on the MABEL and/or NOAEL, whichever is lower and by multiplying the dose by an appropriate safety factor. For other points to consider in conducting the FIH study, the FIH study guidance and the "Questions and Answers (Q & A) on the Guidance For Establishing Safety in First-in-Human Studies during Drug Development" (62) would be useful as a reference.

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