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Regulatory Aspect of Pre-clinical Studies for Regenerative Medicine

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Abstract

Regenerative cell therapy is a discipline in youth, with only limited experience in clinical practice. Once manufacturing protocols of cells have been established *in vitro* studies, the manufactured cells were assigned as candidates for cell-based medicinal products. To judge whether the cell-based medicinal products manufactured according to the established protocols should be valuable for further development for clinical practice, pre-clinical studies shall be conducted. In construction of a pre-clinical study package for cell-based medicinal products, the pre-clinical study package used for chemical compounds will help a lot. The requirements and contents of the pre-clinical studies are classified into toxicity studies (general toxicity studies and special toxicity studies), pharmacological studies (primary pharmacodynamics studies and safety pharmacological studies), pharmacokinetic studies, formulation studies, and others. The pre-clinical studies for the cell-based medicinal products are conducted to evaluate and qualify the efficacy and safety of the candidates. No wonder there is an argument about the selection of assessment parameters in toxicity, pharmacological and pharmacokinetics studies for cell-based medicinal product, because it is well-known that the cell-based medicinal products are different in properties from chemical compounds. Besides parameters used in these studies, the assessment issues may be an assumable minimum consensus, and others should be added on a case-by-case basis, depending on the cell manufacturing processes. In anticipation of the approval as the cell-based medicinal products, the possible requirements should be discussed here in the development process of such products from the standpoint of regulatory science, especially focusing on those in pre-clinical studies.

Keywords: Cell-based medicinal products; Regulatory science; Pre-clinical study; Toxicity study; Pharmacological study; Pharmacokinetics study

Introduction

Recent advances in regenerative medicine have promised us the innovative therapeutic approaches for intractable diseases [1-4]. To move forward regenerative medicine into clinical application, some issues are here. One of them might be regulatory science, which is a common platform for basic research, development, and clinical application of regenerative cell therapy, and regulatory science-based scientific regulation. In anticipation of the approval as the cell-based medicinal products, I will here discuss the possible requirements in the development process of such products from the standpoint of regulatory science, especially focusing on those in pre-clinical studies.

Pre-clinical studies

Definition

Once protocols of cell-cultivation have been established *in vitro* studies, the differentiated cells were assigned as candidates for cell-based medicinal products. To judge whether the cell-based medicinal products manufactured according to the established protocols should be valuable for further development for clinical practice, pre-clinical studies shall be conducted [5].

The pre-clinical study package used for chemical compounds will help a lot in construction of a pre-clinical study package for cell-based medicinal products, although it is well-known that the cell-based medicinal products are different in properties from chemical compounds. To lesson from the package of the chemical compounds, the requirements and contents of the pre-clinical studies of chemical compounds are listed (Table 1) [6]. As shown in Table 1, pre-clinical studies are classified into toxicity studies (general toxicity studies and special toxicity studies), pharmacological studies (primary pharmacodynamics studies and safety pharmacological studies), pharmacokinetic studies, formulation studies, and others. The pre-clinical studies are conducted to evaluate and qualify the efficacy and safety of the candidates for the cell-based medicinal products. Collection of available information to conduct clinical trials such as those about selection of surrogate markers is among the objectives of the pre-clinical studies. Once an unexpected adverse event occurs in the course of clinical trials, the pre-clinical studies should be reviewed to identify its cause, retrospectively. Although all toxicological studies and some safety pharmacological studies are must be conducted in compliance with the Good Laboratory Practice (GLP) Ordinance, not of all these studies are required to have been completed under the GLP Ordinance till stating of the first-in-man trials.

Toxicity studies

Objectives of toxicity studies: The primary objective of toxicity studies is to evaluate the safety of the candidates for cell-based medicinal products by identifying the types of toxicity, and tissues / organs where toxicity occurs. The toxicities of the cell-based medicinal products include the toxicity that is an extension of their primary pharmacological actions and one associated with the properties of the products but with their pharmacological activities. The former example

is myocardial rupture associated with cell products for myocardial infarction with effect on myocardial fibrous tissue resolution as its primary pharmacological effect, whereas the latter example is ossification after administration of the product to cardiac milieu, resulting in arrhythmia [7]. In general, the former events could be predictable, but latter unpredictable, possibly resulting in serious outcomes.

Item		
1. Toxicity studies	1) General toxicity studies	Single dose toxicity studies
		Repeated Dose toxicity studies
	2) Special toxicity studies	Carcinogenic studies
		Antigenicity test
		Genotoxicity test
		Reproduction test
		Others
2. Pharmacological studies	1) Safety pharmacological studies	Core battery studies
		Follow up studies
		Complementary safety pharmacological studies
	2) Pharmacodynamics studies	
3. Pharmacokinetic studies		Adsorption
		Distribution
		Metabolism
		Excretion
		Toxicity
4. formulation studies		

Table 1: Items of pre-clinical studies.

General toxicity studies and cell-based medicinal products: General toxicity studies are categorized into two types; single-dose toxicity studies and repeated-dose ones [8]. These toxicity studies will be mandatory for cell-based medicinal products, as they are required for the approval of a chemical compound as a new drug. Single-dose toxicity studies (acute toxicity studies) are conducted to determine the potential toxicity of the cell-based medicinal products after a single dose. For chemical compounds, a 14-day post-dose observation period is required, and necropsy including gross pathological and histopathological examination is performed when appropriate. For cell-based medicinal products, it is controversial whether toxicity studies should be conducted with the products *per se* or its analogue derived from the test animal species. The use of an analogue for the test animal species in toxicity studies is not appropriate with exemption, although it seems more rational that the human-derived products is administered to immunedeficient/immunocompromised or immunosuppressed animals to assess the potential toxicity of the product itself.

It would be difficult to estimate the lethal dose of the cell-based medicinal product in general toxicity studies, unlike the case of chemical compounds. Originally, single-dose toxicity studies are designed to demonstrate the occurrence of toxicity due to accidental overdose and/or to find doses for appropriate repeated-dose studies [8]. In regenerative cell therapy, accidental overdose, for example 10 times or more over dose, would not happen. Therefore, the toxicity is considered to be adequately evaluated with doses over the clinical dose up to the maximum dose technically possible. For this purpose, a safety dose finding studies should initially be conducted to determine the maximum dose technically possible in conjunction with collection of the basic data on organ toxicity, which should be reflected on single-dose toxicity studies, conducted in compliance with the GLP. Repeated-dose toxicity studies (sub-acute and chronic toxicity studies) are designed to determine the potential toxicity of the cell-based medicinal products after repeated exposure. The entity of these studies is an observation for toxicity accumulation. Based on the results of the repeated-dose studies, the no observed adverse effect level (NOAEL) is estimated, which is reflected on the planning of clinical trials [9]. Many of the cell-based medicinal products would not require the conducting of repeated-dose toxicity studies, because once the cells have survived and resident after a single dose, long-term exposure is expected. However, repeated-dose toxicity studies shall be required for repeated-use products.

No wonder there is an argument about the selection of toxicity assessment parameters for cell-based medicinal product. Besides parameters used in general toxicity studies, assessment of tissue toxicity may be an assumable minimum consensus, and other

assessment parameters should be added on a case-by-case basis, depending on the cell manufacturing processes.

Special toxicity studies and cell-based medicinal products: Special toxicity studies required for low-molecular weight compounds include carcinogenicity studies; antigenicity studies; genotoxicity studies; reproductive and developmental toxicity studies; and, if appropriate, local tolerance studies, dependence studies, and photo toxicity studies [10-12]. Carcinogenicity studies of chemical compounds are to be conducted if (1) the results of genotoxicity studies suggest a concern about carcinogenic potential, (2) a possible risk of cancer in humans has previously been indicated, (3) the structure-activity relationship suggests genotoxic and/or carcinogenic potential, (4) repeated-dose toxicity studies provide evidence of paraneoplastic changes, and/or (5) the parent compound or its metabolite(s) remain in the tissue for a long time, possibly resulting in local histological or pathological changes. Carcinogenicity studies are also required for chemical compounds intended to be used for at least 6 months [13]. The required observation period is 24 to 30 months for rats and 18 to 24 months for mice and hamsters [13]. Antigenicity studies are intended to evaluate antigenic potential of guinea pigs that are challenged repeatedly with the test compound or a compound-protein conjugate, followed by boosting 2 to 3 weeks after challenge.

The issue remaining to be addressed for the implementation of carcinogenicity studies of cell-based medicinal products is how to evaluate possible malignant transformation of the cells themselves after administration. With respect to genotoxicity studies, the question whether cell-based medicinal products induce malignant transformation into tumor cells remains unanswered. There have been no reports of malignant transformation after transplantation in cases of mesenchymal stem cells or other somatic stem cells. In contrast, it has been reported that induced pluripotent stem cells (iPSCs) transformed into tumor stem cells when co-cultured with tumor cells [14]. Consequently, more considerations will be needed for pluripotent stem cell (ESCs/iPSCs)-derived cell products. For reproductive and developmental toxicity studies, cell products do not seem to affect the reproductive glands and seem very unlikely to cause reproductive and developmental toxicity.

A tumorigenicity test will be mandatory for pluripotent stem cell (ESCs and iPSCs)-derived medicinal products, because the cell products were obtained from immortal ESCs and iPSCs. There has been only one guideline, which could be referred, WHO-TRS978 [15]. In the testing protocol (WHO-TRS978), 10 nude mice (immunocompromised mice) are transplanted with test cells at a dose of 1×107 cells/animal and then observed for subcutaneous tumor formation for 16 weeks. This guideline has originally been developed for quality control for master cell bank in the manufacturing of protein products and other biologics. The statement that this test should not be applied for cellular therapy products is included in an Appendix of the 2010 revised version of WHO-TRS878. At present, there are no released guidelines of tumorigenicity studies for cell-based medicinal products. Unlike conventional chemical compounds, possible interactions with surrounding tissues and local administered pluripotent stem cell-derived products should be taken into consideration. Therefore, in practice, the tumorigenicity studies might be performed in conjunction with a chronic toxicity study or disposition study (metabolic fate study) to observe the consequences of interactions between the cell products and surrounding tissues and the potential toxicity of a locally formed tumor to organs or tissues, if formed.

Pluripotent stem cell input studies: We propose a pluripotent stem cells input study to evaluate the safety of iPSCs and other pluripotent stem cells-derived products. The studies should be performed to clear whether un-/low-differentiated and/or differentiation-resistant ESCs/iPSCs would be contaminated and the contaminated cells could form teratomas in situ or not. To fulfill the purpose, first, a residue assay and detection system should be established of contaminated pluripotent stem cells in the product of interest. An amount of the undesired cells beyond the threshold of the system used is mixed with the cell products, and then teratoma formation is assessed after administration or transplantation. A residue assay and detection of differentiation-resistant pluripotent stem cells could utilize nucleic acid amplification technique (NAT) (e.g., *Alu*-PCR technique) or flow cytometry as reported [16]. For example, if 0.01% residue is the threshold of detection in cold run, pluripotent stem cells are mixed at a concentration above 0.1%. It is ideal that pluripotent stem cells are mixed at a concentration 10 times the limit of detection to ensure non-linearity. Pluripotent stem cells themselves may be required to be administered or transplanted as the positive control. A cell-based medicinal product mixed with pluripotent stem cells are transplanted to the intended site of transplantation in clinical practice, and then teratoma formation is assessed. The animal species is selected on a case-by-case basis, depending on the route of administration and others. The required duration of observation, referred to the carcinogenicity studies, may be 24 to 30 months for rats and 18 to 24 months for mice or hamsters. It may be recommended to observe all the animals until death, to obtain a survival curve on the assumption that tumor formation does not necessarily shorten the life span and that prognosis may be even improved as a result of improvement in the disease condition. However, this observation is time-consuming so that some patients miss the opportunity who could receive a benefit from the cell-bade medicinal product. Therefore, in order to assess tumorigenicity, animals should be sacrificed in the course of study, approximately 6 to 9 months after administration of the product mixed with pluripotent stem cells, and a first-in-man study will be warranted if there is no evidence of teratoma formation at this time point. The number of animals sacrificed in the course of the study and duration of observation from dosing to sacrifice can be determined based on available evidence; for example, if no teratomas were formed from iPSCs-based medicinal products for at least 6 months after transplantation, this finding could be the theoretical rationale for determination.

Pharmacology studies

Safety pharmacology studies: Safety pharmacology studies join primary pharmacodynamics studies as the mainstream of pharmacology studies. Safety pharmacology studies, also known as general pharmacology study, are meant to determine the potential undesired pharmacological effects of the cell-based medicinal product on physiological functions. Safety pharmacology studies consist of core battery, follow-up, and supplemental safety pharmacology studies, but some observations can be undertaken together with each other.

The core battery is meant to investigate the unfavorable pharmacological effects of the product on the physiological functions of the vital organ systems, that is, the cardiovascular, respiratory, and central nervous systems (CNS). Cardiovascular parameters include blood pressure, heart rate, and electrocardiogram. Respiratory parameters include various respiratory function tests such as respiratory rate, tidal volume, and Hb oxygen saturation. CNS parameters include motor activity, behavioral changes, and body

temperature. For individual cell-based medicinal products, specific parameters may be included depending on the mode and route of administration. For example, in the case of injection of cell suspension into the coronary artery, cardiac output and ventricular contraction (wall mobility) should be additional parameters, as the injected cells may cause microinfarction. For a cellular product injected into a vein for the treatment of cerebrovascular disorders, observation blood pH and LDH levels are recommended as the injected cells may cause pulmonary embolism resulted in infarction. Behavioral pharmacology, learning, and memory should also be assessed, as the product is intended to treat cerebral infarction. These additional observations are referred to as follow-up studies and should be undertaken if further investigation is considered necessary based on the results of the core battery. Follow-up studies should be conducted in compliance with the GLP Ordinance to the greatest extent feasible, but not required to be fully GLP compliant. It would be recommended that a follow-up study is included in the core battery to be conducted as a combined study because some effects related to the testing of cellular product that would be observed in the core battery can be expected. Supplementary safety pharmacology studies are meant to evaluate potential adverse pharmacodynamics effects on organ system functions not addressed by the core battery or repeated dose toxicity studies when there is a cause for concern.

Primary pharmacodynamics studies: Primary pharmacodynamics studies are intended to support the effects of a test cell-based product in relation to its desired therapeutic target. The study method varies depending on the effects and indication. These studies are conducted to support pharmacological effects not only *in vivo* studies but also *in vitro* ones. Pathophysiological animal models are often used in primary pharmacodynamics studies, whereas healthy animals are used in general toxicity and safety pharmacology studies. It may seem that primary pharmacodynamics studies are essentially the same as primary pharmacodynamics studies conducted in many non-pharmaceutical laboratories. Such studies that are commonly performed by many non-pharmaceutical researchers may be considered as primary pharmacodynamics studies if their reliability is sufficiently assured for new drug applications. It is important to investigate the mechanism of action of the test product as a key factor in determining endpoints or surrogate markers/parameters in clinical trials. In addition, a dose range-finding study to evaluate the efficacy of the test product is required to prevent an excessively high exposure to subjects and patients. A dose-range finding study to evaluate efficacy includes 4 dose groups given either the test product at 1/3 and 3 times the anticipated optimal dose or the control, and the study ensures the linearity of the dose-response relationship within the dose range used. In general, 5 or more measurements are required to ensure the reliability of the standard deviation, although at least 3 measurements are needed to calculate the standard deviation. Thus, primary pharmacodynamics studies include 5 animals, assuming that some deaths may occur in the course of the study. It can be assumed that linearity (continuity) is maintained in the dose range from more than 1/5 to less than 5 times the anticipated optimal dose. Dose-range finding studies to evaluate the potential toxicity after a single dose include a dose level more than 10 times the effective dose to ensure nonlinearity (discontinuity), and further development is warranted if no toxicity has been observed in rodents at these dose levels.

It is important to decide which rodents or non-rodents are included, what kind of pathophysiological animal model should be used, and what parameters should be assessed. For example, in the case of a cell-based medicinal product that is injected into the coronary artery for the treatment of a cardiac disorder, it is difficult to make proper assessments in rodents because of their vascular size. Cardiac function can be evaluated in large animals such as swine, by CT, MRI, or echocardiography. It is also important to elucidate the mechanism of action in primary pharmacodynamics studies. Pluripotent stem cells-derived cardiomyocytes need to be observed for their survival and maturity to myocardium after transplantation to the myocardium; on the other hand, if the proposed pharmacological effect is a cytokine effect, angiogenesis or activation of endogenous stem cells in cardiac milieu should be evaluated. If fibrinolytic effect is the key pharmacological one, comparison of the percentage of the fibrosis area ratio on histological specimens stained with Masson trichrome and Sirius red, or other specific parameters is needed. The results obtained from these pre-clinical studies are also important in terms of intellectual property. In the extrapolation of the results of pre-clinical studies to a first-in-man clinical trial, the continuity of endpoints, surrogate makers, or parameters should be taken into consideration.

Pharmacokinetic studies/disposition studies

Pharmacokinetic studies of chemical compounds are intended to evaluate the absorption, distribution, metabolism, and excretion (toxicokinetic studies) of the compound, and are collectively referred to as ADME (T) studies. The results of pharmacokinetic studies as well as those of toxicity and pharmacological studies are used for the planning of clinical studies. Pharmacokinetic studies are not required to be GLP compliant, but should be conducted in accordance with reliability standard requirements.

Disposition or metabolic fate studies, but not pharmacokinetic studies, are required for drug products for cell-based medicinal products. Disposition studies determine the distribution of a cellular product in organs by the intended mode of administration or transplantation, and are conducted to observe potential organ toxicity if the cell products resident in undesired organs. The timing of the post-dose observation period is controversial, but logic may be constructed on a case-by-case basis, depending on the properties of the cell-based product. For example, a 3-month post-dose observation period may be applied to a cellular product with cytokine effect, because the cells are not expected to survive for a long period. In contrast, a 6-month or more post-dose observation period may be necessary for cardiomyocytes derived from pluripotent stem cells, which are anticipated to survive and have an effect in the cardiac tissues as replacement therapies.

A major issue is the tracking of administered cells. To date, cells have been labeled with indium-111 or other radioisotopes, and the distribution of radioactivity in the organs has been considered to be that of the administered cells. However, the radioisotope method has limitations. First, it does not always track only viable cells after administration and second, the half-life is too short to be used for long-term tracking. Disposition studies of human-derived cells can include cell tracking using immunohistochemical detection with an anti-HLA antibody or using an in situ FISH technique.

We have reported an *Alu*-PCR detection method based on the *Alu* sequence, a repetitive sequence specific to humans [16]. Another issue is which organs should be observed in a disposition study. Observation of only organs with high blood flow (such as the heart, liver, kidney, and brain) is insufficient, although the organs to be observed depend on the route of administration. We have proposed a list of more than 30 organs to be assessed (Table 2) based on the organ panel which is used in marketing application of an antibody drug [17]. We also

reported the finding procedures of abnormality such as mass formation on gross pathological examination and histological examination [18]. For mesenchymal stem cells, it is necessary to examine possible ectopic differentiation to bone, cartilage, and fat, for which histological examinations using staining with von Kossa, Alucian blue, and HE, respectively, may be added. In case of ESCs/iPSCs-derived products, immunohistological examination may be included for the detection of residual un-/low-differentiated pluripotent stem cells.

	Organ/Tissue
Nervous system	Cerebral Cortex(Putamen), Cerebellum, Spine, Dorsal Root Ganglion, Ganglion
Endocrine system	Pituitary, Thyroid, Parathyroid
Cardiovascular system	Heart, Aorta
Respiratory system	Lung, Trachea
Renal Urinary system	Kidney, Urinary Tract, Bladder
Female reproductive organs	Ovary, Fallopian Tube, Uterus And Endometrium, Uterine Cervix
Male reproductive organs	Prostate, Testicle
Digestive tract	Submandibular Gland, Parotid Gland, Esophagus, Stomach, Duodenum, Small Intestine, Colon
Hepatobiliary system and Pancreas	Liver, Gall Bladder, Pancreas
Skin and musculoskeletal system	Skin, Musculoskeletal Muscle
Sensory system	Eyeball
Reticuloendothelial system	Tonsil, Thymus, Spleen
Hematopoietic system	Bone Marrow

Table 2: Organ panel.

Conclusions

Regulatory science has unique objectives and standards of value that may be different from those of basic and applied sciences, which pursue novelty and aim at usefulness, respectively. There are a wide range of issues to be addressed in regulatory science, which are classified into (1) investigational research (regulatory science, regulatory affairs), in which regulations by regulatory authorities, appropriate usage, international harmonization, and management of new drug development as well as their history, courses, and analyses are discussed, and are based on scientific evidence in terms of better understanding and achievement; and (2) experimental research (evaluation research, regulatory research), which develops techniques based on appropriate prediction or verification that improves the quality, effectiveness, and safety of pharmaceutical products to establish their evaluation procedures, and to turn the efforts back to the development, manufacturing, abandonment and/or review for approval. The science required to appropriately regulate the many efforts from different research fields with different directions has unique objectives and standards that are different from those of basic science and applied sciences. Regulatory science regulates knowledge and technology into the right and desirable frame or direction for humans; in other words, it is the science that makes the rules that are crucial to technological development [19-21].

Regenerative cell therapy is a young discipline, with only limited experience in clinical practice. Even for only pre-clinical studies, there have been no definite conclusions about the timing of a study and how to set up the study protocol. This may be partly because of specific circumstances in which advances in technology through the trials and errors of medical scientists have led to scientific development, unlike previous research and development, in which science led to the development of the technology. Under such circumstances and because evaluation for further clinical development has been increasingly needed, regulatory affairs has had to proceed, which may have led to a mutual distrust between researchers and regulatory scientists. The NIH-FDA regulatory science initiative describes regulatory science as a complex integration of regulatory research and regulatory affairs [22]. We are responsible for establishing scientific and rational regulations, so that a brighter future may be possible through the integration of regulatory research and regulatory affairs, making the delivery of useful regenerative cell therapy, among numerous other research advances, as fast as possible for the benefit of patients.

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