（Appendix）

Guidance on evaluation of human (allogeneic) iPS cell-derived corneal epithelial cell sheet

1. Introduction

Basic technical requirements for ensuring quality and safety of products obtained by processing human-derived allogeneic induced pluripotent stem cells (iPS cells) (hereinafter referred to as "Human (allogeneic) iPS cell processed products") are defined in the "Partial Amendment of the 'Ensuring Quality and Safety of Products Derived from Human Processed (allogeneic) iPS (-like) Cell'" (PSEHB Notification No. 0117-11, dated January 17, 2025, of the Pharmaceutical Safety and Environmental Health Bureau, Ministry of Health, Labour and Welfare [MHLW]).

The guidance on evaluation provides, in addition to the above basic technical requirements, considerations specific to a particular class of human (allogeneic) iPS cell processed products that are used as regenerative medical products applied for treatment of corneal epithelium disorders. The term "Regenerative medical products" is defined in Article 2, Paragraph 9 of the Act on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices (Act No. 145 of 1960) (hereinafter, the same definition is applied)

2. Scope of the guidance on evaluation

The guidance on evaluation provides, in addition to the basic technical requirements, points to consider for evaluation of quality, efficacy, and safety of a particular class of human (allogeneic) iPS cell processed products that are used as regenerative medical products applied for treatment of corneal epithelium disorders.

3. Positioning of the guidance on evaluation

The guidance on evaluation, which applies to human (allogeneic) iPS cell processed products currently undergoing remarkable development of technologies, provides only points to consider at the present time, but does not intend to cover considerations comprehensively. It is supposed to be revised in response to further technological innovation and accumulation of knowledge and thus not binding on application data. Product evaluation requires scientifically rational flexibility with full understanding of characteristics of individual products. In addition to the guidance on evaluation, other related guidelines in and outside Japan should be referred to.

For evaluation required for individual products, consultation with the Pharmaceuticals and Medical Devices Agency (PMDA) is strongly recommended.

4. Definitions of terms

(1) Cell sheet: The term refers to a sheet formed by cells or by cells and supporting material, which are bound to each other.

(2) Corneal epithelium: The cornea is a transparent tissue about 11 to 12 mm in diameter that forms the outer wall of the eye with the sclera and functions as a lens that refracts light from the outside. It consists of three layers, of which the top surface is the corneal epithelium followed by the corneal stroma and corneal endothelium in this order. The corneal epithelium further consists of 4 to 5 layers of corneal epithelial cells.

(3) Corneal epithelium disorders: The term refers to any pathological condition of the corneal epithelium with the functions impaired by various factors such as limbal stem cell deficiency (LSCD).

(4) Limbal stem cell deficiency (LSCD): The term refers to severe conditions of corneal epithelium disorders in which corneal epithelial stem cells in the corneal limbus are lost by trauma or disease.

(5) Cell bank: The term refers to a collection of a substantial number of aliquots with uniform composition filled in containers stored under a certain storage condition. That is, each container contains an aliquot of a single pool of cells. (as defined in ICH Q5D "Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products" [PMSB/ELD Notification No. 873, dated July 14, 2000, of the Evaluation and Licensing Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare or MHW])

(6) Cross-contamination: The term refers to unintentional transfer of substance from one sample to another sample. It is also referred to as contamination between samples. It means contamination between raw materials used for manufacturing and between intermediates. Potential cases are, for example, where cells derived from a cell bank are unintentionally transferred into a cell population from another cell bank; and where a pre-virus-inactivation raw material is unintentionally transferred into a batch of the post-virus-inactivation raw material.

(7) Surrogate indicator: The term refers to an indicator that has been correlated to the intended target indicator in advance and thus can be substituted for it in the event where intended measurement is difficult.

(8) Supporting material: The term refers to non-cellular materials used as scaffolds to form cells into a sheet. Whether it is contained in cell-sheet products to be transplanted/administered is disregarded.

(9) Corneal epithelial barrier function: A stratified epithelial cell layer such as the corneal epithelial layer has a surface in which cells are tightly bonded, forming adhesion structure called tight junction and thereby not allowing foreign substances and water to penetrate freely. This function is called barrier function.

(10) Symblepharon: The term refers to partial or complete adhesion between the palpebral conjunctiva and the bulbar conjunctiva.

(11) Slit lamp microscopy: The term refers to a type of biomicroscopy with a narrow beam called slit-shaped light applied to the eye ball. The observation mainly covers the anterior part of the eye, such as the cornea, anterior chamber, iris, and lens. With a dedicated lens, observation of the posterior part of the eye is possible.

5. Points to consider for evaluation

For the time being, the guidance on evaluation is intended to apply to evaluation of sheets containing corneal epithelial cells as human (allogeneic) iPS cell processed products (hereinafter referred to as corneal epithelial cell sheets). Corneal epithelial cell sheets are manufactured at the manufacturing site where human (allogeneic) iPS cells already established as a cell line and used as a source material for regenerative medical products are accepted and processed. To establish human (allogeneic) iPS cells from somatic cells and manufacture regenerative medical products using the established cells as a source material within the same manufacturing site, not only the guidance on evaluation but also the "Guidelines on Ensuring Quality and Safety of Products Derived from Processed Cell and Tissue

(Allogeneic iPS (-like) cells)" (PFSB Notification No. 0907-5, dated September 7, 2012, of the Pharmaceutical and Food Safety Bureau, MHLW) should be referred to.

(1) Raw materials, etc.

Used as raw materials, etc., iPS cells should be from a cell line of human (allogeneic) iPS cells established as a source material for regenerative medical products, which needs to be confirmed or reasonably expected to differentiate into corneal epithelial cells through a certain manufacturing process. For human iPS cells established through genetic reprogramming by transfection with reprogramming genes in somatic cells, presence of residual transgenes should be ruled out if possible. If the presence could not be ruled out, the transgenes should be demonstrated to have no adverse effects on quality or safety of the final product, the corneal epithelial cell sheet.

(2) Matters warranting special attention in the manufacturing process

For manufacture of corneal epithelial cell sheets (final products), the manufacturing method should be clarified and validated for the following items to the extent possible to ensure certain quality.

[1] Presence or absence of lot configuration and specification of lot

Whether the final product and intermediate product are manufactured on a batch basis or not should be clearly stated. If it is manufactured on a batch basis, definition of a batch should be provided.

[2] Manufacturing method

The manufacturing method up to release of the final product should be outlined, including a history of the human iPS cell line to be used as a source material, acceptance of this raw material at the manufacturing site, a process of cell banking where applicable, and generation of adequately differentiated cells. In addition, the treatment, necessary process control, and quality control should be specified in detail.

a) Acceptance inspection

For the human iPS cell line to be used as a source material, test and inspection items for acceptance at the manufacturing site (e.g., visual inspection, microscopic examination, viability, characterization of cells, and tests to deny bacteria, fungi, viruses, etc. contamination) should be specified with the criteria for each item. Where necessary and possible, tests for bacteria, fungi, viruses, etc. should be performed to the extent that would not affect the phenotype, genetic traits, characteristics such as specific functions, cell viability, or quality. If the result is positive, stock of the human iPS cell line and the transportation should be checked for contamination, and a human iPS cell line should be obtained again.

If testing with a partially processed material is appropriate because of a technical reason, the tests should be performed at an appropriate timepoint after acceptance. For example, the tests may be additionally performed before expansion culture of human iPS cell line, which has been accepted in a frozen state based on test and inspection results at the time of manufacture of the source material (certificate of analysis) and then thawed. At a stage prior to the start of a clinical trial, values measured with test samples obtained to date should be presented, and based on them, provisional values should be indicated.

b) Cell banking

For manufacture of corneal epithelial cell sheets, a process of manufacturing the final product from human iPS cell line accepted at the manufacturing site may have two patterns with and without cell banking. If cell banks are generated, methods for the generation, characterization, storage, maintenance, control, and renewal as well as procedures related to other operation processes and tests should be clearly described in detail and justified with reference to ICH Q5D. However, a part of investigation matters may be omitted if justified by evaluation completed in the upstream process.

c) Preparation of cells to be used as a component of the final product

A method of preparing the cells, to be used as a component of the final product, from the human iPS cell line accepted at the manufacturing site or from its cell bank (including a differentiation induction method, methods of isolation and culture of intended cells, medium at each stage of culture, culture conditions, culture period, and yield) should be specified and justified to the extent possible.

d) Measures to prevent mix-up and cross contamination during the manufacturing process

In the manufacture of corneal epithelial cell sheets (final products), prevention of mix-up and cross contamination during the manufacturing process is of importance, and the preventive measures in the process control should be specified.

e) Establishment of process conditions for manufacture at multiple cell culture processing facilities and cell processing in hospitals

If manufacture is completed involving multiple cell culture processing facilities, conditions for transporting intermediate products from one facility to another facility should be pre-determined. Monitoring should be in place to check if conditions for release, acceptance, and transportation of intermediate products are met.

(3) Quality control of products

Points to consider for quality control of corneal epithelial cell sheets (final products) may include, for example, the following (Reference data 1 to 3). However, adoption or addition of other test items should be considered where necessary and appropriate. In this case, a rationale for including each test item and validity of the test method should be explained. If control values for in-process control and acceptance limits for quality specifications are established at a stage prior to start of a clinical trial, values measured with test samples obtained to date should be presented, and based on them, provisional values should be indicated. If technical difficulties preclude tests with the released product itself or a part of it, tests with substitute samples such as products manufactured in parallel should be performed after being justified.

Before quality control of corneal epithelial cell sheets (final products) is established, the transplantation method should be specified. The transplantation method may include, for example, operations of removing a necessary number of corneal epithelial cell sheets (final products) (e.g., 1 sheet for each treatment practice) from a container and attaching them to an appropriate application site (e.g., affected ocular surface after removal of scar). Confirmation of the following items should be considered: a) shape; b) availability of intact cell sheet; c) cell count and viability; d) characteristics specific to corneal epithelial cells; e) content of main component cells; f) functions; and g) absence of undifferentiated cells. Methods are shown below.

a) Shape

For the shape of the final product, visual observation and phase contrast microscopy may be employed to confirm the whole shape (e.g., transparent membranous tissue) and cell morphology specific to epithelial cells (e.g., cobblestone-like cell form).

b) Availability of intact cell sheet

For mechanical suitability of the final product, test operations ranging from removal of the cell sheet from a culture container to its collection may be performed as done for transplantation to confirm that the collected cell sheet is intact.

c) Cell count and viability

For cell count and viability in the final product, criteria should be specified. Cell count may be determined using a cell suspension prepared from a portion of the final product or intermediate product by a measurement method eligible for validation (e.g., a method using hemocytometer or cell counter). Cell viability may be determined by a measurement method eligible for validation (e.g., a trypan blue dye exclusion or fluorescent dye method) to count viable and dead cells.

d) Characteristics specific to corneal epithelial cells

For cells constituting the final product, expression of genes related to corneal epithelium (e.g., Keratin 3, Keratin 12) may be confirmed by immunostaining (Reference data 4 and 5).

e) Content of main component cells

Of cells mainly constituting the final product, nature and proportion should be confirmed. For this confirmation, expression rates of epithelial cell markers (e.g., Keratin 14, pancytokeratin) may be determined, and the results may lead to confirmation that the main component cells are epithelial cells and what percentage they account for (Reference data 1 and 3). The measurement method may use, for example, flow cytometry of a post-immunostaining cell suspension prepared from the final product.

f) Functions

The final product should be confirmed to have functional properties consistent with the intended therapeutic use. Characterization of corneal epithelial cell sheets may cover the following:

・ Stem cells, precursor cells

Corneal epithelial cell sheets (final products) contain epithelial stem cells and precursor cells, which continue supplying new epithelial cells and thereby support repeated turnovers of the cells at a certain interval (Reference data 1). The above mechanism maintains the functional epithelial tissue for a long period of time after transplantation. For confirmation of stem cells and precursor cells, expression of markers reported to represent their properties (e.g., p.63) (Reference data 6) may be checked by immunostaining, etc.

・ Corneal epithelial barrier function

To confirm the barrier function of corneal epithelial cell sheets (final products), expression of markers reported to correlate to this function (e.g., ZO-1) (Reference data 7) may be checked by immunostaining.

g) Absence of undifferentiated cells

Presence of undifferentiated cells in the final product may be assessed based on expression levels of marker genes determined by quantitative RT-PCR. Of the marker genes, *LIN28* gene is highly specific to undifferentiated cells and provided with a highly sensitive quantitative analysis, which can be used as an assessment method. For studies to evaluate tumorigenicity, the nonclinical study section should be referred to.

(4) Stability testing of products

If a cell sheet is transported as the final product, storage conditions and expiration date should be specified by evaluating stability not only during storage but also during transportation (effects of temperature, vibration, atmospheric pressure change, etc.), and an appropriate container, preservation fluid, and transportation form should be selected. The appropriate storage form, temperature conditions, and transportation fluid to maintain the product stability may differ depending on the product form or cell type. For each product, an appropriate combination should be investigated to warrant stability.

(5) Quality and safety of non-cellular materials

Non-cellular materials related to the final product may include those that come in contact with the final product during the manufacturing process (e.g., culture containers used in the cell sheet culture process) and supporting materials (e.g., amnion, fibrin gel). A cell sheet of the final product may be formed without supporting materials. If they are used, knowledge about quality and safety of the materials, including their use, should be clearly presented.

(6) Nonclinical studies

When tumorigenicity of regenerative medical products manufactured by processing human (allogeneic) iPS cells is evaluated, it should be noted that "correlation and causal relationship between tumorigenicity of iPS cells used as a raw material and that of the final product remain to be elucidated." That is, it must always be noted that for clinical application, in tumorigenicity evaluation, the greatest importance is attached to the final product, a human (allogeneic) iPS cell processed product but not human (allogeneic) iPS cells used as a raw material. Tumorigenicity studies should be conducted with the final product. Tumorigenicity evaluation using a study system with the known detection limit is useful. There are two major types of tumorigenicity evaluation of the final product according to the purpose. Tumorigenicity studies are conducted for the purpose of "quality control" (to check an amount of tumorigenic cells present in the final product) or for the other purpose of "nonclinical safety evaluation" (to check whether cells in the final product exhibit tumorigenicity in a microenvironment corresponding to the administration site in humans). Tumorigenicity evaluation must be performed with either of the two purposes specified. For tumorigenicity evaluation, the "Guideline for Tests for Detecting Undifferentiated Pluripotent Stem Cells and Transformed Cells, Tumorigenicity Studies, and Genetic Stability Evaluation of Human Cell-Processed Products" (PSEHB/MDED Notification No. 0627-1, dated June 27, 2019, of the Medical Device Evaluation Division, Pharmaceutical Safety and Environmental Health Bureau, MHLW) should be referred to.

[1] Tumorigenicity studies for quality control of final products

Tumorigenicity studies for quality control may be conducted as subcutaneous dose studies in severe immunodeficient animals (e.g., NOD/SCID/γCnull (NOG) mice, NOD/SCID/IL2rγKO (NSG) mice, and Rag2-γC double-knockout (DKO) mice), which allow highly sensitive *in vivo* evaluation. To detect undifferentiated cells and transformed cells that may contribute to tumorigenicity of the final product, *in vitro* evaluation methods may be used. To detect undifferentiated cells, applicable evaluation methods may include, for example, flow cytometry using undifferentiated cell markers (e.g., *LIN28*, *TRA-1-60*) as indicators, quantitative RT-PCR, and an assessment method in which the final product is returned to the culture condition for iPS cells used as raw materials, etc., and incubated for a certain period. To detect transformed cells, applicable evaluation methods may include, for example, cell growth characterization (Reference data 1) and digital soft agar colony formation assays. Importance should be attached to comprehensive evaluation of a tumorigenicity risk using evaluation methods suitable for the final product.

[2] Studies to evaluate nonclinical safety and efficacy of final products

The purpose of nonclinical studies of a final product is to evaluate safety and efficacy of the final product in the same local environment as the application site in humans. There is a report on the evaluation in which a corneal epithelial cell sheet, the final product, was transplanted in a disease rabbit model, but such evaluation is not considered to represent meaningful evaluation in the same local environment as that in humans, because the product, a heterologous graft, required use of immunosuppressants, which failed to suppress rejection, and resulted in a graft failure in a short term. Furthermore, transplanting a corneal epithelial cell sheet in eyes of immunodeficient small animals (e.g., mice, rats) is technically difficult and has not been reported so far. In view of the current situation where appropriate evaluation using animal models is difficult, transplantation

cases in clinical settings may be given priority as references over nonclinical studies, even if the number of cases is limited. The cornea at the administration site of the final product is an avascular tissue and thus rarely has lesions associated with tumorigenesis, etc. In contrast, in subcutaneous dose studies in severe immunodeficient animals (e.g., mice) used as tumorigenicity studies for quality control, the product is administered into subcutaneous tissue rich in blood vessels, unlike the cornea, which can represent a microenvironment facilitating tumorigenesis. For nonclinical safety evaluation of the final product with a focus on tumorigenicity, results may be estimated from results from the *in vivo* studies used as tumorigenicity studies for quality control to a certain extent.

In any case, nonclinical safety and efficacy evaluation of a corneal epithelial cell sheet, the final product, critically warrants comprehensive discussion and judgement based on scientifically meaningful information available to date.

(7) Clinical studies (clinical trials)

[1] Indication

The product should be indicated for corneal epithelium disorders. Limbal stem cell deficiency (LSCD) is particularly common.

To ensure that a clinical study is conducted in a population suitable for efficacy and safety evaluation, inclusion/exclusion criteria and evaluation criteria should be established with clinical positioning of the investigational treatment clarified, using internationally accepted diagnostic criteria, severity classification, etc.

[2] Sample size determination

The sample size should be determined based on scientific rationale and according to the study objectives, hypotheses to be tested, study success criteria, and study design.

[3] Efficacy evaluation

In general, the primary efficacy evaluation should be performed using endpoints that have been assessed for reliability and validity and internationally accepted. For the evaluation, a change in endpoint from baseline and proportion of patients with improvement at the evaluation timepoint should be used. Secondary efficacy evaluation is useful not only to validate the results on the primary endpoint but also to discuss the results obtained extensively for the clinical significance. For examination items that can be affected by subjective judgement or expected to vary depending on the method of use of measuring instruments, measures such as training of raters should be fully considered to ensure uniform assessment among raters and to minimize variations among them.

In the field of ophthalmology, measurement of corrected visual acuity using the ETDRS visual acuity chart, decimal visual acuity chart, etc. is internationally accepted and thus can be used as an efficacy endpoint. However, patients with corneal epithelial disorders including limbal stem cell deficiency may have concurrent ocular diseases (e.g., corneal stromal opacity, cataract, retinal disease, glaucoma, optic nerve disease) at sites other than the corneal epithelium, which is indicated for sheet transplantation. These concurrent diseases may interfere with recovery of visual acuity even if the transparent corneal epithelium is successfully restored by the sheet transplantation. The above point should be taken into account if corrected visual acuity is used in the efficacy endpoint in a clinical study.

In view of these situations, improvement of, for example, the following items from baseline to a post-transplantation timepoint can be used as efficacy endpoints.

1) Severity of corneal epithelium disorders (limbal stem cell deficiency), etc.
2) Corneal opacity
3) Corrected visual acuity
4) Corneal neovascularisation
5) Symblepharon
6) Subjective symptoms (e.g., eye pain, foreign body sensation, lacrimation, photophobia)

[4] Safety evaluation

An adverse event is defined as any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product (including regenerative medical products; the same applies in this section) and which does not necessarily have to have a causal relationship with this treatment. An adverse event can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal product. If an adverse event is observed, name of the event, severity, outcome, time of onset and outcome confirmed, use status of investigational drugs (including investigational products; hereinafter the same applies in this section) as well as presence or absence of treatment and its details should be recorded in a case report form, and whether it corresponds to a serious adverse event and whether its causal relationship to the investigational drug can be ruled out should be assessed.

In clinical studies of a corneal epithelial cell sheet derived from iPS cells, tumorigenesis and rejection are adverse events of special interest that should be monitored after the transplantation. Slit-lamp microscopy, used in ophthalmic practice, allows direct observation of the site of transplantation and thereby highly sensitive detection of tumorigenesis and rejection.

Corneal epithelium is reported to be less immunogenic (Reference data 8), and transplantation cases both with and without immunosuppressants can occur. If immunosuppressants are used, attention should be paid to adverse events during or after their use as well.

6. Reference data

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