Development of regenerative medical technology working toward practical application

— Construction of human cell processing system in view of safety for the purpose of clinical application —

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Recently, technology of regenerative medicine which utilizes cells after their proliferation and differentiation process has drawn attention. In order to utilize the technology for clinical application, safety issue of the process as well as usefulness of the cells should be confirmed. We analyzed the issues and succeeded in utilizing the cells after proliferation / differentiation process for the purpose of therapeutic applications.

Keywords : Regenerative medicine, cell culture (cell cultivation), cell differentiation, biomaterial, international standard

1 Introduction

With the recent advances in life science technology, application of advanced medical technology enables revolutionary treatment of diseases for which cure was not available. Advanced medical technology makes new treatment a reality even for serious disease where only option for cure was organ or tissue transplantation. For example, treatments for various intractable diseases are being attempted by regenerative medicine using cells. In regenerative medicine, harvested cells normally require the processes of growing (proliferation) and processing (differentiation) by culture. Needless to say, there must be no contamination by bacteria, mold, or virus in the culture process. Moreover, it is mandatory to ensure safety and efficacy of proliferated and differentiated cells in addition to preventing contamination. Various kinds of cells are used in regenerative medicine, and some are at the stage of practical use along with various risks, from basic research using ES cells to treatment using patient's own somatic cells. Recently in news is the possibility of using induced pluripotent stem cells (iPS) developed by Professor Yamanaka *et al* of Kyoto University instead of ES cells which have ethical issues. However, currently both ES and iPS cells cause tumor called teratoma, and their safeties have not been established and therefore they cannot be used in actual treatment. Considering the above background, this paper reviews the issues in development of regenerative medical technology. Also, to promote early clinical application, we established a medical system for bone regeneration technology that can be readily accepted in the society. I shall describe our approach and results.

2 Issues in the development of regenerative medical technology

Regenerative medicine is a branch of medicine specializing in repairing and regenerating the functions of organs or tissues that were lost by disease or injury, through transplantation of cells or tissue derived from cells. Unlike conventional treatment, it involves the processes of growing (proliferation) and processing (differentiation) cells by engineering techniques for cultivation. Therefore, it is necessary to ensure safety in the processes of cell selection and cultivation. For example, for growing human (mammal) cells, cells are cultivated in culture medium containing various amino acids and vitamins. However, if the culture is contaminated by just one bacterium, since the growth rate of bacteria is several times faster than human cells, there will be far more bacteria by the time human cells have grown sufficiently. Infection may occur when such cultured cells are transplanted to the patient. To prevent such infection, cultivation must be conducted in strictly controlled, bacteria-free environment, or cell processing center (CPC) that specializes in growing human cell (Figure 1).

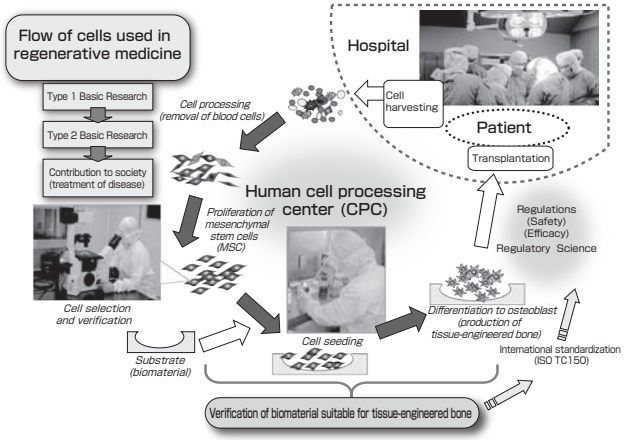

Fig. 1 From culture to transplantation of patient cell.

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While growing and processing cells are conducted in CPC, cells that were harvested from patients in hospitals contain various cells other than target cells. Target cells must be isolated and then grown. To accomplish this, development of cell selection technique and evaluation of whether the selected cells are growing well is necessary. The grown cells are converted to specialized cells after process of differentiation so they may develop into tissues or organs that must be regenerated. Evaluation of whether the differentiated cells possess ability as specialized cells is also necessary. In some cases the grown cells are transplanted directly to patient, but in many cases (for example, in our work of bone and joint regeneration), cells and biomaterials are combined and this hybrid material is transplanted. In this case, it is also necessary to evaluate safety of the biomaterial used as well as efficacy of the cells, such as whether the material supports cell differentiation. Standardizing these evaluation processes will enable application to greater number of patients, and hence the treatment may be accepted by the society. The above discussion can be summarized into four points.

1) Providing environment for cell processing center (CPC)
2) Selection of target cell and verification of proliferative ability
3) Verification of cell differentiation (verification of biomaterial)
4) Standardization of regenerative medicine

3 Our efforts on regenerative medicine issues

3.1 Providing environment for human cell processing center (CPC)

Figure 1 shows the diagram of cultivation process. Cells (bone marrow) are harvested from the patient in hospital. The cells are transported to our CPC where they are grown. Although in some cases the grown cells are directly transplanted, they are differentiated into component cells of tissues or organs after further differentiation process. The differentiated cells are delivered to the hospital and transplanted to the patient in the hospital. Normally, this differentiation process takes place on various biomaterials[1].

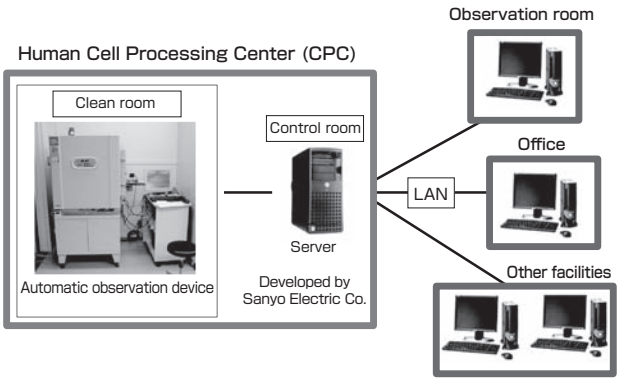

Fig. 2 Providing environment for human cell culture (development of automatic cell observation device).

As mentioned earlier, when bacteria or mold contaminate the culturing processes, bacteria grow along with target cells and the cells become unsuitable for use. Bacteria are present in ordinary environment. Therefore, cell culture maneuvers are conducted in sterile cabinets of the CPC, which is supplied with air from which fine particles are removed by HEPA filter used in clean room in semiconductor plants. Even if sterile environment can be created physically in the CPC, various bacteria exist in human body, and human operator may serve as a source of contamination. However, an operator is absolutely necessary for growing and processing cells in CPC. Also, observation of cell through microscope in the CPC is necessary to check whether the cells are growing and differentiating properly. To minimize entry and exit of operators into the CPC, we developed an automatic cell observation device with Sanyo Electric Co., Ltd.[2]. As shown in Figure 2, using this device, image of any culture dish in any position designated by the user can be observed from remote location via LAN. The cells can be observed without entering the CPC, and sterile environment remains intact. Moreover, culture processing is conducted according to strict quality control procedures, and the work of recording cultured cell data increases the workload of the operator.

Figure 3 shows the images of cell observed at every 24-hour interval using the device. All observations were made for same area (fixed point). The number of cells increases with passage of time, and one can see that the cells are growing properly. There had been no device that allowed remote observation of microscopic field of cell culture at regular time intervals with reproducible results, and thus the significance of this development is high. The device also allows regular recording of observation results as image data. We were able to develop technology that contributes to improvement of quality control and reduction of worker load. Ideally, cell culture that involves no human hands is desirable, and we are working on automatic cultivation device, but I shall not

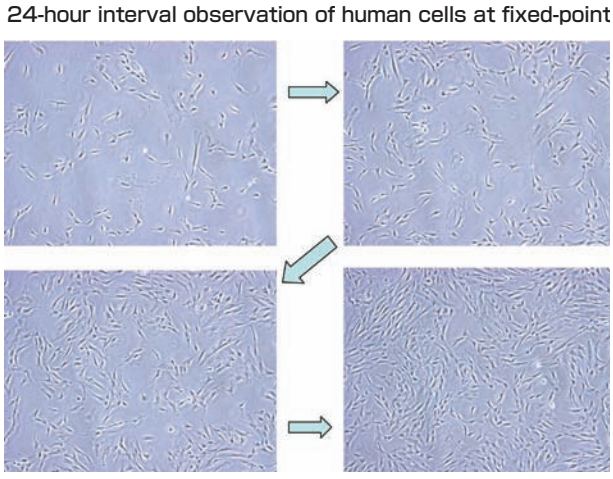

Fig. 3 Fixed-point observation by automatic cell observation device (automatic transportation incubator with cell observation function).

elaborate on this due to space limitation.

3.2 Technology for selecting target cell (verification of cell growth)

Cell growth process is the first process in any regenerative medicine. However, since the harvested cells contain various types of cells, separation of target cell from the mix of cells is necessary. For example, we select and grow mesenchymal stem cell (MSC) from fresh bon marrow that contains hematopoietic cells such as red and white blood cells as well as others. Fresh bone marrow is sowed on culture dish, and the floating blood cells are removed by replacing the culture medium. Thereafter, cells that adhere to the culture surface grow and can be collected. In fact, the cell population collected by this method presents various marker expression usually seen in MSC. However, even at this stage, the cultured cells consists of cell population with different proliferative ability and is not homogenous. This means it is difficult to determine whether the MSC collected at this moment will grow as expected.

In our experience in clinical application, we observed that the growth rate decreases when the nucleus of MSC in culture became thin and the cells flattened in shape. Therefore, we decided to estimate the proliferative ability by measuring this phenomenon quantitatively. We investigated the correlation between the thickness of MSC measured using atomic force microscope and the cell proliferation activity. We found that compared to MSCs with low proliferative ability, the cells with high proliferative ability were smaller and had increased thickness of the nuclear region[3]. However, the atomic force microscope is extremely expensive, difficult to operate, and takes time to make measurements. Therefore, we worked with Olympus Corporation to investigate whether evaluation of cell proliferation activity level in culture was possible by observing the thickness of area of MSC nucleus and the shape of cell (plane) using light microscope image, and then evaluated cell proliferation activity and developed device to measure proliferative activity using these indices. To measure the thickness of cell by light microscope, the phase

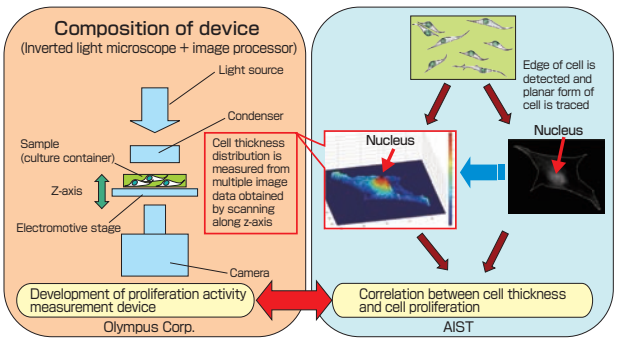

Fig. 4 Development of cell thickness measurement device (evaluation technology for cell proliferation activity and development of the device).

image of MSC that adhered to the culture dish was obtained, and numerical information corresponding to cell thickness and cell surface area were obtained by image processing and analysis software. As shown in Figure 4, the MSC in culture were displayed 3-dimensionally, and the thickness could be measured automatically. Using this device, proliferative ability of the cultured cell could be estimated non-invasively, and it could also check proper growth of the cells. We succeeded in developing technology that allows cell cultivation with higher efficacy. This device can be linked to existing light microscope, and can be accessory device to microscopes that are installed in hospitals and research centers. The device we developed has excellent cost performance, and it is expected that it will be used in various places in the future.

3.3 Verification of cell differentiation (verification of differentiation on material in case cells are hybridized with biomaterials)

In the development of regenerative medical technology, we have worked on the technologies for bone regeneration. Bone regeneration involves the method for regenerative tissue-engineered bone in which MSCs are differentiated into osteoblasts with bone formation ability by cell cultivation, and the bone matrix is formed on biomaterial by these osteoblasts[4][5]. Various types of biomaterials are used to create tissue-engineered bone. Particularly, materials with porous structure to anchor cells are useful. However, evaluation of whether a biomaterial can anchor cells efficiently and whether it has ability to form new bone *in vivo* are necessary. Therefore, we compared the activities of MSC regarding properties of the biomaterial used for tissue-engineered cells, and tried to establish a methodology for assessing new bone formation *in vivo*. To standardize this evaluation method, we employed universal source for cell (in this case bone marrow from rat femur) as well as fixed procedure.

Specific procedures were as follows. Bone marrow of 7-week old rat were cultured in flask to grow MSC, and cell concentration was adjusted to 1×10^6 cell/ml. Porous materials were placed on the culture plate and immersed in adjusted cell suspension. The samples were cultured for 2 weeks using osteogenic culture condition. Detection of differentiated bone cells (osteoblasts) after completion of the culture was conducted by alkaline phostaphase staining. As shown in top photograph of Figure 5, comparing the two materials (porous synthetic hydroxyapatite and hydroxyapatite derived from coral skeleton), bone differentiation was observed only in the pores of surface of the synthetic material. In contrast, cells grew inside the pores in the coral material, and good bone differentiation was observed. The engineered cells were transplanted to rats of same strain. As shown in lower photograph of Figure 5, new bone formation (shown in red) was observed inside the material for coral hydroxyapatite.

Verification of bone differentiation ability in biomaterial used was accomplished by *in vitro* culture and *in vivo* transplantation. We developed an evaluation technique that allows predictable determination of efficacy of biomaterial used in bone regenerative medicine.

3.4 Standards for regenerative medicine

As described above, in regenerative medicine, it is necessary to constantly verify the efficiency of cultivation process as well as check whether the harvested cells, cultured and grown cells, and differentiated cells are performing properly. Considering commercialization of regenerative medicine, it is mandatory to establish the evaluation method as standards for safety and efficacy of the cells used. The standard for evaluation result is built by employing standardized cell evaluation method, and determination of safety and efficacy can be done readily. Standardization will clarify the indices for increasing efficiency of the process, and this will promote designing and production of regenerative medical products. As mentioned in Section 3.3, we are in the process of establishing the evaluation method of biomaterial used in bone regeneration medicine. Therefore, we are considering international standardization of the evaluation method. Currently, about 230 Technical Committees (TC) are active in the International Organization for Standardization (ISO), and TC150 (Implants for Surgery) is in charge of medical devices. TC150 is further broken down into Subcommittees (SC) and Working Groups (WG) where specialists from around the world engage in discussion. For regenerative medicine, standardization proposal for regenerative medical technologies were discussed in WG11 (Tissue Engineered Implants), and in January 2007, the working group was "promoted" to SC7 (Tissue Engineered Medical Products). We submitted the proposal "*In vivo* bone formation in porous materials using rat mesenchymal cell □ Standardization to evaluate bone forming ability of biomaterials," to commence activities toward regenerative medical technology standardization originating from Japan. Figure 5 shows the bone formation in the material conducted according to the proposal.

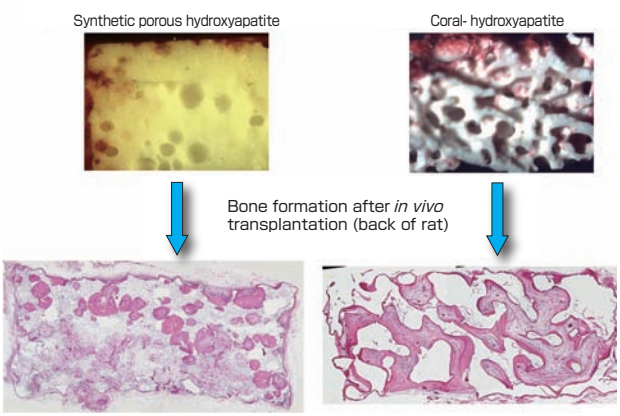

Fig. 5 Verification of biomaterials (areas of osteoblasts location after MSCs seeding).

4 Clinical application of regenerative medical technology

We conducted various technological developments with corporate partners to overcome issues in regenerative medicine. As result, we succeeded in implanting artificial joints formed with tissue-engineered bone to osteoarthritis patient for first time in the world. About 6 years have passed since the first case, and there have been more than 50 cases in total. Although the history is short, there had been no side effects such as inflammation or infection, or "loosening" at the implant site, which is adverse event for artificial joints[6]. Tissue-engineered bones were also transplanted to cases of bone tumor[7] as well as arthropathy patients. According to the survey by Fuji Keizai Co., Ltd., there are about 800,000 arthropathy patients in Japan, of which 200,000 patients are estimated to be candidates of regenerative medicine. Our technology is likely to be indicated for many of these patients. Moreover, it was confirmed that MSC could be differentiated into vascular endothelium and cardiac muscle cells[8], and we started clinical application for heart regeneration in collaboration with the National Cardiovascular Center. As described, we succeeded in developing treatments for heart diseases as well as bone and joint diseases by using cells (bone marrow cells) from patients themselves, and these cells were harvested with minimum invasion (bone marrow aspiration) without sacrificing the patients' tissues. There are estimated 1,600,000 patients with heart disease. Clinical application to wider range of tissue and organ regeneration can be expected by using the cell differentiation ability of mesenchymal cells from bone marrows to various cells and tissues.

5 Discussion (future issues)

As described above, we developed various technologies for regenerative medicine and have conducted applications or clinical studies in patients with various diseases starting with bone regeneration. However, it is necessary for the companies to spend more effort in medical applications before this technology can benefit patients. The studies must undergo the process of clinical trials, and tissue-engineered cells must be marketed as regenerative medical product after receiving approval of the Ministry of Health, Labour and Welfare. In the United States, Genzyme Corporation markets cultivated chondrocytic (cartilage) cells to over 10,000 patients under approval of the Food and Drug Administration (FDA). In Japan, Professor Ochi of the Hiroshima University conducted 3-dimensional culture of cartilage in collagen gel, and developed the cartilage regeneration technology using this cartilage collagen gel hybrid. This technology was transferred to Japan Tissue Engineering Co., Ltd. (JTEC), and the clinical trials have been almost completed but the product is not yet available. At the same time JTEC started cartilage regeneration, Sewon Cellontech Co., Ltd. of Korea started cartilage regeneration business, received approval of

the Korean Food and Drug Administration (KFDA), and used the product in nearly 3,000 patients.

In skin regeneration, which has longer history than cartilage regeneration, various products are available abroad. However, JTEC only recently obtained approval for regenerative medical product in Japan. The commercialization of regenerative medicine in Japan is obviously slow compared to other countries. The slowness of authorization and approval in Japan is evident. In the future, to promote commercialization of regenerative medicine, the government must work to establish the scientific basis for safety and efficacy of regenerative medical products.

Currently, the Medical Affairs Law regulates the Japanese medical system in business phase. For example, drugs and medical devices must undergo the process of clinical trial as designated by the Medical Affairs Law before they can be marketed. This law is based on assumption that the product will be sold to the general public. However, regenerative medicine where cells are isolated from a patient, cultured and grown, and then transplanted back to the same patient, is a technique in which the patients' own cells (autologous cell) are used. It is medical treatment for specific individual, and therefore, the Pharmaceutical Affairs Law that targets the general public may not be applicable for the treatements. Moreover, in regenerative medicine, the physician must harvest the cells from patient, and one-to-one relationship between physician and patient is established before the actual transplantation of cells, and thorough information are given on the risk and benefit of regenerative treatment using autologous cell before patient's consent is obtained. Regenerative medicine using autologous cell is clearly different from treatment using someone else's (allogenic) cell, and new approval system must be considered for this medical technology[9]. New system that is not bound by conventional concept must be created to deal with new technological development including regenerative medicine.

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Discussion with reviewers

1 Need for technology utilizing MSC

Question (Hiroshi Kuriyama)

I think it is necessary to mention briefly why it is necessary to use patient-derived MSC in the Introduction. I think it is also necessary to describe the danger of iPS cell (as response to common question why iPS, which is frequently highlighted in the media, cannot be used).

Answer (Hajime Ohgushi)

In the Introduction, I added the description that iPS cell may cause tumor called teratoma when it is transplanted.

2 Importance of bone and cartilage treatment

Question (Hiroshi Kuriyama)

Perhaps you should explain how many cases require bone and cartilage regeneration treatment where this technology can be applied, as well as projection of demand in Japan and other countries in Section 4. Also, I think if you include the numbers of other cases and patients who may benefit from this medical technology, the efficacy of this technological development may become clearer (how about showing the patient figures in table?). Although the data may be available in Reference #6, maybe you should indicate the actual case figures and treatment results for bone and cartilage treatment.

Answer (Hajime Ohgushi)

I added the number of patients with arthropathy and the projected number of patients to whom regenerative medicine may be indicated. I also added the figures for patients with heart disease. The actual number of regenerative treatment for arthropathy that we conducted is 50 or so cases, so I described it as over 50 patients. Detailed description of the treatment result increases the number of words and departs from the main subject, so I simply stated that we have not observed “loosening” at implant site which is adverse event for artificial joint and that good result is maintained.