Review Article

Toward using iPS cells to treat spinal cord injury: Their safety and therapeutic efficacy

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The spinal cord, which is part of the central nervous system, has been considered a typical example of an organ in which regeneration is difficult. However, since the report of recovery of function in a spinal cord injury (SCI) model as a result of cell transplantation of rat-fetus-derived neural stem/progenitor cells (NS/PCs), stem cell transplantation therapy has attracted great hope of restoring and replenishing lost neurons and glia.

In recent years induced pluripotent stem (iPS) cells that possess embryonic stem (ES)-cell-like pluripotency and proliferative capacity have been produced by introducing several different genes into somatic cells. Rapid progress is currently being made in research on iPS cells with the aim of enabling cell transplantation therapy, and reports of the development of methods of inducing human iPS cells to differentiate into a variety of somatic cells and cases of treatment of murine models with mouse iPS cells have appeared one after another. However, when viewed from a safety standpoint, problems that arise because ES cells and iPS cells are both pluripotent stem cells and many problems unique to iPS cells, which have been artificially reprogrammed, still remain unresolved, and there is a desire for further progress in research. In this paper we outline these issues and report the latest findings in regard to application to the treatment of spinal cord injury.

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Introduction

According to the rapid progress of iPS cell researches[1-6], on June 30, 2010, a revision of the Japanese Ministry of Health, Labour and Welfare's Guidelines for Clinical Research Using Human Stem Cells was adopted by a committee, and clinical research involving the use of induced pluripotent stem cells (iPS cells), irrespective of whether homologous or heterologous, is now covered by the guidelines. The revised guideline was launched on November 1, 2010. That appears to be a major step in terms of the future development of regenerative medicine in Japan. However, fetal stem cells and embryonic stem (ES) cells are still not covered by the guidelines. The clinical trials of human ES cells-based therapy has already started in the United States in October, 2010.

The central nervous system (CNS), including brain and spinal cord, has been considered a representative example of organs in which regeneration is difficult. However, the situation has been changing based on the fact that several groups, including our own, have demonstrated that stem cells are also present in the adult CNS of mammals, including humans, and that neurogenesis occurs in the brains of adults[7-9]. The regeneration of CNS regeneration means three things: (i) axon regeneration, (ii) replenishment of cells that have been lost as a result of disease, and (iii) functional recovery[10]. It seems that a strategy based on the fundamental concept of inducing recapitulation of developmental process will definitely be necessary in order to realize the CNS-regeneration. In the fall of 2006, a Keio University–Kyoto University Joint Research Team was established as a result of the close collaboration between our research group and Professor Shinya Yamanaka's research laboratory, with the aim of applying iPS cells to the treatment of SCI. In this paper we describe basic research and the current situation in relation to iPS cells with the aim of regenerative medicine for the CNS, and we discuss future prospects.

(1) Tasks common to both iPS cells and ES cells

a) Tasks related to tumor (teratoma) formation by undifferentiated cells that persist after induction of differentiation

ES cells are endowed with both semipermanent proliferative capacity and pluripotency, i.e., the ability to differentiate into a variety of different cell types, and iPS cells possess properties that resemble ES cells in these two respects. Transplanting cells obtained after inducing these pluripotent stem cells to differentiate into the target cells is regarded as the usual method of applying them to cell transplantation therapy. However, a problem arises when this is done, because of teratoma formation caused by undifferentiated cells that remain after the induction of differentiation. We produced neurospheres containing neural stem/progenitor cells (NS/PCs) from mouse ES cells and iPS cells and assessed their safety by transplanting them into the striatum of non-obese/severe combined immunodeficient (NOD/SCID) mice[11]. The results showed that teratomas had been formed by intermingled undifferentiated cells in 10% of the group of mice into which ES-cell-derived neurospheres had been transplanted and in 40% of the group of mice into which iPS-cell-derived neurospheres had been transplanted. It has been reported that teratoma formation can be avoided by transplanting cells induced to differentiate into NS/PCs from mouse ES cells after using expression of the neural stem/progenitor cell marker Sox1 to purify them[12]. Jaenisch et al. reported that no teratoma formation occurred when they induced the formation of dopaminergic neurons from mouse iPS cells and transplanted them after eliminating cells that were positive for theundifferentiated-cell-marker SSEA-1 with a flowcytometer[5]. It seems important to establish methods of inducing differentiation that yield highly pure target cells, and to develop flowcytometers that comply with Good Manufacturing Practice (GMP) grade

and target cell purification methods that make full use of them.

b) Tasks regarding the safety of differentiated cell transplantation

Even if it is possible to avoid the risk of teratoma formation as discussed in (i), the possibility of tumor formation after transplantation of differentiated cells must be assessed long-term and carefully. In 2008, the development of a transplanted-cell-derived brain tumor was reported in a boy with ataxia telangiectasia who had undergone intracerebral transplantation of human fetal neural stem cells[13]. There are great expectations of trials of transplantation of NS/PCs differentiated from human iPS cells or ES cells as a method of treating SCI, Parkinson's disease, etc., but, depending on the circumstances, assessment of treatments that use growth-arrested cells, or novel methods to ensure safety may become necessary, e.g., introducing a suicide gene such as HSV-TK in advance so that it is possible to eliminate the transplanted cells in the worst case scenario.

(2) Tasks peculiar to iPS cells

a) Tasks from a safety standpoint as a result of introducing genes

iPS cells were initially produced by introducing four factors, i.e., Oct3/4, Klf4, c-Myc, and Sox2, by means of retrovirus vectors. c-Myc is well known as a proto-oncogene, and the other three factors are genes that are known to be highly expressed in cancer. The probability that tumors would develop in chimeric mice and their progeny produced as a result of using these four-factor iPS cells was found to be approximately 20%[14]. Because expression of c-Myc transgene that had been integrated into the genome of iPS cells with the retrovirus became reactivated in these tumors, the risk resulting from the transduction of c-Myc became a problem. However, it was later discovered that, although the rate is low, iPS cells could be generated by introducing the other three factors without c-Myc[15], and no tumors were detected in the chimeric mice produced with the iPS cells derived with the three factors even when they were observed for more than 100 days. At that point the production of iPS cells without using c-Myc had been achieved, but the problems associated with the other three factors having been inserted into the genome with retroviruses had not been resolved. Retroviruses and lentiviruses are often integrated in proximity to a gene promoter, and there is the risk that they will alter the state of expression of endogenous genes in the vicinity and cause tumorigenesis. Development of leukemia in 2 of 10 patients with X-linked severe combined immunodeficiency (X-SCID) who underwent gene therapy with a retrovirus vector has actually been reported[16]. There has been rapid progress in research on this problem in recent years, and success without using retroviruses or lentiviruses has been reported in regard to production of so called "integration free iPS cells" (i.e., iPS cells into whose genome no exogenous genes have been inserted) by using the transducing proteins[17,18], a plasmid or an episomal vector[19, 20], Sendai virus vector[21] and in vitro synthesized RNA[22]. It appears to be desirable to use the integration-free iPS cells, when using cell transplantation therapy. Nevertheless, in the future detailed comparisons of their properties will be necessary to determine whether the integration-free iPS cells produced by these methods possess pluripotency and in vitro differentiation capacity comparable to that of iPS cells produced with retroviruses.

b) Tasks from a safety standpoint associated with the reprogramming of somatic cells and the type of original cells

Somatic cell nuclear transfer (SCNT) to oocytes is known as other reprogramming methods besides induction of iPS cells. The birth rate of clone mice produced by SCNT is very low, and such abnormalities as placental hyperplasia, obesity, and a short life span

have been discovered and are thought to be attributable to inadequate reprogramming of the somatic cell nuclei. On the other hand, hardly any differences in gene expression or DNA methylation status have been found between ntES (ES cells produced from cloned blastocysts after SCNT)[23] and ordinary ES cells, and production of tetraploid chimeras is also possible. Production of tetraploid chimeras from iPS cells has also been reported recently[24], and in that respect iPS cells appear to have capacities that are fairly close to those of ES cells. However, gene expression in iPS cells and ES cells is not the same, and there are even reports that part of the gene expression patterns of the cells from which they were derived persists[25]. Moreover, we recently discovered that the response of iPS cells to inductive signals of differentiation and tumorigenic propensities vary with the type of somatic cells from which they were derived (see below). Based on these findings there is a strong possibility that some of the properties of the original somatic cells persist in iPS cells, and when they are used for cell transplantation therapy the possibility of causing tumor formation or some other form of abnormality cannot be ruled out. Because many genes are thought to be involved in somatic cell reprogramming, it may be possible to produce better quality iPS cells by increasing the number of reprogramming factors. There are high hopes that future progress in research will reveal the optimal composition of the reprogramming cocktail.

We recently demonstrated that the responsiveness of iPS cells to neural differentiation and their safety after transplantation vary greatly according to differences in somatic cell origin at the time they were generated[11]. We induced differentiation of neurospheres by using 36 mouse iPS cell clones that had previously been generated in our laboratory, and then evaluated their differentiation capacity in vitro and safety after transplantation by transplanting them into the striatum of the brains of NOD/SCID mice. The results showed that almost all of the iPS cell lines analyzed were capable of differentiating into neurospheres. However, a detailed analysis by flowcytometry showed large differences in the percentages of Nanog-EGFP-positive undifferentiated cells that remained in the neurospheres according to the type of somatic cells from which the iPS cells had been derived. A mouse embryonic fibroblast (MEF)-derived iPS cell clone showed responsiveness to induction of differentiation that was comparable to that of ES cells, and hardly any undifferentiated cells remained in the neurospheres. Teratoma formation after transplantation in a group of mice transplanted with neurospheres derived from this MEF-iPS cell clone was infrequent and minor, and it was comparable to the results in a group of mice transplanted with ES-cell-derived neurospheres. Moreover, no teratoma formation was observed during a 16-week observation period in a group transplanted with two iPS cell clones that had been produced from adult gastric epithelial cells (Stm). On the other hand, an iPS cell clone derived from adult tail-tip fibroblasts (TTFs) showed statistically significant resistance to differentiation, and many undifferentiated cells remained in the neurospheres after inducing differentiation. Formation of significantly larger teratomas was observed in the group of mice into which these TTF-iPS-derived neurospheres had been transplanted, and many of the mice soon became debilitated or died. The responsiveness to induction of differentiation and the tumor formation capacity of an adult hepatocytes (Hep)-derived iPS cell clone were intermediate between that of the MEF-iPS cell clone and TTF-iPS cell clone. On the other hand, whether c-Myc had been introduced or screening of the reprogrammed cells by means of reporter proteins had been performed at the time they were generated had no effect on the responsiveness of the iPS cells to induction of differentiation or on their safety after transplantation. It appears that persistence of the gene expression patterns of the original somatic cells from which they were derived, as stated

above, may be the reason why variations in the differentiation capacity of the iPS cells emerged according to differences in the cells from which they were derived. In connection with this, according to a recent report, early-passage iPS cells obtained by reprogramming adult mouse tissue by means of transcription factors, leave behind traces of characteristic DNA methylation in the original somatic cell tissue. They show a tendency to differentiate along cell lineages similar to the donor cells, and choices of any other cell fates are narrowed. This sort of donor tissue "epigenetic memory" has been reported to play a large role in the properties of iPS cells. Moreover, Dr. Kazuhiro Sakurada has proposed calling genes whose expression pattern in iPS cells and ES cells is different "reprogramming-recalcitrant genes"[26]. This name is based on these genes showing resistance to the same transcription status being induced as in ES cells. Identifying "reprogramming-recalcitrant genes" that are the cause of tumor formation by neural cells derived from iPS cells is definitely important in the future.

(3) Transplantation of "safe" mouse iPS-cell-clone-derived neural stem cells to treat SCI

The therapeutic effects of NS/PCs-transplantation in the treatment of SCI has been reported many times at the research level. Our own laboratory has also previously reported the effectiveness of rodent fetal NS/PCs or ES-derived NS/PCs in the treatment of SCI[27-30] and the effectiveness of human NS/PCs-transplantation in a primate common marmoset model of SCI[31-32]. Because the cells used for transplantation in these studies were derived from fetuses, it is difficult to proceed with clinical application because of the ethical issue. It appears that this ethical issue can be avoided by using iPS-cell-derived NS/PCs.

We therefore first assessed safety by in vivo transplantation into the brains of the NOD/SCID mice described above, and then conducted a study of effectiveness by transplanting neurospheres derived from a mouse iPS cell clone whose safety had been confirmed into a model of contusive SCI[33]. Neurosphere transplantation was performed in the subacute stage on day 9 after the injury. The results of bioimaging with luciferase showed that approximately 20% of the transplanted neurospheres had survived in the injured spinal cord, and they had differentiated into the three neural lineages. Severe atrophic change and demyelination had occurred in the injured spinal cord after the contusive injury, but these changes were significantly prevented in the group transplanted with iPS-cell-derived neurospheres. When hindlimb motor function was evaluated by the Basso mouse scale, significantly better recovery of function was seen in the group transplanted with iPS-cell-derived neurospheres than in the control groups (phosphate-buffered saline [PBS] transplanted group and fibroblast transplanted group). This recovery of function appeared to have been due to such effects as promotion of axonal growth in the raphespinal serotonergic fibers, which are associated with locomotor functions of hindlimbs, and remyelination by the transplanted cells-derived oligodendrocytes, in addition to the prevention of atrophic change and demyelination described above.

Next, we conducted a similar transplantation experiment using adult-tissue (TTF)--derived iPS cell clones as a more realistic clinical application model. Only one of the six TTF-derived iPS cell clones used in the safety study described above was safe, and after inducing differentiation into neurospheres of this one "safe" TTF-derived iPS cell clone and two "unsafe" TTF-derived iPS cell clones that had been found capable of tumor formation, we transplanted them into a mouse model of SCI. The results showed that although recovery of function was seen in the all of the groups into which TTF-iPS-clone-derived neurospheres had been transplanted, the function recovery that had been temporarily attained in the groups transplanted with

"unsafe" TTFiPSclonederived neurospheres was suddenly lost 6 weeks after the injury due to the tumor mass effects, and most to the mice suddenly died. Furthermore, no tumor formation was seen in the group into which "safe" TTFiPS-clone-derived neurospheres had been transplanted, and recovery of function recovery had obviously been achieved. Based on these findings, it was shown that although there is a great deal of variation among iPS cell clones in regard to safety after transplantation, if safety is rigorously assessed in advance, they are capable of serving as a source of cells that are useful for treating SCI.

(4) Future tasks and prospects

As shown above, iPS cells can be said to have great potential as a cell transplantation source for the treatment of SCI. However, their safety, i.e., the "quality" of iPS cells, must be evaluated very carefully.

At present, the methods that are generally used to evaluate iPS cells that have been generated consist of (1) transplanting cells in an undifferentiated state subcutaneously or into the testes of mice and assessing their ability to differentiate into cell types of the three embryonic germ layers in vivo by allowing them to form teratomas, (2) assessing their degree of contribution to chimeric mice and their degree of contribution to germline transmission by transplanting undifferentiated cells into blastocysts (impossible to apply to human iPS cells), (3) analysis of global gene expression by means of microarrays, and (4) genome analysis by karyotyping or comparative genomic hybridization (CGH) arrays. However, it may be impossible to adequately evaluate the safety of iPS cells by these methods alone. Our research has revealed that evaluation by in vitro differentiation induction systems is also very important. iPS cell clones that contribute to the germline of chimeric mice are not always highly responsive to induction of differentiation in vitro. On the other hand, almost all iPS cells clones with poor responsiveness to induction of differentiation and that form large teratomas after transplantation have been able to contribute to chimeric mice. These findings clearly show that it is impossible to evaluate the quality or safety of iPS cells by means of just one evaluation system, and evaluation from multiple angles is essential. iPS cells have the advantage of being able to conveniently establish many clones, but at the same time it has been becoming clear that there are large variations in quality between the clones. It appears that picking out the highest quality clone from a number of iPS cell clones that have been established from individual patients is a necessary task, but evaluating each of the clones from many different aspects including karyotypes, flowcytometric analysis, gene expression profile, whole genome methylation patterns and tumorigenic propensities of NS/PCs derived from them, is extremely unrealistic in terms of both time and cost. It seems that a screening system that makes it possible to conveniently separate out good quality clones will need to be established in the future. It was recently reported that there is a correlation between the potential of ES/iPS cells for germline transmission and ability to contribute to tetraploid chimeras and the activity of the Dlk1-Dio3 imprinted gene cluster on mouse chromosome[12, 34, 35]. However, as stated above, there is a strong possibility that the markers of potential for germline transmission and ability to contribute to chimera mice and the markers of ability to differentiate in vitro are different. Moreover, which differentiated cells to produce and what to use them for, and the criteria that they will have to meet are expected to differ even more according to their intended purpose. It seems that it will be necessary to search for a variety of markers that will serve as indicators of responsiveness to induction of differentiation that is suited to each individual purpose.

The generation of iPS cells is an extremely large first step toward making cell transplantation therapy with self-derived pluripotent stem cells a reality. In view of the cur-

rent rate of progress in iPS cell research worldwide, we have the feeling that the day when iPS cells will actually be applied to transplantation therapy of SCI is not very far off. However, as stated in this article, many tasks regarding safety when iPS cells are applied to transplantation therapy remain, and when they have been resolved it will be possible to administer them to humans for the first time. It is important to proceed with research in a multifaceted manner while conducting careful evaluations. In addition to research directly linked to clinical applications and to basic research with a view to clinical applications, it appears important to freely proceed with a variety of basic research, to vigorously debate the information obtained, and to integrate it.

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