The therapeutic potential of ex vivo expanded CD133+ cells derived from human peripheral blood for peripheral nerve injuries

Laboratory investigation

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Object. CD133+ cells have the potential to enhance histological and functional recovery from peripheral nerve injury. However, the number of CD133+ cells safely obtained from human peripheral blood is extremely limited. To address this issue, the authors expanded CD133+ cells derived from human peripheral blood using the serum-free expansion culture method and transplanted these ex vivo expanded cells into a model of sciatic nerve defect in rats. The purpose of this study was to determine the potential of ex vivo expanded CD133+ cells to induce or enhance the repair of injured peripheral nerves.

Methods. Phosphate-buffered saline (PBS group [Group 1]), 105 fresh CD133+ cells (fresh group [Group 2]), 105 ex vivo expanded CD133+ cells (expansion group [Group 3]), or 104 fresh CD133+ cells (low-dose group [Group 4]) embedded in atelocollagen gel were transplanted into a silicone tube that was then used to bridge a 15-mm defect in the sciatic nerve of athymic rats (10 animals per group). At 8 weeks postsurgery, histological and functional evaluations of the regenerated tissues were performed.

Results. After 1 week of expansion culture, the number of cells increased 9.6 ± 3.3–fold. Based on the fluorescence-activated cell sorting analysis, it was demonstrated that the initial freshly isolated CD133+ cell population contained 93.22% ± 0.30% CD133+ cells and further confirmed that the expanded cells had a purity of 59.02% ± 1.58% CD133+ cells. However, the histologically and functionally regenerated nerves bridging the defects were recognized in all rats in Groups 2 and 3 and in 6 of 10 rats in Group 4. The nerves did not regenerate to bridge the defect in any of the rats in Group 1.

Conclusions. The authors’ results show that ex vivo expanded CD133+ cells derived from human peripheral blood have a therapeutic potential similar to fresh CD133+ cells for peripheral nerve injuries. The ex vivo procedure that can be used to expand CD133+ cells without reducing their function represents a novel method for developing cell therapy for nerve defects in a clinical setting.

Abbreviations used in this paper: CMAP = compound muscle action potential; FACS = fluorescence-activated cell sorting; PBS = phosphate-buffered saline.

This article contains some figures that are displayed in color online but in black-and-white in the print edition.

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The fresh CD133+ cells were cultured with serum-free blood (lot no. PCA1521B, AllCells) for these experiments. The purity of the CD133+/CD34+ cells in the freshly isolated CD133+ cells was assessed using a FACSCalibur analyzer (Becton Dickinson and the CellQuest Pro software program 4 times (Becton Dickinson Immunocytometry Systems).

Cells were stained with phycoerythrin-conjugated anti–CD34 antibodies (clone 581, BD Pharmingen) and allophtocyanin-conjugated anti–CD133 antibodies (clone 293C3, Miltenyi Biotec). After washing, the cells were resuspended in staining medium supplemented with 7-amino-actinomycin D. Stained cells were analyzed by FACS, and dead cells stained with 7-amino-actinomycin D were excluded from the analysis.

Experimental Animals

Eight-week-old female athymic nude rats (F344/NJcl-rnu/rnu, CLEA Japan, Inc.), each weighing between 130 and 160 g, were used in this study. The rats were fed a standard maintenance diet and were provided with water continuously.

Surgical and Grafting Procedures

The sciatic nerve defect was induced in rats as described previously. In brief, the rats were anesthetized by intraperitoneal administration of ketamine and xylazine (60 and 10 mg/kg body weight, respectively). The left sciatic nerve was exposed, a 10-mm section was excised at the center of the thigh, and a 15-mm defect was prepared. This defect was bridged with a silicone tube that was 1.5 mm in internal diameter and 17 mm long. The proximal and distal stumps of the nerve were inserted 1.0 mm into the tube and were connected to the tube using 3 stitches through the epiperineurium and the outer wall of the silicone tubes by using 8-0 monofilament nylon threads, leaving a 15-mm gap between the exposed ends. The rats underwent transplantation with silicone tubes infused with PBS (PBS group [Group 1]), 10^6 of freshly isolated CD133+ cells (fresh group [Group 2]), 10^6 of ex vivo expanded CD133+ cells (expansion group [Group 3]), or 10^4 of fresh CD133+ cells (low-dose group [Group 4]) that were embedded in 1% atelocollagen gel (Koken) to analyze the effects of ex vivo expanded CD133+ cells and the proper numbers of cells in comparison with the previous experiment.

Macroscopic Observation

We observed the grafted sites macroscopically through the silicone tubes to confirm the continuity and the thickness of the regenerated nerve. If there was continuity in the grafted sites, the sites were removed for electrophysiological research.

Electrophysiological Studies

As a functional evaluation of the regenerated tissue, the regenerated axons were electrophysiologically assessed in each group. The bilateral sciatic nerves were exposed, and needle electrodes were placed in the gastrocnemius muscle using the tendon-belly method. The nerves were stimulated with constant currents at supramaximal intensity (0.2-msec square-wave pulses) using bipolar electrodes. The stimuli were applied to the left sciatic nerve proximal to the silicone tube and to the right sciatic nerve at the same level as the left side. The CMAPs were recorded after stimulation using a commercially available system (Viking Quest, Nicolet Biomedical) after they traversed a bandpass filter of 0.5–2000 Hz. An epoch of 25 msec after stimulation was digitized at a 5-kHz sampling rate. The onset latency and peak-to-peak amplitude of the CMAPs from the experimental side were compared with those recorded from the contralateral normal side. All rats were euthanized with an overdose of sodium pentobarbital at the end of electrophysiological studies.
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Morphometric Evaluation

The regenerated tissues in the tubes were removed and immersion-fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. The specimens were postfixed in 1% OsO₄ in 0.1 M phosphate buffer (pH 7.4) for 3–4 hours at 4°C, dehydrated with an ascending series of ethanol, longitudinally oriented, and embedded in epoxy resin. Transverse semi-thin sections (2 μm) were cut, stained with 0.5% toluidine blue in 0.5% borate, and viewed using an Olympus B51 light microscope (Olympus Optical Co.).

Digitized images were imported into a personal computer using the Photoshop software program (Adobe Systems, Inc.). A computer analysis of this digitized information, based on gray and white scales, was used to measure the total number of fibers and the total fascicular area in sections at the midpoint of the tube. This analysis was performed using the Image analysis software program (ImageJ, National Institutes of Health). At 1000 magnification, 6 randomly selected fields per nerve were evaluated for myelin width and axon diameter. Based on these data, additional calculations of the total number of myelinated fibers and the percentage of neural tissue (100 × neural area/inafascicular area) were made. An observer blinded to the experimental groups performed all measurements.

Statistical Analysis

Data in this study are presented as the mean ± SD. One-tailed ANOVA was used to determine the differences between groups for histomorphometric analysis. If the difference was significant, the Tukey-Kramer test was performed to compare groups. Statistical significance was established at p < 0.05.

Results

Expansion Efficiency of Human Peripheral Blood CD133+ Cells

After 1 week of expansion culture, the number of cells increased 9.6 ± 3.3–fold (Fig. 1A). Based on the FACS analysis, it was demonstrated that the initial freshly isolated CD133+ cell population contained 93.22% ± 0.30% CD133+ cells and 91.16% ± 0.37% CD133+/CD34+ cells, and it was further confirmed that the expanded cells had a purity of 59.02% ± 1.58% CD133+ cells and 32.22% ± 0.74% CD133+/CD34+ cells (Fig. 1B).

Macroscopic Observations of the Grafted Site

The macroscopic findings were as follows. Extremely poor continuities were formed, with scarlike tissues, in 2 rats in the PBS group (Group 1). The other 8 rats in Group 1 did not have any continuity bridging the defect (Fig. 2A). Nervelike tissues bridging the defects were demonstrated in all animals grafted with fresh CD133+ cells (10⁵ cells; Group 2) (Fig. 2B). Similarly, in the expansion group (Group 3), nervelike tissues bridging the defects were recognized in all cases (Fig. 2C). However, in the rats that were transplanted with a low concentration of fresh CD133+ cells (10⁴ cells, Group 4), there were moderate nervelike tissues in 6 rats (Fig. 2D) and slight continuities in 4 rats (Fig. 2E).

Functional Axonal Regeneration of Excised Sciatic Nerve

In the electrophysiological evaluations, the CMAPs were observed in all cases in Groups 2 and 3 (Fig. 3A). In Group 4, CMAPs were detected in 6 rats in which continuities bridging the nerve defects were recognized (Fig. 3A). However, clear CMAPs were not detected in the other 4 rats in Group 4 or any of the 10 rats in Group 1 (Fig. 3A). The mean latency of CMAPs and the mean amplitude, respectively, were 1.7 ± 0.1 msec and 11.9 ± 4.1 mV in Group 2, 1.7 ± 0.2 msec and 10.7 ± 2.8 mV in Group 3, and 1.8 ± 0.1 msec and 5.6 ± 2.9 mV in Group 4. The mean latency was 1.5 ± 0.2 msec and the mean amplitude was 23.6 ± 3.5 mV in the contralateral normal nerve. Although the latencies in the 3 groups transplanted with fresh CD133+ cells or expanded cells were longer than those in the contralat-
eral normal nerve, there was no significant difference in the latency values among these groups. On the other hand, the amplitude values in all of the regenerated nerves were significantly smaller than those in the contralateral normal nerves. However, the amplitudes in Groups 2 and 3 were significantly larger than those in Group 4. In addition, there was no significant difference in the amplitude values between Groups 2 and 3 (Fig. 3B).

Histological Axonal Regeneration of Excised Sciatic Nerves

The morphological recovery of excised sciatic nerves in each group was evaluated by histological examination. Using representative cross-sections, morphological regeneration was investigated by toluidine blue staining. From low-magnification photomicrographs of each group, atelocollagen was observed in the center of the tubes, and tissues were newly formed around the atelocollagen gel (Fig. 4). At high magnification, statistical analysis showed the following indices for histological neural regeneration: the total number of myelinated fibers (Group 1, 9.17 ± 12.33; Group 2, 985.58 ± 485.21; Group 3, 829.92 ± 438.40; and Group 4, 430.92 ± 511.62 [p < 0.01 for Group 2 vs 1 or 4, Group 3 vs 1, and p < 0.05 for Group 3 vs 4, Group 4 vs 1]), the axonal diameter of regenerated nerves (Group 1, 1.36 ± 0.29; Group 2, 2.78 ± 1.11; Group 3, 2.55 ± 1.10; and Group 4, 2.53 ± 1.12 [p < 0.01 for Group 2 vs 1, Group 3 vs 1, and p < 0.05 for Group 4 vs 1]), the width of myelin (Group 1, 0.83 ± 0.28; Group 2, 1.39 ± 0.45; Group 3, 1.31 ± 0.38; and Group 4, 1.28 ± 0.38 [p < 0.01 for Group 2 vs 1, Group 3 vs 1, and p < 0.05 for Group 4 vs 1]), and the percentage of neural tissue (Group 1, 0.47 ± 0.55; Group 2, 12.26 ± 3.50; Group 3, 9.80 ± 3.46; and Group 4, 5.21 ± 2.51; [p < 0.01 for Group 2 vs 1 or 4, Group 3 vs 1 or 4, and Group 4 vs 1]) (Fig. 5). In all parameters of the histological examination, the values in the Groups 2, 3, and 4 were significantly greater than those in Group 1. In addition, none of the parameters showed any significant differences between Groups 2 and 3. However, the values in Groups 2 and 3 were significantly higher than those in Group 4 with regard to the total number of myelinated fibers and the percentage of neural tissue.

Discussion

The expansion culture of the endothelial progenitor cells, including CD133+ cells, has been established with various growth factors/cytokines. To improve the quality and quantity of expanded cells, Masuda et al.13 tried several growth factor/cytokine combinations and demonstrated the most optimal expansion culture method. Therefore, we chose the same culture method, because we think that this expansion culture method is the most suitable to maintain the revascularization function and the purity of CD133+ cells.

In the present study, our findings demonstrated that the number of human peripheral blood CD133+ cells increased approximately 10 times by ex vivo expansion. Although the purity of CD133+/CD34+ cells decreased from 91.16% ± 0.37% to 32.22% ± 0.74% on average after expansion, the transplantation of expanded cells is equally efficacious for the treatment of peripheral nerve injury in comparison with freshly isolated CD133+ cells.

However, despite the fact that mononuclear cells contain small numbers of CD133+ cells, in our previous study we reported that transplantation of mononuclear cells could not bring about the regeneration of peripheral nerve injuries.9 Therefore, it is necessary for the expanded cells to retain some degree of purity. Although a higher purity of CD133+ cells is considered to be better, about 30% of purity of CD133+ cells is sufficient for the expanded cells to function properly.

Our previous study of a model of sciatic nerve defect in athymic rats showed that CD133+ cells derived from human peripheral blood could promote functional recovery after peripheral nerve injury.9 In addition, in the pres-
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We demonstrated that the transplantation of ex vivo expanded CD133+ cells, as well as fresh CD133+ cells derived from human peripheral blood, was successful for repairing a peripheral nerve gap in the same model. Furthermore, the transplantation of a low concentration of fresh CD133+ cells was less effective in promoting the regeneration of injured peripheral nerves. These findings suggest that ex vivo expanded cells retain the capacity to promote regeneration of peripheral nerves equal to that of CD133+ cells before expansion, and that increasing the number of CD133+ cells by ex vivo expansion is beneficial for the treatment of peripheral nerve injuries.

We selected peripheral blood–derived CD133+ cells as clinical candidates for transplantation to address neural defects because these cells are very accessible human cells and therefore pose no serious ethical or technical concerns. However, acquiring a sufficient number of CD133+ cells to obtain an effective treatment using peripheral blood–derived CD133+ cell therapy is a clinically limiting factor because mononuclear cells contain only small numbers (0.04%) of CD133+ cells. To obtain this rare population of cells, administration of growth factors, such as granulocyte-colony stimulating factor, is required in the clinic. We were able to obtain approximately 10^8 CD34+ cells from human peripheral blood using granulocyte-colony stimulating factor mobilization and 10 L of apheresis from young adults, although the number of CD133+ cells that can be harvested is much lower. However, it is necessary to consider the side effects and invasiveness associated with the acquisition of CD133+ cells using growth factor mobilization and apheresis. In addition, more than 10^8 cells might be needed in the clinical setting, depending on the size of the nerve defects. Moreover, the same number of cells cannot be isolated from each patient because of interindividual differences in cell populations. Therefore, it is probable that

Fig. 3. Compound muscle action potentials recorded in the gastrocnemius muscle as the functional axon regeneration of the excised sciatic nerve. A: Representative CMAP waves. “Control” refers to the contralateral normal side. B: The peak-to-peak amplitudes of CMAPs. There is no significant difference in the amplitude values between the fresh group (Group 2) and the expansion group (EXPAN, Group 3). However, the amplitudes in the low-dose group (Group 4) are significantly smaller than those in Groups 2 and 3. *p < 0.05; **p < 0.01.
Fig. 4. Representative light micrographs of cross-sectional views in the midportion of harvested tissues stained with toluidine blue. **A and B:** Photomicrographs of tissue sections obtained from Group 1. Myelinated fibers are shown; however, they are few and small in diameter. **C and D:** Photomicrographs of tissue sections obtained from Group 2. Myelinated fibers with a large diameter are shown. The fibers are surrounded with myelin. **E and F:** Photomicrographs of tissue sections obtained from Group 3. They are approximately equal to those in Group 2. **G and H:** Photomicrographs of tissue sections obtained from Group 4. Myelinated fibers with a moderate diameter are shown; however, they are sparse. Bar = 50 μm.

Fig. 5. Bar graphs showing the results of the histomorphometric evaluation of nerve regeneration among the groups. All data were analyzed using an image analyzer. *p < 0.05; **p < 0.01.
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the therapeutic effect would be different for each patient. For example, older patients, or those who have atherosclerotic risk factors, including hypertension, diabetes, smoking, a family history of coronary artery disease, and high levels of low-density lipoprotein cholesterol, have quantitative and qualitative deterioration of their endothelial progenitor cells. Iwasaki and colleagues reported that human CD34+ cell transplantation may have dose-dependent potential for vasculogenesis and cardiomyogenesis, with functional recovery from myocardial infarction. The present results also suggest that CD133+ cell transplantation may have dose-dependent potential for functional recovery from peripheral nerve injury. Therefore, it is important to use as many cells as possible, and this can be achieved by using the serum-free expansion culture method. For all of these reasons, ex vivo expansion of CD133+ cells was found to be useful for the clinical treatment of peripheral nerve injury.

However, the mechanisms by which the transplantation of CD133+ cells or ex vivo expanded CD133+ cells promotes peripheral nerve regeneration remain unclear. Our previous study suggested that transplanted cells are able to contribute to neovascularization by the secretion of angiogenesis factors, and they also partially contribute to peripheral nerve regeneration by differentiating into Schwann cells. However, the number of transplanted cells that differentiated into Schwann cells was quite small in comparison with the number of regenerating axons. In the present study, we confirmed peripheral nerve regeneration by ex vivo expanded CD133+ cells. Although the number of cells increased approximately 10-fold by the expansion culture, the purity of CD133+ cells was reduced by about 39% after expansion. This indicates that the initial stem/progenitor cell fraction, which had the potential to differentiate into Schwann cells, was reduced by the expansion protocol. We therefore speculate that the transplantation of ex vivo expanded cells promotes both endogenous angiogenesis and axonal regeneration, predominantly through their paracrine effects.

In this experiment, we did not compare our results with those obtained with an autologous nerve graft, but we consider that our results are by no means inferior to those of autologous nerve graft if the nerve defect is short, as is the case in our current study. However, if a nerve defect is longer, it is likely that the results will not be as good. We do not know whether the transplantation of ex vivo expanded CD133+ cells can aid in the repair of a long nerve defect such as an autologous nerve graft can. Therefore, it is necessary to increase the number of transplanted cells or to try other methods. At least for a short nerve defect, nerves bridging the defects were recognized in all cases in the expansion group. As a result, the transplantation of ex vivo expanded CD133+ cells is useful for the regeneration of the peripheral nerve.

Future challenges for the clinical application of this strategy include a conclusive demonstration of the safety of ex vivo expanded cells, improvement of the culture efficiency, and further elucidation of the peripheral nerve regeneration mechanism to achieve better regeneration. However, we believe that the development of an artificial nerve in the clinical setting will be advanced by the results reported in our present study.

Conclusions

Our results show that the therapeutic potential of ex vivo expanded CD133+ cells derived from human peripheral blood is similar to that of fresh CD133+ cells for peripheral nerve injuries. This procedure can therefore be used to expand the number of CD133+ cells without reducing their functions or abilities, thereby providing a potential new cell-based therapy for nerve defects in the clinical setting.

Disclosure

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References


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