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BRIEF COMMUNICATION

Improvement of hind-limb paralysis following traumatic spinal cord injury in rats by grafting normal human keratinocytes: new cell-therapy strategy for nerve regeneration

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Abstract Somatic (adult) stem cells are thought to have pluripotency, just as do embryotic stem (ES) cells. We investigated the possibility that grafted epithelial keratinocytes could induce spinal cord regeneration in an animal model of spinal cord injury (SCI). Normal human keratinocytes were cultured by the routine technique, and normal human dermal fibroblasts were cultured by a similar method as a control group. SCI model was prepared by dropping a 10-g weight onto the exposed spinal cord of rats from a height of 25 mm, and 8 days later, the cultured cells were grafted into the injury site. Motor function was significantly improved in the cultured-keratinocyte-grafted group compared with that in the fibroblast-grafted group. After functional observation, human nestin- and nuclei-positive cells were found at the grafted spinal cord. Grafted cultured keratinocytes induced in vitro morphological changes in the neural induction medium. These results indicated one possibility that some of the grafted cultured keratinocytes survived and could have contributed to neural regeneration. On the other hand, it should be noted that the grafted cultured keratinocytes secreted a large amount of enzymes and/or growth factors. Therefore, another possibility is that the grafted-keratinocyte-derived factors could induce survived cell growth and endogenous neural differentiation of spinal-nerve-derived stem cells surrounding the injured spinal cord, leading to functional recovery. Epithelial stem cell therapy may be applied clinically in the near future to treat SCI.

Keywords Spinal cord injury · Cultured keratinocytes · Nerve regeneration · Cell therapy · Regenerative medicine

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Introduction

Various techniques have recently been tried in an attempt to treat spinal cord injury (SCI) [1–3]. Nerve protection against acute-phase inflammatory response is important as an early-stage intervention in treating central nervous system (CNS) injuries, but its only objective is to protect normal tissue in the surrounding region [4]. Even if various drugs, such as so-called radical scavengers, are used as acute-phase therapy for CNS damage, no repair or regeneration of the injured region can be expected. Although

applications of embryonic stem (ES) cells and bone marrow stromal (stem) cells have been devised as a form of regenerative medicine for treating CNS damage, the technique requires considerable time before its clinical application because the mechanism of regulating their differentiation cannot be reliably cleared [5]. In particular, implementation of ES cell technology will be a lengthy process unless definite differentiation induction techniques are established [5], and the ethical issues surrounding ES cell use have not been resolved.

On the other hand, bone marrow stromal/stem cells have been confirmed to be pluripotent and are being used for regeneration of both the spinal cord and myocardium [6]. Autologous stem cells contained in bone marrow and adipose tissue, both of which are mesenchymal tissues, have been used experimentally to treat paralysis associated with SCI [7, 8]. The reason autologous mesenchymal stem cells, such as bone marrow stromal cells and adipose-tissue-derived stem cells, are being used is that they can be collected in large numbers without any ethical problems. Bone marrow stromal cells are capable of differentiating into liver and nervous system as well as into bone, cartilage, fat, and myocardium tissues [9, 10], meaning that bone marrow stromal (stem) cells, which are part of the mesenchymal system, can convert into an ectodermal system. Thus, if the target organ or tissue for differentiation induction were the same ectodermal system, the stem cells of the same germinal system might be able to transform into the target tissues or organs more effectively. Oshima et al. [11, 12] reported that keratinocytes in hair follicles have stem-cell-like multipotency despite being dermal components, and we actually observed acceleration of granulation and healing of refractory skin ulcers after grafting cultured keratinocytes.

In this study, we investigated the possibility of cultured keratinocytes differentiating into nervous tissue, which is an ectoderm-derived tissue, by performing an experiment on a model of SCI in the rat.

Materials and methods

Cell source

Normal human surplus skin was collected aseptically during plastic surgery. The skin was cut into 2-mm fragments, and the fragments were sterilized by immersion in povidone iodine for 2 min at ambient temperature. The skin fragments were digested for 18 h at 4°C with phosphate-buffered saline (PBS) containing 0.25% trypsin and then gently stirred for 60 min at ambient temperature in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). To separate free cells from the digested skin, the suspension was filtered with Cell-Strainer (Japan B.D. Co., Tokyo Japan). The filtrate was used for keratinocyte culture, and the digested skin residue was used for fibroblast culture. This investigation was approved by the Bioethics Committee (No. 1295) based on the Guidelines for Clinical Experimentation of St. Marianna University School of Medicine.

Keratinocyte culture

Normal human keratinocytes were cultured by the method described by Rheinwald and Green [13]. The filtrate described above was centrifuged for 5 min at 400×*g* at 4°C, and the cell pellet obtained was resuspended in F-12/DMEM (1/3) containing both 10% FBS and a cocktail of various growth factors [insulin, T3, human epithelial growth factor (hEGF), transferrin and cholera toxin]. The cell suspension obtained was seeded onto gamma-ray-irradiated cultured 3T3 feeder cells. The cells were incubated for the first 4 days at 37°C under an atmosphere containing 10% carbon dioxide, and the medium was changed every 2 days until the cells reached 80% confluence under the same culture conditions. To remove the 3T3 cell remnants from the cultured keratinocytes, culture dishes were thoroughly rinsed with PBS containing 0.02% ethylenediaminetetraacetate (EDTA), and the keratinocytes were isolated by routine trypsin treatment. The keratinocyte suspension was adjusted to 5×10^7 cells/ml with PBS and used in the experimental model of SCI. In this case, the keratinocytes used in this study were not cultured from specific dissected hair follicle or other niche region. Rochat et al. [14] defined the excellent proliferated cells cultured under the above condition as keratinocytes. Therefore, these cultured cells are classified into the transient amplified keratinocytes. In this study, these cultured cells were also named keratinocytes.

Fibroblast culture

A few fragments of the enzyme-digested skin residue were placed on culture dishes, and sterilized cover glasses were placed on the residue to prevent floating. The skin fragments were cultured under an atmosphere containing 5% carbon dioxide until fibroblasts migrated onto the culture dish at 37°C. After the fibroblasts were treated by the routine method, they were subcultured on DMEM containing 10% FBS until cells reached 80% confluence. The fibroblast suspension was adjusted to 5×10^7 cells/ml with PBS and used in the experimental model of SCI.

SCI model in rats: cell transplantation, and evaluation of motor function

Adult female Sprague-Dawley (SD) rats weighing 249.0 ± 7.07 g (Japan SLC, Shizuoka, Japan) were deeply

anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). A dorsal laminectomy was performed at the thoracic level T9–10 vertebra. A 10-g weight was dropped onto the exposed spinal cord from a height of 25 mm, inflicting a moderate contusion injury [15, 16]. The animals were allowed to recover from the anesthesia on a heating pad maintained at 36.5°C. To prevent wound infection, penicillin-G (1 mg/kg s.c., Meiji Seika Co., Tokyo, Japan) was used as an antibiotic. On the eighth day after injury, human keratinocytes (5×10^6 cells/5 ml) were grafted at the site of injury with a microsyringe [17]. As a control, human fibroblast cells (5×10^6 cells/5 ml) were transplanted. Cyclosporine A (3 mg/kg/day) was injected subcutaneously for 4 days before and after the cell graft. Motor function was assessed using the Basso Beattie Bresnahan (BBB) [15, 16] open-field locomotor scale. Animals were allowed to walk around freely in a circular field (1.2 m in diameter) for 10 min while movements of the hind limbs were closely observed. The BBB locomotor scale includes frequency and quality of hind-limb and fore-limb/hind-limb coordination. From these observations, the animal received a score between 0 and 21, where 0 indicates total absence of movement, and 21 indicates normal movement. Two researchers were trained as observers to evaluate the animals on the BBB scale. All observers were unaware of the group identity of the animals. Rating of each animal was later checked by slow-playback video recordings of each animal walking. Scores for the left and right extremities were averaged. All animals were housed in an animal room maintained at 23 ± 1°C and 50–60% humidity under a 12-h light/dark cycle, with free access to food and water. This investigation was approved by the Animal Care and Use Committee, based on the Guideline for Animal Experimentation of St. Marianna University Graduate School of Medicine.

Immunostaining

Animals were perfused with cold physiological saline and 4% paraformaldehyde under anesthesia, after which the segment of spinal cord (1.5-cm long) containing the center of the damaged region was removed and further fixed with 4% paraformaldehyde for 24 h. Tissue was cryoprotected by immersion in 10% sucrose phosphate-buffered saline (PBS) for 1 h and overnight in 30% sucrose-PBS at 4°C. Spinal cord specimen was then frozen in embedding compound (Tissue-Tek, Sakura, Torrance, CA, USA) and sliced into 20-μm thickness on a cryostat and placed on poly-L-lysine-coated glass slides. Goat anti-human nestin antibody (Santa Cruz Biotechnology Co., CA, USA) or mouse anti-human nuclei monoclonal antibody (Chemicon International Co., CA, USA) was used to identify the grafted cells. Fluorescein isothiocyanate (FITC)-conjugated anti-goat immunoglobulin (Ig)G (Chemicon international) or rhodamine-conjugated anti-mouse IgG (Rockland Immunochemicals Co., Gilbertsville, PA, USA) was used as the secondary antibodies. In vitro neural induction was carried out and cultured in neural induction medium [DMEM/F-12 (GIBCO) containing N2 Plus Media Supplement, EGF, and basic fibroblast growth factor (bFGF)]. After fixing the cells, immunocytochemical staining was performed. Mouse anti-β-III-tubulin (Promega Co., Tokyo) and mouse anti-glial fibrillary acidic protein (GFAP, Sigma-Aldrich Co. MO, USA) were used as the primary antibodies. FITC-conjugated anti-mouse IgG (Sigma-Aldrich) or rhodamine-conjugated anti-mouse IgG was used as the secondary antibodies. Before mounting, each section was counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies Japan, Tokyo).

Statistical analysis

All data are expressed as mean ± standard deviation (SD). The statistical significance was evaluated using the Mann–Whitney U test, and $P < 0.05$ was used as the criterion for significance.

Results

Transplantation of cultured human keratinocytes to SCI rats (Fig. 1)

The contusive spinal cord resulted in complete paralysis. The BBB score of functional behavior was 3.70 ± 1.95 (mean ± SD, $n = 5$) on day 8 after injury, whereas that of another group was 3.10 ± 2.07 ($n = 5$). There was no significant difference between the two. Human keratinocyte transplantation promoted recovery from hind-limb locomotor dysfunction. Three days after cell transplantation, the BBB score was 6.70 ± 2.6 when compared with that prior to cell graft. Human-fibroblast-grafted animals (control) showed gradual but significantly less potent recovery (Fig. 1a). After 4 weeks, some animals in the human-keratinocyte-grafted group could walk with fore-limb/hind-limb coordination and could perform integrated voluntary motor movements. Those in the control group also recovered motor function, but some crawled persistently and moved without weight bearing. There was a significant difference between groups from day 6 after transplantation. The BBB score of all SCI animals reached a plateau 4 weeks after transplantation, when grafted animals had significantly better locomotor function (12.8 ± 1.9) compared with controls (9.4 ± 0.8) ($P < 0.01$).

After functional observation, histological analysis was performed. There were human-nestin-positive cells around

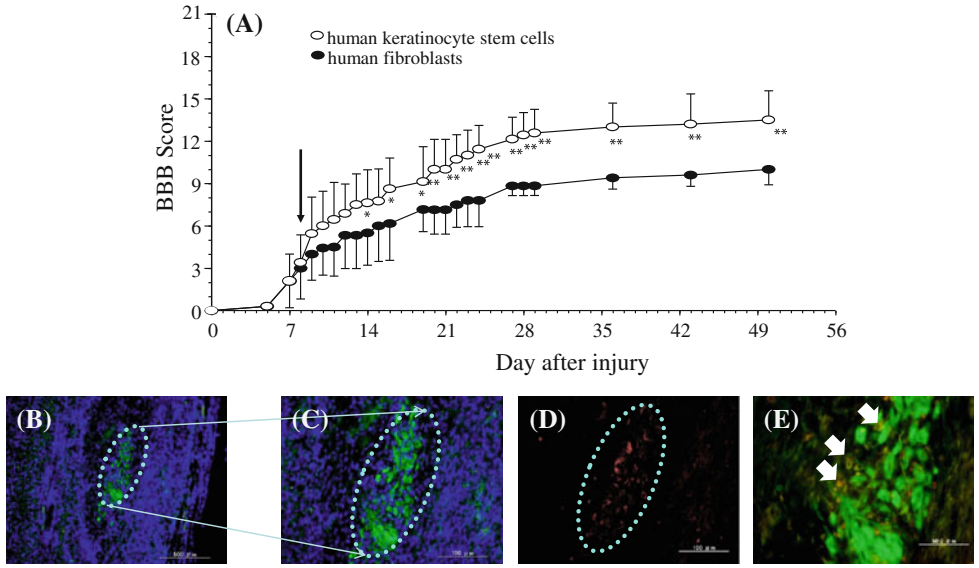

Fig. 1 Transplantation of keratinocyte stem cells to rats with spinal cord injury (SCI). *Upper* functional recovery from SCI-induced motor dysfunction. **a** On the eighth day after injury, human keratinocyte stem cells, and fibroblasts as control (5×10^6 cells), were grafted at the center of the injured region (*arrow*). *Open circle* keratinocyte group. *Closed circle* fibroblast group (control). Basso Beattie Bresnahan (BBB) scores were monitored before and after cell transplantation (mean ± standard deviation, $n = 5$). $^*P < 0.05$, **0.01: compared with data obtained from control group. **b**, **c** Nestin-like immunoreactants (*green*) expressed in injured region of the rat spinal cord. **d** Human nucleus (*red*) was also stained in grafted region. **e** Some were double-positive cells (*arrow*). **b** *Scale bar* 500 μm. **c**, **d** *Scale bar* 100 μm, **e** *Scale bar* 50 μm

the injured spinal cord (Fig. 1b, c), some of which were positive for human nuclei (Fig. 1d).

Morphological change of keratinocytes in vitro

When human keratinocytes (Fig. 2a) were cultured in the neural induction medium, morphological change was observed (Fig. 2b–e). They were positive for GFAP and β-III-tubulin neural markers.

Discussion

A new category of regenerative medicine, adult stem cell therapy, has recently been devised to repair damaged organs and tissues. In general, bone marrow stromal cells and adipocyte stem cells are used for stem cell therapy [18, 19]. This study demonstrated that grafted human keratinocytes improved the hind-limb motor function of SCI rats and that the improvement was significantly greater than in the fibroblast-grafted group (control). As another control, the vehicle used to suspend the cultured human keratinocytes should have been used. In this study, the human-cultured fibroblasts were chosen as a control instead, as there was concern that nonspecific stimulation of the grafted cells might have influenced the results. Therefore, this finding might indicate the possibility of autologous cell therapy for CNS damage. Although keratinocyte stem cells are present in hair follicles and basal cell layer of the epidermis [20], neural differentiation of stem cells has never been reported.

Whether the grafted cultured keratinocytes are stem cells is of great importance. Rochat et al. [14] defined that the cells with excellent proliferation rate under the same culture condition as implemented in our study were keratinocyte stem cells. On the other hand, Ohyama et al. [21] obtained CD200-positive cells purified from human bulge stem cells. CD200-positive cells are considered to be important as markers of keratinocyte stem cells, and further study would be needed as to whether the grafted cultured keratinocytes were positive for CD200. In this investigation, cells containing keratinocytes were collected from full\-thickness skin of the forearm and then cultured. The skin specimens were enzyme digested directly by trypsin without skin dissection. As Amoh [22] reported, we did not dissect hair follicles only from the skin specimen. However, it would be important that cells obtained from the nondissected skin specimen have the potential to promote functional recovery following cell graft.

Human-derived cells (human nuclei and nestin-positive cells) were found in the grafting region, indicating that the grafted human keratinocyte in the rat spinal cord survived. Nestin-positive stem cells in hair-follicle bulges have been reported to have the potential to differentiate into keratinocytes and neurons [23]. Nestin, a specific intermediate filament protein, is expressed in ectodermal neural

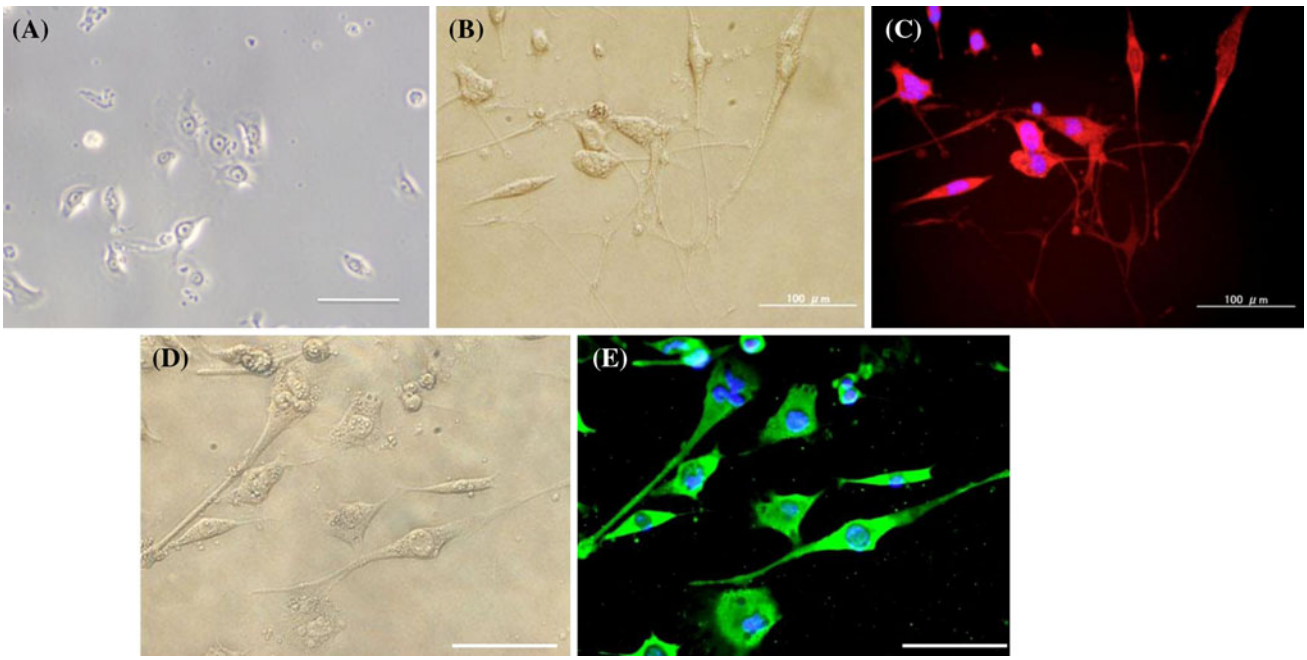

Fig. 2 Morphological change of keratinocytes. **a** Typical phase-contrast image of keratinocytes cultured in normal medium. *Scale bars* 100 μm, **b–e** morphological change after culture with neural induction medium for 3 days. *Scale bars* 100 μm. **b**, **d** Typical phase-contrast image. Immunocytochemical staining for (**c**) anti-glial fibrillary acidic protein (GFAP) and (**e**) *β*-III-tubulin

progenitor cells and is specifically expressed during CNS formation. In vitro morphological changes were observed when grafted keratinocytes were cultured in the neural induction medium. They expressed neuron-specific markers. Therefore, one possibility would be that some of the grafted keratinocytes might have differentiated into neuron-like cells or existed in the form of stem-cell-like keratinocytes. Li [23] showed that nestin-positive cells are present in hair follicles, whereas Amoh [24] demonstrated that hair-follicle stem cells, which have pluripotency, are capable of differentiating in to neurons, glial cells, and keratinocytes. Hoffman [25] reported that hair-follicle stem cells showed their pluripotency, such as neurons, and were valuable for various therapeutic purposes Another possibility of keratinocyte-mediated neurogenesis would be that many kinds of neurotrophic factors and glycosidase secreted by the grafted keratinocyte might have contributed to re-forming the neural network system surrounding the injured spinal cord. Actually, we already reported that keratinocytes secrete various glycosidases, proteases, and growth factors [26]. Secreted glycosidase might have hydrolyzed the proteoglycan of the damaged neural site. Neurotrophic factors could have induced survived cell growth and endogenous neural differentiation of spinal-nerve-derived stem cells surrounding the injured spinal cord, leading to functional recovery. We are now investigating these possibilities.

In clinical application, although tissue collection from patients is indispensable to organ- or tissue-regenerative medicine, it is important to minimize surgical invasion. Collection of bone marrow stromal cells is more invasive than collection of small amounts of adipose tissue or skin fragments. As large numbers of keratinocytes can be easily cultured from small skin fragments, in the future, stem cell therapy may be applied to treating SCI as well as to treating refractory skin ulcers.

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