

Original

**A New Regenerative Approach to Fetal Myelomeningocele
by Cell Sheet Transplantation**

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Abstract: Myelomeningocele (MMC) is the most common form of congenital neural tube defect. Current fetal surgical repair performed to prevent the exposed spinal cord from being injured until delivery cannot reverse those injuries already inflicted in utero. “Cell sheet” technology has been adopted successfully for the regeneration of diverse organs and tissues, although this promising modality has not yet been used for fetal therapy. This study thus tested our hypothesis that fetal MMC tissue histologically injured in utero could be regenerated using cell sheet technology. We used the L6 myoblast cell line derived from rat skeletal muscle for the cell sheet engineering. A fetal MMC model was also obtained from pregnant Sprague-Dawley (SD) rats fed orally with retinoic acid (60 mg/kg, embryonic day 10). Cell sheets were then transplanted onto the fetal MMC lesion (embryonic day 19) following maternal anesthesia, laparotomy, and hysterotomy. The incisional wound of the uterus was kept open for 4 hours under anesthesia with the MMC lesion maintained outside the body with the transplanted cell sheet. Subsequently, the experimental fetuses were sacrificed for histological (HE stain) and immunohistochemical studies to evaluate viability and differentiation potential based on cell markers of the transplanted L6 myoblasts. Immunohistochemical studies clearly demonstrated cell-sheet markers specific to neurons, skeletal muscle, and myoblasts within the treated MMC lesions, confirming that the cell sheet was biologically implanted within 4 hours after the procedure. Cell sheet technology could be useful for intrauterine regeneration of the fetal rat spinal cord injured by an associated MMC.

Key words: cell sheet, fetal myelomeningocele, transplantation, tissue regeneration

Introduction

Myelomeningocele (MMC) is a congenital anomaly associated with potentially lifelong neurological disabilities that are closely related to the so-called “two-hit” hypothesis¹⁾. The first hit involves early-stage embryological abnormalities that result in the MMC morphology and the

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second hit involves environmentally acquired damage to the exposed spinal cord that lasts in utero until delivery. Although prenatal surgical repair of the MMC could prevent the second hit, this procedure is limited to morphological correction of the spinal cord and does not heal those injuries acquired before surgery¹⁾.

Regenerative medicine is increasingly expected to yield promising results in the field of prenatal medicine. Among diverse regenerative technologies, we used animal models of fetal MMC to focus on surgically transplanting a “cell sheet” onto the MMC lesion with potential full recovery of the fetal spinal cord architecture before birth. We previously found that such cell sheets could be biologically implanted and incorporated quite early after surgical application onto the fetal MMC²⁾. In this article, we present the outcomes of our experimental study and the future prospects of cell sheet technology in the field of prenatal medicine.

Materials and methods

Experimental animals: All animals were treated in accordance with the guiding principles for care and use of animals (National Center for Child Health and Development, Tokyo, Japan). Time-dated pregnant Sprague-Dawley rats (E-10 days; Sankyo Labo Service Corporation, Inc., Tokyo, Japan) were gavaged with retinoic acid dissolved in olive oil (60 mg/kg; Wako Pure Chemicals, Osaka, Japan). Both the retinoic acid-fed and non-fed fetuses were harvested on E-19 or 21 days. The retinoic acid-fed fetuses were further divided into MMC-positive and -negative animals. In our study, the MMC-positive fetuses were likely to have no tail, thus we statistically assessed the coincidence of two findings: “having MMC” and “no tail development”.

Cell culture and preparation of the cell sheet: L6 cells (rat myoblast cell line derived from the skeletal muscle; American Type Culture Collection, Rockville, MD, USA) were grown to near confluence in DMEM (Gibco, Brooklyn, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Shrewsbury, NJ, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Brooklyn, NY, USA) on 25-cm² tissue culture flasks (BD Biosciences, Franklin Lakes, NJ, USA). The L6 cells were then transferred to a “thermo-responsive culture dish”, which is coated with a temperature-responsive polymer such that a simple temperature change from 37°C to 32°C readily alters the dish surface from hydrophobic to hydrophilic. This in turn allows a sheet of cultured L6 myoblast cell clusters (cell sheet) to detach with cell connections and cell-to-cell communications well preserved.

Cell sheet transplantation (CST): On E-19 days, pregnant rats were anesthetized with somnopentyl (Kyoritsu Seiyaku Corporation, Japan) and subjected to laparotomy. A small hysterotomy was made following a 6-0-silk purse-string suture placed on the uterine wall. Then, after an amniotomy, the MMC lesion was exposed through the uterine wall. The cell sheet was then directly placed to cover the entire fetal MMC lesion, and after filling the uterine cavity with warm saline, the hysterotomy site was closed for 4 h, during which the animals were maintained under anesthesia. Thereafter, the fetuses were harvested and fixed in neutral buffered 10% formalin (Sigma-Aldrich, USA) for gross and microscopic studies including immunohistochemistry.

Histological and immunohistochemical studies : After sequential processing, the fetuses were embedded in paraffin wax with the transplanted cell sheet and sectioned for histological analysis (5- μ m-thick sections). The sections were then dewaxed, rehydrated, and HE stained. To evaluate viability and differentiating potential of the transplanted L6 cells, the sections were further analyzed immunohistochemically based on L6 myoblast markers specific to neurons, skeletal muscles, and myoblast cells³⁾. To do this, the sections were incubated with primary rabbit antibodies against polyclonal beta 3 tubulin or polyclonal anti-nerve growth factor (NGF), and mouse monoclonal anti-smooth muscle actin (SMA) (Abcam, Cambridge, UK ; dilution 1:100). Immunostained sections were examined using a conventional fluorescence microscope (Zeiss, Jena, Germany).

Results

Experimental animals : There was no statistically significant correlation in the incidence of MMC between the fetuses observed at E-19 and E-21 days. Fig. 2B shows that fetuses with no tail had a significantly high incidence of MMC. Accordingly, to accurately diagnose a fetus as having MMC without hysterotomy, it was practical and quite helpful to identify the fetal tail during these procedures by palpating the uterus from the outside.

Histological and immunohistochemical studies of L6 cells and cell sheets transplanted onto fetal MMCs : Cultured L6 myoblast cells microscopically showed fibroblast-like and spindle-shaped morphology, which was unaffected by the length of culture. The HE staining shown in Fig. 3A indicates the viability of L6 cells in the transplanted cell sheet. The immunostaining shown in Figs. 3B, C, and D reveals that the cultured L6 cells expressed SMA, NGF, and beta tubulin 3 markers, respectively. Fig. 4A shows HE staining of the transplanted cell sheet attached to the fetal MMC lesion, and the accompanying immunostaining in Figs. 4B, C, and D shows positive reactions for SMA, NGF, and beta tubulin 3 in the attached cell sheet in the area where the cell sheet was transplanted.

Discussion

Our animal experimental model of MMC indicated that the sheet-like and viable L6 cell clusters (cell sheet) were likely to become attached biologically shortly after surgical transplantation onto the fetal MMC lesions, preventing its further histological aggravation in utero. This finding suggests that future human fetal MMC treatment using cell sheet technology could potentially promote prenatal regeneration of the exposed neural tissues injured in utero and thus further improve the lifelong neurological outcomes currently achieved with fetal surgical repair⁴⁾. Our conclusion is supported by the HE staining of tissue from the whole fetus in revealing that the adhering transplanted cell sheet remained viably attached to the fetal spinal cord at 4 hours after transplantation, with a well-preserved ECM. Positive immunostaining of the attached cell sheet for the intrinsic neuromuscular growth factors NGF, SMA, and beta-tubulin 3 further strengthened the potential success of this technique by suggesting that the L6 cell sheet could enhance early regeneration of the neural tissue at the site of cell-sheet transplantation.

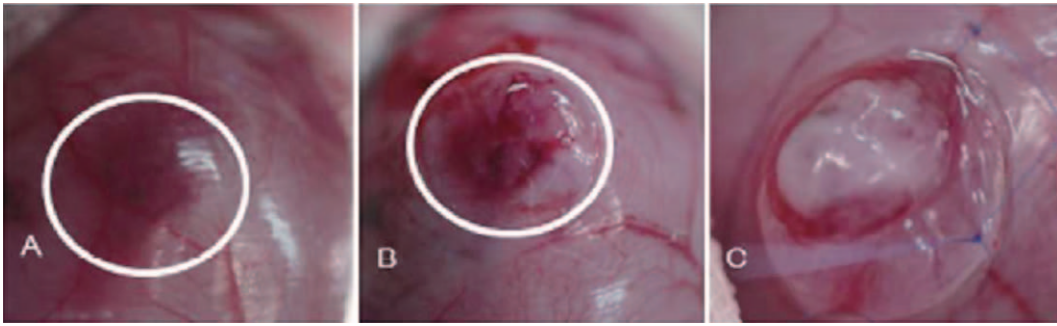


Fig. 1. A) MMC area of a fetus from outside the uterus. B) MMC inside the uterus after incision. C) Cell sheet transplantation on the MMC area. 254 × 190 mm (96 × 96 DPI).

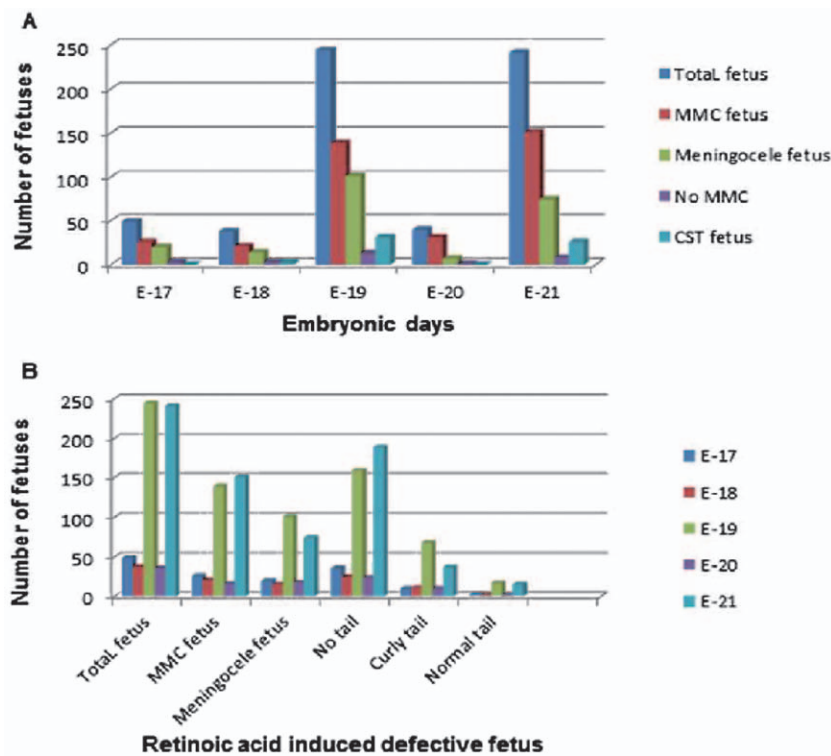


Fig. 2. A) Number of fetuses on different embryonic days after retinoic acid induction. B) Number of defective fetuses after retinoic acid induction. 254 × 190 mm (96 × 96 DPI).

Cell sheet technology as investigated here has already been reported experimentally and clinically to promote diverse tissue regeneration, and has been successfully employed in a variety of medical fields. For example, in a study using rabbit models, transplanted cell sheets enhanced epithelialization of laser-ablated tissues within 5 minutes, leading to complete re-epithelization in 3 to 5 days, while in therapeutic ophthalmology, this technology enabled reduction in corneal haze after excimer laser keratectomy⁵⁾. In addition, a cell sheet derived from a patient's oral mucosal epithelium was transplanted onto their eyes with improved clinical outcomes⁶⁾. In adult

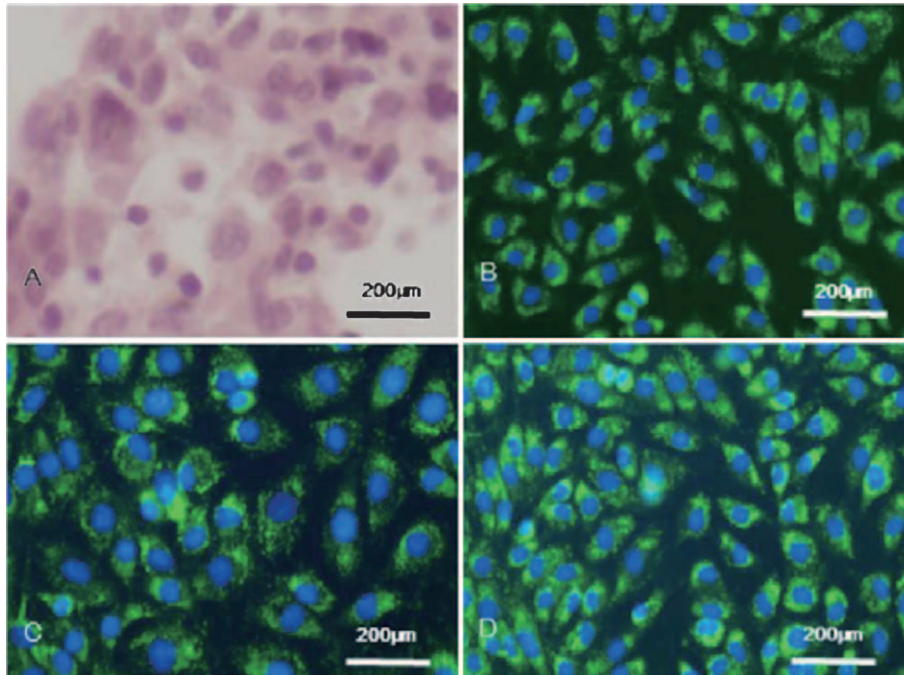


Fig. 3. A) H-E staining of the cell sheet. B, C, D) Immunostaining of smooth muscle actin, nerve growth factor, and beta tubulin 3, respectively, in L6 myoblast cells (green – cell, blue – nucleolus). 254 × 190 mm (96 × 96 DPI).

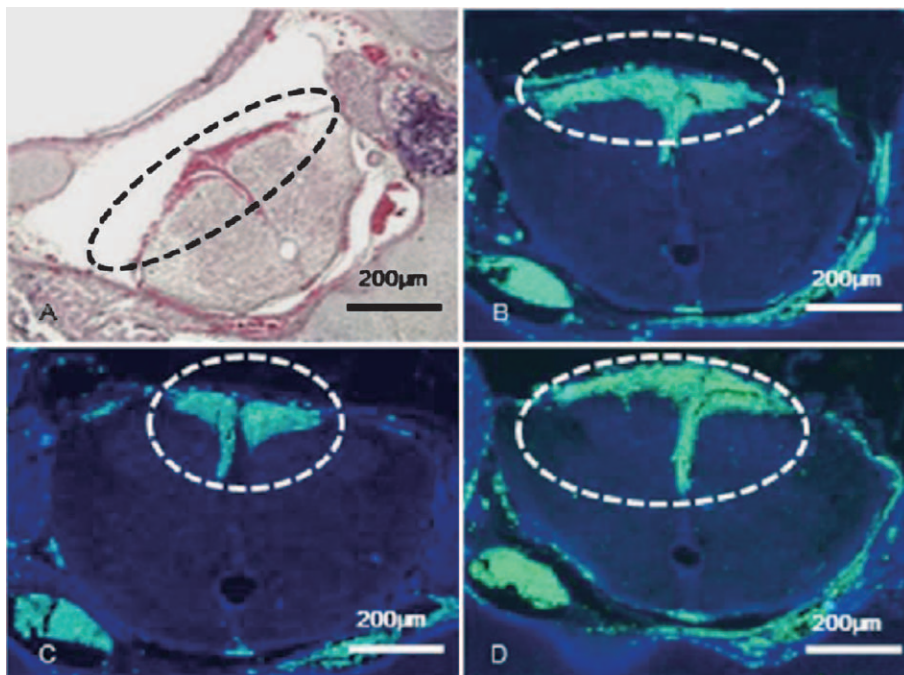


Fig. 4. A) H-E staining of the spinal cord at 4 hours after the cell sheet transplantation in an MMC-defective rat fetus. B, C D) Immunostaining of NGF, SMA, and BETA3 of the transplanted cell sheet in the defective area, respectively. 254 × 190 mm (96 × 96 DPI).

dogs with experimentally induced dilated cardiomyopathy (DCM), transplanting of a myoblastic sheet onto the left ventricle attenuated myocardial remodeling in 4 weeks with resultant improvement in cardiac function⁷⁾. Indeed, there are several clinically successful cases of cell sheet technology reported for DCM^{8, 9)}. Interestingly, mesenchymal stem cells (MSCs) prenatally injected into the MMC cord with growth factor (s) could also stimulate tissue regeneration¹⁰⁾. However, while this MSCs injection technique is seemingly less invasive, the cell sheet used herein contains its own built-in ECM without the need for extrinsic growth factor (s), making it superior in preventing the constituent L6 cells from being scattered. Furthermore, the sheet-like structure sealing the exposed MMC lesion should hopefully minimize aggravation of the in utero mechanical and chemotoxic injuries to the neural tissues (“second hit”).

Our experimental study had three major limitations. First, without hysterotomy, we could not accurately diagnose a fetus as having MMC. In this regard, maternal laparotomy for locating the fetal tail was actually helpful in observing MMC through the stretched thin uterine wall. Second, it was difficult to keep the cell sheet attached to the MMC lesion securely within the amniotic fluid environment; however, in our experiment, the cell sheet transplanted onto the exposed spinal cord remained histologically attached in utero for at least 4 hours and seemed to promote tissue regeneration much sooner than we had expected. In addition, it seems encouraging that the combined use of the gelatin-based scaffold and bioactive proteins reportedly promotes cellular ingrowth that could be accompanied by spinal cord regeneration¹¹⁾, because the sheet-like cluster of L6 cells might also work as a kind of durable scaffold owing to the inherent ECM component. Lastly, we could not easily identify the best prenatal timing for cell sheet transplantation, but we believe that the clinical timing of fetal surgical repair of between 19 and 26 weeks of gestation could be used as a reference¹²⁾.

Conclusion

This feasibility study on the prenatal use of cell sheet technology suggests that sealing up the MMC lesion using an adherent cell sheet together with surgical repair could lead to more effective prenatal MMC repair. Further study of such techniques is needed to facilitate actual improvement in fetal treatment of MMC.

Acknowledgements

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Conflict of interest disclosure

The authors declare that they have no conflict of interest.

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