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Remyelination of the nonhuman primate spinal cord by transplantation of H-transferase transgenic adult pig olfactory ensheathing cells

Christine Radtke^{*,†}, Yukinori Akiyama^{*}, Jane Brokaw[†], Karen L. Lankford^{*}, Konstantin Wewetzer[‡], William L. Fodor[†], and Jeffery D. Kocsis^{*}

^{*}Department of Neurology, Yale University School of Medicine, New Haven 06516; Neuroscience Research Center, Veterans Affairs Medical Center, West Haven 06516

[†]Alexion Pharmaceuticals Inc., Cheshire, Connecticut 06410

[‡]CTR. Anatomy, Hannover Medical School, Hannover, Germany

Abstract

Olfactory ensheathing cells (OECs) have been shown to mediate remyelination and to stimulate axonal regeneration in a number of in vivo rodent spinal cord studies. However, whether OECs display similar properties in the primate model has not been tested so far. In the present study, we thus transplanted highly-purified OECs isolated from transgenic pigs expressing the $\alpha 1,2$ fucosyltransferase gene (H-transferase or HT) gene into a demyelinated lesion of the African green monkey spinal cord. Four weeks posttransplantation, robust remyelination was found in 62.5% of the lesion sites, whereas there was virtually no remyelination in the nontransplanted controls. This together with the immunohistochemical demonstration of the grafted cells within the lesioned area confirmed that remyelination was indeed achieved by OECs. Additional in vitro assays demonstrated 1) that the applied cell suspension consisted of >98% OECs, 2) that the majority of the cells expressed the transgene, and 3) that expression of the HT gene reduced complement activation more than twofold compared with the nontransgenic control. This is the first demonstration that xenotransplantation of characterized OECs into the primate spinal cord results in remyelination.

Keywords

OEC; HT; transplantation

Olfactory ensheathing cells (OECs) are being considered as a potential donor cell source for a cell-based spinal cord injury therapy. This consideration is based on work showing that OECs transplanted into the injured rodent spinal cord can enhance axonal regeneration (1–4) and remyelination (5,6). Moreover, improved behavioral function in spinal cord injury models has been reported after transplantation of OECs (7–9). Although autologous OECs could in principle be derived from a biopsy of a patient's olfactory mucosa, harvesting a sufficient number of OECs from human tissue for clinical studies remains problematic. Xenogeneic neural tissue provides another potential source of cells for cell-based transplantation therapies. Transgenic pigs that express a human complement inhibitor, CD59 (10), H-transferase (HT; 11), or both transgenes (12) are designed to escape humoral-mediated hyperacute rejection

(HAR) and could serve as an abundant source of neural tissue. OECs isolated from transgenic pigs expressing a human complement inhibitor, CD59, have been shown to support axonal regeneration and remyelination in transected rodent spinal cords (4).

HT transgenic pigs offer another potential source of OECs as expression of HT leads to the expression of the universally accepted human O blood group phenotype that in turn reduces Gal α 1,3Gal expression resulting in reduced human antibody reactivity and complement-mediated cell lysis (11). As a first step to examine the feasibility of using HT OECs in transplantation studies, we developed methods to harvest OECs in high purity from HT transgenic pigs. We determined that the HT transgenic OECs indeed expressed the transgene and displayed antigenic properties similar to the well-characterized rodent OECs, including S100, glial fibrillary acidic protein (GFAP), and p75 nerve growth factor receptor (NGFR) expression. The OECs were characterized in culture and could be consistently prepared in large number, thus allowing transplantation of cells that displayed consistent and identified phenotypic properties. Moreover, an in vitro functional assay indicated that the porcine HT OECs have reduced complement deposition on the cell surface when exposed to human serum as compared with nontransgenic control OECs. The repair potential of OECs transplanted into the injured rodent spinal cord has been demonstrated; however, the ability of OECs to repair the nonhuman primate spinal cord has not been tested. We demonstrate that transplanting phenotypically characterized pig OECs expressing the H-antigen into the demyelinated nonhuman primate spinal cord results in remyelination with a morphological architecture characteristic of peripheral myelin. Thus, the remyelination potential observed in rodents with transplantation of OECs can be extrapolated to the primate, and the expression of the HT transgene does not appear to affect the repair potential of the OECs.

METHODS

Cell harvesting and culture conditions

The generation of HT transgenic pigs was described previously (11). Pigs (postnatal 4-8 wk) were deeply anesthetized (20 mg/kg ketamine, 2 mg/kg xylazine), exsanguinated, and the olfactory bulbs were removed. The isolated olfactory bulbs were placed in a 10 cm petri dish containing Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corporation, Carlsbad, CA). With the use of aseptic technique and a dissection microscope (Zeiss, OpMi-I), the olfactory nerve and glomerular layers (ONGL) were dissected from the bulb by peeling off the gray matter from the underlying white matter. This microdissection step was included to diminish the amount of contaminating glia cells. ONGL tissue was then processed essentially as described previously (13). Cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/ml penicillin; 100 μ g/ml streptomycin) at 37°C, in a 5% CO₂ incubator.

Immunocytochemistry on cultured cells

OEC cultures were immunocytochemically labeled with anti-human-p75 NGFR monoclonal antibody (dilution 1:100; 3 day supernatant from 196 hybridoma cells, clone 132: gift from Eugene Johnson), anti-human GFAP (1:500; Sternberger Monoclonal Inc., Lutherville, MD), and S100 (mouse anti-human S100B at 1:400; Dako, Carpinteria, CA) using standard techniques. The cells were then incubated for 90 min (room temperature) in Alexa Fluor 594 goat anti-mouse IgG for p75 NGFR or Alexa Fluor 488 goat anti-mouse IgG for GFAP or S100 immunostaining. We detected the carbohydrate structures Gal α 1,3Gal and the H epitope using the FITC conjugated lectins GS-IB4 (10 μ g/ml, EY Laboratories, Inc. San Mateo, CA) and UEAI (10 μ g/ml, EY Laboratories), respectively. Photographs were taken with a Spot RT Color CCD.

Flow cytometry

OECs were analyzed by flow cytometry after direct immunofluorescent staining of the cell surface carbohydrate epitopes with fluorescein isothiocyanate-(FITC)-conjugated lectins: GSIB4 (10 µg/ml, EY Laboratories) and UEAI (10 µg/ml, EY Laboratories). Porcine histocompatibility SLA class I antigen and porcine CD44 were detected with PT85A and BAT31A, respectively (VMRD, Inc., Pullman, WA), followed by FITC-conjugated goat anti-mouse IgG (Zymed Laboratories, Inc. San Francisco, CA). Cell surface expression was measured on a Becton-Dickinson FACSsort flow cytometer. The average of the mean FI-1 was calculated from flow cytometry data on independent cell isolates (HT, $n=14$; control, $n=7$). Statistical analysis was performed using the unpaired Student's t test. Error bars represent the standard error of the mean (SE).

C3 complement deposition assay

OECs isolated from HT transgenic pigs or control littermates were examined for cell surface complement activation as described previously (14) with the following modification. Cultured OEC isolates were incubated first with purified human anti- α -gal antibody at 4°C for a minimum of 30 min, followed by C8-depleted human serum (Quidel, San Diego, CA) at 37°C for 30 min. The cells were then analyzed for deposition of the activated complement component C3d with an anti-C3d monoclonal antibody (5 µg/ml, Quidel) followed by goat anti-mouse IgG secondary antibody (Zymed Laboratories). Flow cytometry analysis ($n=3$) was performed on a Becton-Dickinson FACSsort. Statistical analysis was performed using the unpaired Student's t test. Error bars represent the SE.

Lesion induction and cell injection into the spinal cord

Adult female African green monkeys (3.5-5.0 kg) were prepared for surgery under sterile conditions. They were anesthetized by intramuscular injection with ketamine (15 mg/kg) and glycopyrrolate (0.0015 mg/kg) followed by isoflurane gas anesthesia after tracheal intubation. Demyelinating lesions were made at either T7 and T9 (animal number=10; lesion site number=20), or at T7 ($n=4$) or T9 ($n=4$) alone. A lesion site was defined as the spinal cord level at which the lesion was made (e.g., T7 or T9). Thus, a total of 28 lesions were made in 18 monkeys. When lesions were made at both T7 and T9, one level was used for cell injection and the other for control using vehicle injection. When single spinal cord level lesions were made, some were used for cell injection ($n=6$) and the others for vehicle alone injections ($n=2$). The demyelinating lesion was induced by the direct injection of ethidium bromide (EB) or lysophosphatidyl choline (LPC, Sigma-Aldrich, St. Louis, MO) into the dorsal column via a drawn glass micropipette. Injections of 0.5-1.0 µl of 0.3 mg/ml EB or 0.5% LPC in saline were made at depths of 1.2, 0.8, and 0.4 mm at three longitudinal distances separated by 2.0 mm each along the midline of the dorsal funiculus. At each spinal cord level, three small openings (~100 µm) separated by 2.0 mm were made in the dura. A sterile micropipette with a tip diameter of ~40 µm was mounted to a micromanipulator that was fixed to a stable base. OECs or control vehicle (DMEM) were injected 3 days postlesion induction. Typically, 0.5 µl of cell suspension or vehicle alone was injected at each of three depths identical to the injections made to create the lesion (3.0×10^4 cells/µl, total cells injected ~2.5-3.0 $\times 10^5$). Both control and cell transplant recipients were immunosuppressed daily with cyclosporine A (CsA, 15 mg/kg/day im, Sandoz Pharmaceuticals) 1 day before spinal cord transplantation and throughout the course of the experiment.

Histology and immunohistochemistry

The animals were prepared for histological preparation between 3 to 5 wk after transplantation. Intracardiac perfusion with saline was followed by 4% paraformaldehyde, and the tissue was postfixed overnight. Plastic embedded sections for light and electron microscopy were

prepared as described previously (4). Briefly, 2.0 mm spinal cord blocks (coronal) were postfixed in osmium (1%) for 4 h. Tissue blocks were then embedded in epon. For light microscopy, semithin sections (1 μ m) were cut and counterstained with methylene blue and azure II (0.5% each in 0.5% borax). Ultrathin sections were cut for electron microscopy. To identify the transplanted porcine OECs, immunohistochemistry for p75 NGFR expression was performed on 6 μ m frozen sections from the perfusion-fixed monkey spinal cords. Negative controls were performed on lesioned monkey spinal cords without OEC transplantation. The sections were preblocked in normal blocking serum before incubation with the primary anti-human p75 NGFR monoclonal antibody (1:50) followed by incubation with a biotinylated secondary antibody and avidin-biotin-complex (Vectastain ABC Kit ELITE, Vector Labs, Burlingame, CA) and visualized with diaminobenzidine (DAB, Vector Labs).

RESULTS

Demyelinating lesion induction in the monkey spinal cord

Normal intact dorsal funiculus of the monkey is shown in Fig. 1A and B. Note the large number of myelinated axons of varying sizes (Fig. 1B). At 4-5 wk after lesion induction, the central region of the dorsal funiculus at the targeted lesion spinal level (Fig. 1C) was extensively demyelinated. The lesion area is marked by large numbers of demyelinated axons and phagocytic cells (Fig. 1D). Lesions in the primate spinal cord were more variable in size than similar lesions in the rodent, and the tendency for axon loss was greater in the monkey. In control lesions, vehicle alone in the absence of cell transplantation, there was generally no endogenous myelin repair ($n=12$) studied at 3-5 wk postlesion induction. However, in one control lesion, a small area of Schwann cell-like remyelination was observed superficially near the dorsal root entry zone (data not shown). The lack of endogenous remyelination at 4-5 wk in this nonhuman primate model spinal cord after chemically induced demyelination is different than in the rodent spinal cord where robust endogenous remyelination occurs within the lesion zone by 2-3 wk. It is uncertain if endogenous repair is delayed in the monkey and would be observed at later survival times.

Transplantation of HT transgenic pig OECs into the monkey spinal cord

To examine the potential of the transgenic pig OECs to remyelinate axons in vivo, we transplanted HT OECs into demyelinated lesions by microinjection 3 days after lesion induction. All monkey recipients were immunosuppressed with CsA (15 mg/kg/day im) beginning 1 day before transplantation and continuing throughout the course of the experiment. The transplanted spinal cords were prepared for histological analysis between 3-5 wk postinjection. Evidence of remyelination was detected in 10 of 16 (62.5%) transplant sites as determined by histological analysis of serial sections through the lesion area. A representative spinal cord from a HT OEC recipient 4 wk posttransplantation illustrated the area of the lesion and the transplantation site within the dorsal funiculus (Fig. 2A). The area of the dorsal funiculus inside the white dashed lines was densely remyelinated (Fig. 2B). The positions of b, d, e, and f in Fig. 2A correspond to positions in the spinal cord from which photomicrographs for B, D, E, and F, respectively, were obtained. A higher power photomicrograph of a field within the transplant zone (boxed area in Fig. 2B) revealed extensive remyelination of axons of varying sizes (Fig. 2C). A number of the remyelinated axons are clearly associated with ensheathing cells that contain large cytoplasmic compartments and large nuclei (arrows, Fig. 2C). We also observed that remyelination did not extend across the entire cross-sectional area of the lesion. Analysis of the border between the transplant zone and the demyelinated region revealed demyelinated axons (left, Fig. 2D) adjacent to remyelinated axons (right; Fig. 2D). Another finding in some of the transplant recipients was the presence of axon-deficient regions that contained cells with dark staining nuclei (Fig. 2E). The few axons that were present within this zone were remyelinated with a peripheral myelination pattern. These cells did not extend

into the surrounding remyelinated regions or outside the lesion area and did not obviously distort the spinal cord. Finally, areas outside of the central remyelination zone remained demyelinated (Fig. 2F).

Electron micrographs of the OEC-transplanted spinal cord indicated that the remyelinated axons have a cytoplasmic region surrounding the myelin and some are associated with large nuclei (Fig. 3A). A higher power electron micrograph shows a remyelinated axon with an outer cytoplasmic rim associated with a basement membrane (arrows) and extracellular collagen deposition (Fig. 3B). The presence of a basement membrane and extracellular collagen is a hallmark feature of peripheral myelin (15) and distinguishes the myelin-forming cells from oligodendrocyte-mediated central nervous system (CNS) myelin. The number of myelin wraps surrounding the remyelinated axons (13 ± 2 , $n=28$) was less than axons obtained from corresponding normal spinal cord white matter (21 ± 4 , $n=22$; SE: $P < 0.0004$) and suggests that these axons are undergoing repair. The morphological profile observed in the remyelinated axons is characteristic of peripheral myelin produced by either Schwann cells (15) or OECs (5-7,16,17).

Immunohistochemical analysis for p75 NGFR on frozen sections demonstrated that the peripheral pattern of remyelination was induced by the transplanted OECs. We used an anti-human p75 antibody that recognizes the porcine homologue and does not cross-react with monkey spinal cord tissue (18). Immunohistochemical analysis revealed p75 expression and indicated that p75 immunoreactivity is limited to the transplant zone (Fig. 4A). A higher power micrograph within the area of the transplant zone reveals intense p75 immunoreactivity (Fig. 4C). A high power micrograph of the lateral funiculus, outside of the transplant zone, shows no immunoreactivity (Fig. 4B). These results indicate the presence of p75-expressing pig OECs within the area of remyelination.

Morphology and purity of transgenic pig OECs in culture

OECs were prepared from pig olfactory bulbs and maintained in culture for 5-7 days. The prevalence of p75 NGFR (Fig. 5A)-labeled cells confirmed the identity and purity of the OECs, because they are the only cell type in the olfactory bulb that expresses p75 NGFR (19). The cultures were counterstained with nuclear yellow (Hoechst 33250) to identify individual cells, and the vast majority (~98%) of the cells were p75 NGFR-positive (Fig. 5A). In addition to p75 expression, OECs are known to express both S100 and GFAP (19). To determine if the HT transgenic pig OECs express both S100 and GFAP, we double labeled the cells for p75 NGFR and S100 or p75 NGFR and GFAP (Fig. 5B and C, respectively). There is a high degree of coexpression of S100 and GFAP immunoreactivity with p75 NGFR immunoreactivity, but a small number of cells were p75 NGFR⁽⁻⁾ and S100⁽⁺⁾ as has been reported for rodent OECs (19). These data indicate that similar to rodent OECs, the cultured HT transgenic pig OECs express p75 NGFR, S100, and GFAP. However, unlike rodent OECs, the porcine OECs could be harvested at high purity without additional purification strategies such as immunopanning.

Expression of the HT transgene in transgenic pig OECs

The Gal α 1,3Gal epitope is expressed on all mammalian cells except humans and other Old World primates (20) and can be detected with the lectin GS-IB4. Nontransgenic (wild type) pig cells express high levels of Gal α 1,3Gal as demonstrated by a high level of GS-IB4 binding (Fig. 5E) and negligible H epitope expression as detected with the lectin UEA1 (Fig. 5D). In contrast, the H epitope is expressed at high levels in the HT OECs (Fig. 5G) and the Gal α 1,3Gal epitope is down-modulated in HT transgenic OECs (Figs. 5H and 6B-C). This observation is consistent with previous studies where a reciprocal staining pattern for UEA1 and GS-IB4 (inverse intensity of staining between the 2 lectins in wild type vs. HT transgenics) was observed in HT transgenic and nontransgenic cells (11). A control cell surface adhesion

molecule, CD44, has been shown to be expressed on pig mesencephalic neurons (18) and was used as a positive control on both nontransgenic (Fig. 5*F*) and transgenic pig OECs (Fig. 5*I*).

OECs isolated from nontransgenic and HT transgenic pigs were also analyzed for transgene expression by flow cytometry (Fig. 6*B* and *C*, respectively). The reciprocal expression pattern observed in the flow cytometric profiles correlated with the immunocytochemical analyses. To further illustrate the inverse relationship between Gal α 1,3Gal antigen and H antigen expression between nontransgenic and HT transgenic pig OECs, we performed a semiquantitative analysis by calculating the mean fluorescence intensity (FL-1) from several flow cytometry experiments (Fig. 6*C*). These data demonstrate that a 100-fold increase in HT expression leads to about a sevenfold reduction in Gal α 1,3Gal expression (Fig. 6*C*). We also monitored the expression level of two control antigens, SLA class I and CD44, to ensure comparability in the flow cytometry analysis (Fig. 6*C*). There was no difference in the expression levels of the porcine SLA class I or CD44 between the nontransgenic and the transgenic OECs, and indicated that changes in the carbohydrate phenotype did not alter the expression of other cell surface molecules in the transgenic OECs.

Complement activation on HT transgenic and control porcine OECs

To determine the effect of HT expression on complement activation in the transgenic OECs, we analyzed the HT and nontransgenic OECs in a complement activation assay. This assay activates complement on the target cell surface without lysing the cell and allows for the detection of the activated human complement component C3d on the cell surface using an anti-human C3d monoclonal antibody and flow cytometry. Complement activation was reduced more than twofold in the HT OECs as compared with the nontransgenic cells (Fig. 6*D*), indicating that the expression of HT in the transgenic OECs leads to protection from antibody reactivity and complement activation.

DISCUSSION

The capacity of OECs for neural repair has been demonstrated in a number of studies (1–9, 17,21–22). Whether the experimental evidence gathered so far, however, will have an impact on the therapy of human disease cannot be decided to date. This is, at least in part, due to the fact that most of the experimental work was done on the rodent system and that OEC preparations of varying purity and cellular composition were applied (for review see ref 23). Since the cell yield of the rodent olfactory bulb is limited because of its size, purification protocols usually require an antibody-based purification step together with the mitogenic stimulation of the cultures. While possible, attainment of high yields of human OECs is problematic. Here we demonstrated that high yields of pure OECs can be obtained from transgenic pigs designed to resist humoral immunity in humans and that these cells can remyelinate the nonhuman primate spinal cord.

Specifically, we performed a thorough characterization of the cultures and show that 1) pig-derived OECs can be isolated at high purity without growth factor expansion, 2) the cells have phenotypic properties similar to the previously characterized rodent OECs (1–2,16,19), and 3) the HT transgene is expressed in the porcine OECs. The morphology of the pig OECs harvested in culture for up to 1 wk was similar to comparable cultures of rodent OECs. The vast majority of cells (98%) were of a spindle-shaped morphology and coexpressed typical OEC marker molecules, such as p75 NGFR, S100, and GFAP. Only a few cells displayed a fibroblast-like morphology and did not express p75 NGFR. It has to be emphasized that OECs were cultured in the absence of any growth factor or forskolin supplements, thereby excluding the possibility that such a treatment may have altered the properties of the cells and their potential for neural repair. The relative ease of the preparation of porcine OECs in high purity and large number may be due to the large size of the pig olfactory bulb, which allows a precise

dissection of the olfactory bulb outer nerve layer and its accurate separation from the surrounding meninges.

The matter of cell yield has another serious implication. Recent studies have questioned the regenerative potential of OECs for neural repair speculating that meningeal-derived Schwann cells may account for the observed effects (24). In our study separation of the olfactory bulb from the meninges was facilitated due to size and the preparations yielded a high number of cells without any growth factor expansion. Thus, it can virtually be excluded that residual contaminating Schwann cells were overgrowing the cultures. This, in turn, together with other evidence (see below) is a further confirmation that it was actually the OECs that mediated the observed in vivo effects. Taken together, our data demonstrate that the pig model system serves as a convenient and efficient source for obtaining highly purified OEC preparations suitable for transplantation.

Expression of the H antigen on OECs from HT transgenic pigs

Expression of HT leads to the expression of the universally accepted human O blood group phenotype that in turn reduces Gal α 1,3Gal expression resulting in reduced human antibody reactivity and complement-mediated cell lysis (11). We found that OECs isolated from HT transgenic animals express the H antigen and exhibit a sevenfold reduction in Gal α 1,3Gal epitope expression that led to reduced complement activation by human serum, which is consistent with observations on other cell types derived from the HT transgenic pigs (11,12). Although anti- α Gal antibody reactivity and complement activation certainly contribute to xenogeneic organ rejection (25,26), cells transplanted into the CNS are also susceptible to humoral-mediated rejection (27). Cicchetti et al. (27) showed that an induced anti- α Gal antibody response to cells transplanted in the CNS was a parameter for tissue rejection and that the induced anti- α Gal response was similar to the response observed in whole organ transplantation, i.e., three- to five-fold increase within the first 2 wk after transplantation. In addition, an induced anti- α Gal antibody response was completely absent in an HT neuron transplant recipient. The OEC transplants described here are comparable to those tissue transplants in that the nonvascularized OEC cell preparations are transplanted into the white matter of the primate spinal cord. Serum antibody titers were taken from a few recipients in this study, and a similar pattern was observed (data not shown). We did observe an absence of an induced antibody response from two recipients that received HT transgenic OECs after transplantation. However, not all recipients underwent blood collection and serum analysis and therefore a complete set of data are not possible.

Demyelination in the nonhuman primate spinal cord

Chemically induced remyelination in rats results in robust remyelination with both a central (oligodendrocyte) and peripheral (Schwann cell-like) remyelination at 2-3 wk postlesion induction. If the rat spinal cords are focally X-irradiated for several centimeters before focal injection of ethidium bromide, persistent demyelination is present for at least 6-8 wk (6). The focal X-irradiation results in a localized radiation lesion of the skin over the irradiated spinal cord region. Although this lesion is manageable in the rodent and does not present a long term medical problem, for ethical reasons we chose not to X-irradiate the primates where the lesion would be larger and possibly more problematic with regard to the comfort and health of the animal. We found that chemically induced demyelination alone consistently resulted in demyelination with virtually no endogenous repair in control lesions studied at 3-5 wk postlesion induction, suggesting either an absence or delay of endogenous myelin repair in the vervet. These control lesion data in the primate spinal cord indicated that if the remyelination potential of transplanted OECs was studied within this time window, observed remyelination was quite likely the result of the donor cells.

Adult transgenic pig OECs remyelinate the demyelinated monkey spinal cord

Transferring basic research to clinical studies requires the use of model systems as close to the human system as possible. OECs have been shown to mediate remyelination and to support axonal regeneration in the central nervous system in a variety of studies. However, without exception, this work was based on the rodent system as a recipient (23). Since it is known that there are substantial differences in the spinal cord and its reaction to injury (e.g., size, fiber tract location, endogenous repair potential) between the rodent and the primate system, the question arises as to whether the evidence gathered so far can be extrapolated to human therapeutic approaches.

In the present study, we, therefore, used well-characterized and highly purified OECs for transplantation into the demyelinated monkey spinal cord. This is the first demonstration that OECs are able to remyelinate the adult nonhuman primate spinal cord. In 62.5% of the transplantation sites, robust myelination was observed at 3-4 wk posttransplantation, whereas there was virtually no remyelination in the operated but nontransplanted control sites at this time. This is in contrast to the rodent system where endogenous remyelination is known to occur by 3 wk after demyelination and further argues for the use of primate systems. It is important to note that remyelination was not observed in six transplant sites. We believe that given the difficulty of the model related to the irregularity of the lesions that many of the negative animals were negative due to surgical difficulties in getting the cell transplants into the lesion site. Although we cannot rule out immune rejection, we observed very little immune infiltrate in any of the sections and believe the CsA therapy was effective in modulating the immune response to these cells. Our data thus imply that in spite of species-specific differences in the reaction to injury, the well-established repair potential of OECs for the rodent system is also true for the primate. To demonstrate that the cells transplanted were actually responsible for the effects, we used antibodies against p75NGFR that selectively labeled the grafted pig OECs. We show that p75-positive cells resided within the area of remyelination.

The myelin morphology and the pattern of axonal remyelination induced by the transplanted OECs were similar to that reported for rodent OEC transplant studies (5-7,16,17). In particular, individual myelinated axons were associated with an ensheathing cell that had an extensive cytoplasmic compartment and a large nucleus. The morphology observed is characteristic of peripheral myelination (15). Virtually all remyelinated axons observed by electron microscopy were associated with a basement membrane, which is not present in axons myelinated by oligodendrocytes. Although large areas of remyelination were observed, the peripheral margin of the lesions often remained demyelinated. This suggests that either the number of cells injected was not sufficient for a complete repair or the migration of the OECs with the lesioned area was limited.

The development of porcine cell- and tissue-based therapies to treat human diseases has gained much attention, and currently there are several potential clinical applications for the use of porcine tissues. Examples include the treatment of Parkinson's disease patients with embryonic porcine mesencephalic neurons (28) and the use of porcine hepatocytes for liver support systems in cases of acute liver failure (29). Many porcine cell types potentially useful for transplantation, such as cardiomyocytes for damaged heart muscle (30) or OECs for axonal repair (4), would be expected to trigger a humoral and cellular immune response (25-27). Therefore, the demonstration that genetically engineered pig OECs, modified to resist humoral immunity, exhibit characteristic OEC phenotypic properties and can repair demyelinated primate spinal cord lesions reinforces the potential utility of transgenic porcine tissues for human cell therapy.

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Figure 1. Dorsal funiculus of control and demyelinated monkey

Low (**A**) and high (**B**) power light photomicrographs of control dorsal funiculus of the monkey spinal cord of plastic-embedded (1 μ m sections) stained with methylene blue and azure II (see Methods). Note the densely packed myelinated axons in **B**. **C**) Low power micrograph showing extent of demyelinated lesion in the monkey spinal cord induced by ethidium bromide injection. **D**) High power micrograph shows densely packed demyelinated axons. Lowercase b and d in **A** and **C**, respectively, indicate the positions in the dorsal funiculus from which the photomicrographs in **B** and **D** were obtained. Scale bar shown in **D**: **A** and **C** = 0.7 mm, **B** and **D** = 10 μ m.

Figure 2. Transplantation of transgenic pig OECs into the demyelinated monkey spinal cord
A) Low power photomicrograph of lesion after cell transplantation in the dorsal funiculus (DF) from sections prepared as in Fig. 1. Remyelination was observed within the white dashed lines and most of the dorsal funicular region outside of the dashed lines remained demyelinated. The positions of b, d, e, and f in **A** correspond to positions in the spinal cord from which photomicrographs for **B**, **D**, **E**, and **F**, respectively, were obtained. **B)** Central core (b from **A**) of the lesion was densely remyelinated. **C)** Expansion of the boxed area in **B**, showing myelinated axon profiles exhibiting a peripheral pattern of remyelination. **D)** At the edge of the densely remyelinated zone (d in **A**) a transition from demyelinated (left) to remyelinated axons (right) can be seen. **E)** Area marked e in **A** has only a few intact axons but is replete with cells with dark nuclei. **F)** Micrograph (obtained from f in **A**) of a region outside the remyelination zone illustrating demyelinated axons interspersed with phagocytic cells. Scale bar shown in **F**: **A** = 0.5 mm, **C** = 50 μ m, **B**, **D**, **E**, **F** = 125 μ m.

Figure 3. Ultrastructure of the remyelinated axons

A) Electron micrograph showing a field of remyelinated axons within the central core of the transplantation zone. Note the large nuclei and cytoplasmic regions surrounding many of the remyelinated axons. **B)** High power electron micrograph illustrates the presence of a basement membrane surrounding a remyelinated axons. Scale bar in **B**: **A** = 3.0 μm ; **B** = 200 nm.

Figure 4. Human p75 immunoreactivity within the transplantation zone

A) Low power micrograph of a cryosection through the dorsal funiculus (DF) and dorsal horn (DH) of a HT transgenic OEC transplanted monkey. The section was immunostained using an anti-human p75 antibody. **B)** High power micrograph from the lateral funiculus where no lesion was made and without transplant did not stain. **C)** High power micrograph at the edge of the remyelination area showing intense immunoreactivity for p75 on the right (area of remyelination) and reduced staining on the left (demyelinated area). Dashed lines in **A** indicate the margins of the lesion and in **C** the transition zone between demyelinated and remyelinated axons. Arrowheads delineate the outer margins of the dorsal funiculus. Scale bar in **A**: **A** = 400 μm , **B** = 50 μm .

Figure 5. Purity and characterization of porcine olfactory ensheathing cells in culture

A) Cultured OECs immunolabeled at 5 days with anti-human p75 NGFR and counterstained with nuclear yellow (Hoechst 33250). **B** and **C)** A field of cultured OECs showing p75 immunoreactivity co-localized with either S100 (**B**) or GFAP (**C**). UEAI and GSIB4 lectin fluorescence labeling of control (**D**, **E**) and of HT (**H**, **G**) pig OECs. CD44 immunocytochemical analysis of control (**F**) and HT (**I**) pig OECs. Cells were stained while alive in culture and photographed with an inverted microscope. Scale bar shown in **G** = 50 μm .

Figure 6. Flow cytometry and complement activation analyses of transgenic (HT) and nontransgenic (NT) pig OECs

Flow cytometry analysis of Gal α 1,3Gal and H-epitope carbohydrate cell surface expression on NT (A) and HT (B) OECs as determined with reactivity to the lectins GS-IB4 and UEA1, respectively. C) Bar graph summarizing the expression pattern for the α -Gal and H carbohydrates and for 2 control cell surface proteins, SLA class I and CD44. Mean fluorescence intensity (FL-1) was calculated from flow cytometry experiments on control NT ($n=7$) and HT ($n=14$) OECs. Reduction of Gal α 1,3Gal expression on the HT OECs was significantly lower than in nontransgenic control animals, $P \leq 0.05$. D) Cell surface complement activation on HT transgenic ($n=3$) and NT control ($n=3$) olfactory ensheathing cells was determined by flow cytometry using an antibody to the activated complement component, human C3d. Mean fluorescence intensity (FL-1) was calculated from 3 independent cell isolates ($P=0.0036$).