Morphological integration and functional assessment of transplanted neural progenitor cells in healthy and acute ischemic rat eyes

Sinisa D. Grozdanic a,*, Allison M. Ast b, Tatjana Lazic a, Young H. Kwon d, Randy H. Kardon d, Ioana M. Sonea c,1, Donald S. Sakaguchi b,c,*

a Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA
b Neuroscience Program and Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, USA
c Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA
d Department of Ophthalmology and Visual Sciences, University of Iowa Hospitals and Clinics, Iowa City, IA 52242, USA

Received 28 April 2005; accepted in revised form 24 August 2005
Available online 5 October 2005

Abstract

We have functionally and morphologically characterized the retina and optic nerve after neural progenitor cell transplants to healthy rat eyes and eyes damaged by acute elevation of intraocular pressure (IOP). Green fluorescent protein-expressing adult rat hippocampal progenitor cells (AHPCs) were transplanted by intravitreal injection into healthy eyes and eyes damaged with acute ocular hypertension. Pupil light reflexes (PLR) and electroretinograms (ERGs) were recorded preoperatively and postoperatively. Eyes were subsequently prepared for immunohistochemical analysis and confocal imaging.

Transplanted AHPCs were found in 8 of 15 (53%) acute ischemic eyes 62 days after surgery and 5 of 10 (50%) healthy eyes 32 days after grafting. Analysis of PLR and ERG function in acute ischemic eyes revealed no statistically significant difference compared to controls after transplantation for all observed functional parameters. Transplant into healthy rat eyes revealed no PLR or ERG amplitude deficits between transplanted and non-transplanted (control) eyes. Morphological and immunohistochemical analysis revealed that transplanted AHPCs survived and differentiated in both normal and injured retinal environments. Morphological integration occurred primarily within the inner retinal layers of the acute ischemic eyes. AHPCs were found to express neuronal and glial markers following transplantation.

Transplanted AHPCs have the ability to integrate and differentiate in ischemia damaged retinas. PLR and ERG analysis revealed no significant difference in functional outcome in transplant recipient eyes.

Keywords: electrotetrogram; pupillometry; optic nerve; stem cells; retinal ischemia

1. Introduction

The death of retinal neurons is a hallmark feature of glaucoma, acute ocular hypertension, diabetic retinopathy and other ocular diseases characterized by ischemic insults. Traditionally, retina and optic nerve damage has been considered irreversible in humans and animals due to the lack of the regenerative capacity of the mammalian central nervous system (CNS). The retina, as a part of the CNS, is the target of many degenerative diseases with blindness as a final common outcome. Retinal regeneration has been a field of interest for more than 50 years (Stone, 1950). Recent studies have shown that some regenerative activity and the potential re-establishment of visual processing can be achieved by different transplantation techniques (Girman et al., 2003; Lund et al., 2001; Sagdullaev et al., 2003; Woch et al., 2001). However, some transplantation procedures are primarily based on the availability and use of fetal retinal tissue, which is associated with major ethical and practical issues. Alternative strategies are needed in order to provide an unlimited supply of transplantation substrate.

Recent discoveries in the field of neural stem/progenitor cell biology offer new hope for treatment of incurable chronic neurodegenerative diseases (Kim et al., 2002; Ourednik et al., 2002) and also potentially provides an alternative to the use of fetal tissue (Cameron and McKay, 1998). It is important to note
that studies examining the transplantation of ‘neural stem cells’, are likely grafting mixed populations of cells, some of which may be ‘true’ neural stem cells, but that also contain cells that are more differentiated and therefore these cells are best-termed neural progenitor cells.

Neural stem or progenitor cells are primordial cells postulated to give rise to the array of more specialized cells of the CNS (Cameron and McKay, 1998). They are defined by their ability to differentiate into cells of all CNS lineages (neurons, oligodendroglia and astroglia), to give rise to new progenitors with similar potential, and to populate developing and/or degenerating CNS regions (Gage et al., 1995). These cells have been isolated from adult, developing and embryonic brain and in vitro studies have revealed that they possess the ability to adopt a variety of cellular fates (Gage et al., 1995; Shatos et al., 2001; Svendsen et al., 1999).

Studies in fish and amphibians have shown that new retinal cells are continually formed from retinal progenitor cells during the life of the animal (Fernald, 1990). Furthermore, probably the most remarkable examples of the potential of stem cells is their ability to regenerate the CNS and is based on the studies of retina and optic nerve regeneration in lower vertebrates (Raymond and Hitchcock, 1997; Reh and Nagy, 1987). While identification of naturally occurring retinal progenitors in adult mammalian eyes has given hope for the existence of regenerative mechanisms in humans (Coles et al., 2004; Tropepe et al., 2000) so far there is no evidence of spontaneous retinal regeneration in injured and/or diseased mammalian eyes. However, the application of stem cell based therapies might offer the possibility to replace lost neurons or at least protect disease affected neurons in pathological eye conditions. While multiple studies have demonstrated the capability of neural progenitors to survive and morphologically differentiate into neuron-like cells in ischemic (Guo et al., 2003; Kurimoto et al., 2001) and traumatized (needle-injured) retinas (Chacko et al., 2000; Nishida et al., 2000), there are only two published reports, which have demonstrated some preservation of visual function after neural progenitor transplantation in laser-damaged eyes (Zhang et al., 2004) and eyes with retinal degeneration (Klassen et al., 2004). In order to validate the efficacy of stem cell based therapy it is essential to determine: whether neural progenitors are capable of survival in an environment which is continuously exposed to potential neurotoxic factors such as retinal ischemia and whether transplantation of neural progenitors can provide protection and recovery of compromised retinal neurons.

The principal purpose of this study was to determine whether transplanted green fluorescent protein-expressing adult rat hippocampal progenitor cells (AHPCs) could survive, integrate, differentiate and protect (or recover) function in acute ischemic eyes. Furthermore, we were interested to observe whether transplantation of neural progenitors would interfere with function and morphology of healthy rat eyes.

2. Materials and methods

2.1. Animals

All animal studies were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and procedures were approved by the Iowa State University Committee on Animal Care. Adult Brown Norway rats (n=31) were used in the study.

2.2. Culturing procedures

Adult rat hippocampal progenitor cells (AHPCs) were obtained from F.H. Gage (Laboratory of Genetics, The Salk Institute). The AHPCs were originally isolated from the brains of adult Fischer 344 rats as reported by Palmer et al. (1997). The AHPCs were maintained in polyornithine/laminin-coated tissue culture flasks (T75; Fisher Scientific, Pittsburgh, PA) in Dulbecco’s Modified Eagle’s Medium/Ham’s F12 (DMEM/F12, 1:1 Gibco BRL, Gaithersburg, MD) supplemented with N2 (1%; Gibco-BRL), 20 ng/ml basic fibroblast growth factor (bFGF; Promega Corp., Madison, WI) and L-glutamine (2.5 mM; Gibco BRL). To harvest the AHPCs for in vitro analysis, they were detached from the flasks using ATP solution (Gibco BRL), collected and pelleted by centrifugation at 800 RPM for 4–5 min.

2.3. Transplantation procedure

AHPCs were transplanted by injection using a bevelled glass micropipette attached, via a saline (0.9% NaCl) filled polyethylene tube, to a 20 μl Hamilton syringe. Animals received intraocular injections of AHPCs through the superior-lateral aspect of the pars plana. Two microliter of cell suspension (∼50,000 cells/μl) were slowly injected into the vitreal chamber of the eyes. An aliquot of cells used for each transplant session were subsequently plated into sterile culture dishes and visualized using fluorescence microscopy to verify GFP expression and the viability of the transplanted cells.

2.4. Acute ischemia model in rats

A previously published procedure to generate an ischemia-reperfusion insult in rats was used (Grozdanic et al., 2003a,b). Briefly, adult Brown Norway rats (n=21) were anesthetized and the anterior chamber was cannulated with a 25-gauge needle connected to a reservoir containing 0.9% NaCl. The intraocular pressure in experimental eyes was controlled by the height of the reservoir to maintain a pressure of 110 mmHg for 60 min. To prevent potential infection, antibiotic ointment (neomycin+polymyxin B+bacitracin; Bausch & Lomb Pharmaceuticals Inc; Tampa, FL) was applied topically after the procedure. Ten days after ischemia-reperfusion insult 15 rats received AHPC transplant, while six rats received transplant of non-viable cells (repeatedly frozen and thawed AHPCs which were exposed to UV illumination for 10 min).
Sixty-two days after ischemia-reperfusion insult rats were euthanized and tissue was collected for histological analysis.

2.5. Healthy rats

Ten adult Brown Norway rats received intravitreal transplant of the AHPCs in the right eye. The left eye was non-injected and served as a control. Thirty-two days after transplantation, rats were euthanized and tissue was collected for histological analysis.

2.6. Functional monitoring

2.6.1. Computerized pupillometry

The pupil light reflex was evaluated with a custom-made computerized pupillometer (University of Iowa, Iowa City, IA) as we previously described (Grozdanic et al., 2002, 2003a, 2004). Briefly, rats were anesthetized and a light plane of anesthesia was maintained with 1% halothane, 30% NO and 70% O2 to avoid suppression of the pupil light reflex response as detected with the use of higher doses of anesthet. The computerized pupillometer was attached to two infrared sensitive CCTV video cameras for simultaneous visual monitoring of both pupils. However, one channel computerized pupillometer was used to record the movement of the pupil from the control (non-operated) eye, while the stimulus light was alternated between the control and operated eye.

2.6.2. Electroretinography

To quantify damage to the retina due to chronic elevation of the IOP, a simultaneous recording of electroretinogram from both eyes (control and operated) was performed with two Ag–AgCl electrodes as previously reported (Grozdanic et al., 2002, 2003a, 2004). The lumiance of the Ganfeld dome surface was measured with a J17 LumaColor TM photometer equipped with a J1803 luminance head (Tektronix, Willsonville, OR). Measured lumiance in our system was 1600±200 cd/m^2. A flash ERG routine was delivered at a 0.2 Hz frequency (10 averaged signals per recording session, sensitivity 100 μV/division, low-cut frequency 0.5 Hz, high-cut frequency 10 kHz, analysis time 500 msec). Oscillatory potentials were recorded by delivering light stimuli at a 0.2 Hz frequency (10 averaged signals per recording session, sensitivity 100 μV/division, low-cut frequency 50 Hz, high-cut frequency 500 Hz, analysis time 100 msec). Isolated cone responses were recorded from previously light adapted eyes by delivering stimuli at 20 Hz (50 averaged signals per recording session, sensitivity 50 μV/division, low-cut frequency 0.5 Hz, high-cut frequency 10 kHz, analysis time 500 msec).

2.7. Histological and immunohistochemical examination

Immunohistochemical examination was performed as described previously (Van Hoffelen et al., 2003). Briefly, after performing functional recordings at the appropriate survival periods, the rats were deeply anesthetized with halothane and perfused transcardially with 4% paraformaldehyde in 0.1 m PO4 buffer. Eyes were removed, post-fixed and then cryoprotected in 30% sucrose in 0.1 m PO4 buffer. Tissue was embedded, (Tissue-Tek OCT compound, VWR International, West Chester, PA) frozen and sectioned coronally at 20–40 μm thickness using a cryostat (American Optical, Buffalo, NY). Sections were thaw mounted onto Superfrost microscope slides (Fisher Scientific) and stored at −20 °C until processed.

Specific primary antibodies (see Section 2.8) were used to identify proteins associated with differentiated cell phenotypes. For antibody staining, tissue sections were washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na2 HPO4, 1.47 mM KH2PO4) and incubated in blocking solution (5% goat serum; 0.4% bovine serum albumin, BSA; Sigma, and 0.2% Triton X-100, Fisher Scientific, in PBS). Primary antibodies were diluted in blocking solution and preparations were incubated overnight at 4°C in a humid chamber. On the following day the preparations were rinsed with PBS and incubated with an appropriate secondary antibody for 90 min at room temperature. Slides were then rinsed and cover-slipped using Vectashield Fluorescence (Vector Laboratories, Burlingame, CA) mounting media.

2.8. Antibodies

Antibodies and concentrations used in this analysis were as follows: anti-microtubule associated protein (MAP) 2ab (mouse IgG; 1:500; Sigma) was used as a marker of inner retina neurons (ganglion cells and the inner plexiform layer) (Caceres et al., 1986); anti-neurofilament antibody (mouse IgG; 1:200; Developmental Studies Hybridoma Bank, Iowa City, IA) was used as a marker of retinal ganglion cell axons and the optic nerve; anti-class III beta tubulin (TUJ1) (mouse IgG; 1:200; Chemicon International, Temecula, CA) was used as an immature neuronal marker; anti-Calretinin (rabbit; 1:3,000; Chemicon) was used to identify this calcium binding protein which has been used as a marker of a subclass of horizontal cells, amacrine cells, and ganglion cells (Massey and Mills, 1999; Volgyi et al., 1997); anti-synaptic vesicle protein 2 (SV2) (mouse IgG; 1:100; Developmental Studies Hybridoma Bank) was used to identify the synaptic layers in the retina (Buckley and Kelly, 1985). Anti-glial fibrillary acidic protein (GFAP)(mouse IgG; 1:200; ICN Immunobiologicals, Costa Mesa, CA) was used as a marker of astrocytes and reactive Müller glia of the retina (Debus et al., 1983) and anti-RIP (mouse IgG; 1:100; Developmental Studies Hybridoma Bank) was used as a marker for oligodendrocytes (Friedman et al., 1989).

After labelling with primary antibodies, the specimens were rinsed with PBS and incubated with secondary antibodies diluted in blocker solution. Antibody and fluorochrome concentrations used in this study were: Alexa 546-conjugated goat anti-mouse IgG (1:200, Molecular Probes), rhodamine isothiocyanate (RTIC)-conjugated goat anti-mouse IgG (1:200, Southern Biotechnology, Birmingham, AL), Cy5-conjugated goat anti-mouse IgG (1:400, Jackson ImmunoResearch),
Monitoring of PLR amplitudes revealed no significant difference between acute ischemic rat eyes in which AHPCs were detected by histological analysis (n=8) and the control group, which received non-viable cells (Fig. 1). We previously demonstrated spontaneous, but temporary recovery of some function between 30 and 35 days postoperatively (ERG and PLRs) in our model of acute retinal ischemia (Barnett and Grozdanic, 2004; Grozdanic et al., 2003b). As such, we wanted to be certain that possible changes in function were most likely due to the effect of the transplanted cells and not to intrinsic host retinal mechanisms, and therefore, we selected time points identical to the data from our previous studies.

Values for the PLR ratio (ratio = indirect/direct PLR) are illustrated in Table 1. There was no statistical significance between different groups after cell injection (p > 0.1).

### Table 1

<table>
<thead>
<tr>
<th>Time</th>
<th>Pupil light reflex ratio (%)</th>
<th>NV</th>
<th>AHPC</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preop</td>
<td>65.9 ± 3.9</td>
<td>74.2 ± 3.9</td>
<td>74.4 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>9 d</td>
<td>4.5 ± 2.9*</td>
<td>20.7 ± 8.4</td>
<td>26.8 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>35 d</td>
<td>32.5 ± 8.5</td>
<td>24.2 ± 8</td>
<td>29.6 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>42 d</td>
<td>17.7 ± 8.7</td>
<td>21.7 ± 7.8</td>
<td>26.5 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>60 d</td>
<td>21.3 ± 9.1</td>
<td>28.1 ± 9.4</td>
<td>36.4 ± 6.5</td>
<td></td>
</tr>
</tbody>
</table>

The group of rats that received AHPCs, but did not have histological evidence of viable cells at the end of the experiment had significantly greater amplitudes at 9 days postoperatively (time point before transplantation). NV, group which received non-viable cells; AHPC group which had cells detected on histology; ND, group which received AHPC, but no cells were detected on histology. (*p < 0.05; Kruskal–Wallis test with Dunn’s post-test).
Table 2
Analysis of PLR velocity ($\Delta$Velocity = velocity$_{ctrl} -$ velocity$_{operated}$) showed no significant difference among different experimental groups ($p > 0.1$; Kruskal–Wallis test with Dunn’s post-test).

<table>
<thead>
<tr>
<th>TIME</th>
<th>NV</th>
<th>AHPC</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preop</td>
<td>0.38±0.09</td>
<td>0.30±0.07</td>
<td>0.35±0.04</td>
</tr>
<tr>
<td>9 d</td>
<td>2.4±0.1</td>
<td>1.8±0.3</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>35 d</td>
<td>1.8±0.2</td>
<td>1.9±0.3</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>42 d</td>
<td>2.0±0.4</td>
<td>1.8±0.2</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>60 d</td>
<td>1.8±0.3</td>
<td>1.8±0.3</td>
<td>0.98±0.2</td>
</tr>
</tbody>
</table>

NV, group which received non-viable cells; AHPC, group which had cells detected on histology; ND, group which received AHPC, but no cells were detected on histology.

Kruskal–Wallis test with Dunn’s post-test), however there was significant difference between the group of rats which received non-viable cells and the group that received transplanted cells, but did not have detectable cells at the 9 days postoperative time point (time point prior to the transplantation procedure).

We analysed velocity of the PLR ($\Delta$velocity = velocity$_{ctrl} -$ velocity$_{operated}$) and detected no significant difference among the different experimental groups ($p > 0.1$; Kruskal–Wallis test with Dunn’s post-test, Table 2).

By subtracting latency time values ($\Delta$latency = latency$_{operated} -$ latency$_{ctrl}$) we determined significantly increased latency time deficits at 42 and 60 days postoperatively (Table 3). At 42 days postoperatively, the latency deficit was significant in the group of rats which received non-viable cells, compared to animals which received transplants but did not have any AHPCs detected histologically at the termination of the experiment ($p < 0.05$ Kruskal–Wallis test with Dunn’s post-test). At 60 days, the control transplant group which received non-viable cells ($p < 0.01$) and the group which received AHPCs and had morphologically detectable cells ($p < 0.05$), had significantly prolonged latency times when compared to animals which received transplants, but did not have any cells detected histologically (Kruskal–Wallis test with Dunn’s post-test).

Pupillometric analysis of the transplanted acute ischemic eyes revealed no significant beneficial effect on the constriction amplitude. Furthermore, eyes that had morphologically detectable AHPCs had more significant latency deficits at 60 days postoperatively compared to the eyes that received viable cells, but did not have morphological evidence of AHPC survival.

Analysis of scotopic ERG amplitudes (62 days postoperatively) revealed no statistically significant difference between groups that received AHPCs and those animals that received non-viable cell transplants for any of the tested parameters (Figs. 2 and 3).

The latency time of a-waves was not significantly different in AHPC transplanted eyes (50.8±0.5 msec) compared to eyes, that received AHPCs but were not histologically detected (51.3±0.6 msec) and which received non-viable cells (50.5±1.2 msec, $p = 0.8$, Student’s t-test). The latency time of b-waves was not significantly different in AHPC transplanted eyes (48±1.6 msec) compared to eyes, that received AHPCs but were not histologically detected (53.7±3.3 msec) and those eyes that received non-viable cells (48.9±5.5 msec, $p = 0.9$, Student’s t-test).

The photopic flicker ERG amplitude ratios were 1.8±1.2% (AHPC eyes), 0% (AHPC transplanted, but not detected) and 22.1±13.9% in eyes that received non-viable cells ($p > 0.1$, One Way ANOVA with Bonferroni’s Multiple Comparison Test). The latency time of the flicker ERG was not significantly different in AHPC transplanted eyes (62.9±0.1 msec) compared to eyes that received non-viable cells (61.4±1.1 msec, $p = 0.09$, Student’s t-test). Oscillatory potentials were not detected in any of the experimental groups postoperatively due to severe inner retinal damage that occurs in this model of acute retinal ischemia. While there was no statistical difference in the ERG amplitudes between experimental groups, we observed a trend of better b-wave amplitudes in animals that had morphologically detectable AHPCs.

Table 3
Analysis of PLR latency ($\Delta$latency = latency$_{ctrl} -$ latency$_{operated}$) showed significantly larger latency deficits at 42 and 60 d postoperative time points for the group of animals which received non-viable cells when compared to animals which received AHPCs, but showed no evidence of cell survival.

<table>
<thead>
<tr>
<th>TIME</th>
<th>$\Delta$Latency (msec)</th>
<th>NV</th>
<th>AHPC</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preop</td>
<td>13.8±5.1</td>
<td>18.8±4.9</td>
<td>16.6±3.6</td>
<td></td>
</tr>
<tr>
<td>9 d</td>
<td>144±16.4</td>
<td>137.2±15.6</td>
<td>114.2±14.8</td>
<td></td>
</tr>
<tr>
<td>35 d</td>
<td>147.1±18.4</td>
<td>116.6±16.2</td>
<td>88.9±8.4</td>
<td></td>
</tr>
<tr>
<td>42 d</td>
<td>133.2±10.5*</td>
<td>116.5±13.1</td>
<td>76.6±6</td>
<td></td>
</tr>
<tr>
<td>60 d</td>
<td>144.1±11.8**</td>
<td>121.4±12.5</td>
<td>50±14.3</td>
<td></td>
</tr>
</tbody>
</table>

Interestingly, rats that received AHPCs, but had evidence of the morphological survival of the transplanted cells, had significantly smaller latency deficits compared to the rats which received AHPCs and had morphological evidence of cell integration. NV, group which received non-viable cells; AHPC, group which had cells detected on histology; ND, group which received AHPC, but no cells were detected on histology. ($^*p < 0.05$; **$p < 0.01$; Kruskal–Wallis test with Dunn’s post-test).

3.2. Assessment of retina and optic nerve function of healthy rat eyes after AHPC transplantation

To test whether neural progenitor cells can survive and differentiate after grafting, and to determine whether or not they would alter the electroretinogram and pupil light reflex, we transplanted AHPCs into normal healthy adult rat eyes. Transplanted AHPCs were detected in 5 of 10 transplanted healthy rat eyes 30 days after transplant. As such, our functional analysis focused on this group of animals. Monitoring of PLR amplitudes revealed no significant difference between preoperative and postoperative values (Fig. 4(A)). Preoperative values for the PLR$_{ratio}$ (ratio = indirect/direct PLR, Fig. 4(A)) were 78.2±4.1 (mean±s.e.m; %). Fifteen days after transplantation the PLR$_{ratio}$ was 73.3±8.6 and was not significantly different compared to preoperative values ($p > 0.1$, repeated measures ANOVA with Bonferroni post-test, $n = 5$). Thirty days after transplantation the PLR$_{ratio}$
was 79.3 ± 2.3 and again was not significantly different compared to preoperative values \( (p > 0.1, \text{repeated measures ANOVA with Bonferroni post-test}) \). We analysed latency time and velocity of the PLR and detected no significant decrease of the PLR velocity \( (p > 0.1, \text{repeated measures ANOVA with Bonferroni post-test}) \) or the latency time \( (p > 0.1, \text{repeated measures ANOVA with Bonferroni post-test}) \) 15 and 30 days after transplantation. Calculation of the velocity parameters \( (\text{velocity}_\text{ctrl} - \text{velocity}_\text{transpl}) \) revealed the following values: preoperative 0.26 ± 0.04 mm/s \( (\text{mean} \pm \text{S.E.M}; n = 5) \), 15 days after transplantation the velocity deficit increased 0.46 ± 0.03 mm/s, but the difference was not statistically significant when compared to the pre-transplant values \( (p > 0.1, \text{repeated measures ANOVA with Bonferroni post-test}) \). Thirty days post-transplant the velocity was 0.26 ± 0.12 \( (p > 0.1, \text{repeated measures ANOVA with Bonferroni post-test}) \).

By subtracting latency time values \( (\text{latency time}_\text{oper} - \text{latency time}_\text{ctrl}) \) we determined the interocular difference in latency values at the following time points: preoperative = 20 ± 6.2 msec \( (\text{mean} \pm \text{s.e.m}; n = 5) \), 15 days postoperatively 26.7 ± 4.1 msec \( (p > 0.1, \text{repeated measures ANOVA with Bonferroni post-test}) \) and at 30 days 20 ± 9.7 msec \( (p > 0.1, \text{repeated measures ANOVA with Bonferroni post-test}) \).

Analysis of ERG amplitudes revealed no significant difference between transplanted and non-transplanted (control) eyes 30 days after transplantation \( (\text{Figs. 4(b) and 5}) \). The a-wave amplitudes were 191 ± 7.3 μV \( (\text{control eyes}) \) and 163 ± 16.9 μV \( (\text{transplanted eyes}; p = 0.24, n = 5, \text{Paired } t\text{-test}) \). However, latency time of a-waves was significantly prolonged in transplanted eyes \( (23.9 \pm 0.74 \text{msec}) \) compared to control eyes \( (22.4 \pm 1.81 \text{msec}; p = 0.0479, \text{Paired } t\text{-test}) \). The b-wave amplitudes were 498 ± 23.8 μV \( (\text{control eyes}) \) and 491 ± 38.4 μV \( (\text{transplanted eyes}; p = 0.86, n = 5, \text{Paired } t\text{-test}) \). Latency time of b-waves was not significantly prolonged in transplanted eyes \( (14 \pm 0.8 \text{msec}) \) compared to control eyes \( (14.1 \pm 0.6 \text{msec}; p = 0.71, \text{Paired } t\text{-test}) \).

Isolated cone response \( (\text{flicker ERG}; \text{Fig. 5(B)}) \) revealed no deficits in transplanted eyes 30 days after surgery with average amplitude of 12 ± 5.6 μV \( (\text{control eyes}) \) and 9.6 ± 2.2 μV \( (\text{transplanted eyes}; n = 5, p > 0.1, \text{Paired } t\text{-test}) \) and latency time of 50.8 ± 1.6 μV \( (\text{control eyes}) \) and 50.5 ± 1.8 μV \( (\text{transplanted eyes}; n = 5, p > 0.1, \text{Paired } t\text{-test}) \).

Functional analysis of the majority of ERG and PLR parameters revealed no dramatic negative effects of the AHPC transplantation in healthy rat eyes. The only exception was minimal, but significantly increased latency time of the a-wave amplitudes, which may be attributed to the reactive host glial response to the presence of transplanted cells \( (\text{Fig. 6(E)}) \).
3.3. Morphological analysis of adult hippocampal progenitor cells following transplantation into the acute ischemic and healthy rat eyes

After the final pupillometry and ERG recordings, the eye tissue was prepared for immunohistochemical analysis (62d postop—acute ischemic eyes, 32d post-transplant—healthy rat eyes). The AHPCs were reliably identified following grafting based on their GFP fluorescence. Green fluorescent protein-expressing AHPCs were observed throughout the posterior segment of the eye. Limited intra-retinal integration (predominantly inner retinal layers) was observed in acute ischemic rat eyes (Fig. 6(A)–(C)). Transplanted AHPCs were primarily found along the inner limiting membrane (ILM), in the vitreous and around the lens (Fig. 6(D)–(F)) in the healthy rat eyes.

Specific antibodies were used to investigate the ability of grafted AHPCs to morphologically differentiate within the environment of transplanted eyes. Co-expression of GFP and one of the phenotypic antibody markers was used to evaluate neural differentiation of the AHPCs.

Subpopulations of GFP expressing AHPCs transplanted into the acute ischemic and healthy environment of the eye expressed the markers for MAP2ab and GFAP. Within the retina, MAP2ab immunoreactivity (-IR) is normally found within neurons of the inner retina (Caceres et al., 1986). MAP2ab-IR was observed in AHPCs integrated within the inner retina, within the ILM and GCL (Fig. 6(A) and (D)). In addition, some MAP2ab-IR AHPCs appeared to have processes extending into the inner plexiform layer (IPL) (Fig. 6).

An antibody against GFAP was used to determine if grafted cells expressed this glial marker. GFAP is normally expressed in the astrocytes along the inner retina and in addition is expressed in reactive Müller glial cells. Extensive GFAP-IR was observed within GFP-expressing AHPCs located within the vitreal chamber, along and in the ILM and in the GCL (Fig. 6(B), (C), (E) and (F)). Many of the GFP-expressing AHPCs possessed processes that were strongly immunoreactive for the GFAP antibody marker (Fig. 6(F)).

As illustrated in Fig. 6, extensive GFAP-IR in the radially oriented Müller glial cells, as well as in the astrocytes along the inner retina was observed in transplanted eyes. The transplanted AHPCs tended to be restricted vitreal to the ILM in healthy rat eyes and extensive co-expression of GFAP by many AHPCs integrated in the inner retinal layers was...
clearly evident in acute ischemic rat eyes (Fig. 6(B) and (C)). Although several other cell-specific antibodies were used in our analysis, only occasional transplanted AHPCs were observed labelled with the neuronal markers anti-neurofilament or anti-SV2 antibodies and no APHCs were observed labelled with antibodies directed against calretinin (neuronal), beta III class tubulin (neuronal), recoverin (photoreceptor) or RIP (oligodendrocytes).

Morphological analysis revealed a similar percent of survival of transplanted AHPCs in the healthy (50%) and acute ischemic eyes (53%). Immunohistochemical analysis for the neuronal marker (MAP-2ab) revealed that 17% (acute ischemic eyes) and 13% (healthy eyes) of the transplanted AHPCs were labelled with this neuronal marker. Analysis for the glial marker (GFAP) revealed that 49% (acute ischemic eyes) and 31% (healthy eyes) of the detected AHPC were positively immunoreactive. This immunohistochemical analysis of transplanted AHPCs suggests that the majority of cells were differentiating toward a glial rather then a neuronal lineage.

4. Discussion

A number of studies have examined the survival and morphological integration of transplanted neural progenitor cells in injured or healthy rat retinas (Chacko et al., 2000; Guo et al., 2003; Kurimoto et al., 2001; Nishida et al., 2000; Takahashi et al., 1998; Wojciechowski et al., 2002; Young et al., 2000). However, to date no studies have performed detailed functional analysis of transplanted eyes to demonstrate whether transplanted cells integrated in the retinal circuitry or recovered function of eyes damaged by acute elevation of the intraocular pressure.

A previous study reported that transplanted neural progenitors can establish synaptic-like contacts (based on morphological criteria) with host neurons in mechanically injured retinas (Nishida et al., 2000). We were interested to examine whether transplanted neural progenitors can morphologically integrate, differentiate and recover function in damaged eyes. Since acute retinal ischemia damaged retinal ganglion cells, we wanted to investigate whether intravitreally transplanted
progenitors will predominantly integrate in the RGC layer. In a previous study (Guo et al., 2003), which used a similar model of acute retinal ischemia and GFP modified AHPCs, the authors demonstrated robust integration in different retinal layers after subretinal injection. However, since retinal ischemic diseases usually affect RGCs and the inner nuclear layer, it was our intention to use an approach which would deliver transplanted cells in close proximity to these layers and avoid possible complications and additional injury (retinal detachment) related to the subretinal delivery of cells. Indeed, the majority of transplanted cells were located in close proximity to the retinal ganglion cells, which is consistent with the possible presence of cues produced in the damaged inner retina which favoured predominant integration in the most damaged layer (RGC layer). Functional analysis using various pupil light reflex parameters (amplitude, velocity and latency time) revealed only significantly smaller latency deficits at 42 and 60d postoperatively in animals which received AHPC transplantation in eyes damaged by acute ischemia, however, amplitude and velocity were not significantly different among different tested groups during the course of the study. The absence of PLR deficits in healthy transplanted rat eyes is highly supportive of the rather passive role of the transplanted neural progenitors in healthy rat eyes, since we observed no disturbances in the retina and optic nerve physiological transmission which would be detected by a potential decrease in any of the tested PLR parameters (amplitude, latency or velocity) as it is usually the case in conditions where retina and/or optic nerve might be damaged (Grozdanic et al., 2003a,b, 2004).

The electroretinogram is an extracellular response, which arises during retinal activity because cell membranes become hyperpolarized or depolarised in response to photic stimulation. Any pathological event, which may affect electrophysiological properties of retinal cells, will affect the amplitude or the latency time (time between the onset of the light stimulus and the occurrence of electrical signals). We previously demonstrated the presence of significant ERG deficits in the rat model of acute ocular ischemia (Grozdanic et al., 2003b). In this study, we did not detect any significant positive effect of neural progenitor cell transplantation on ERG function in acute ischemic rat eyes. However, transplantation of the neural progenitors in healthy rat eyes caused only mild, but significant a-wave latency time deficits in transplanted eyes ($p = 0.0479$). Since, we did not detect significant integration of the neural progenitors in the outer nuclear layer it is difficult to speculate that direct contact of transplanted cells interfered with photoreceptor function. Although in some transplant recipient retinas we observed a radial pattern of GFAP-IR, indicative of reactive Müller glial cell labelling. It has been previously demonstrated that reactive Müller cells up-regulate production of bFGF (Harada et al., 2002), which can attenuate the transmission of the photoreceptor response to inner retinal cells (Gargini et al., 1999). There is also a possibility that transplanted cells induced immune reaction in recipient eyes as previously described in some retina transplantation experiments (Anosova et al., 2001). However, recent data revealed that these neural stem/progenitor cells do not express the class II MHC molecules, essential for the CD4+ mediated rejection of the transplanted tissue (Hori et al., 2003; Klassen et al., 2003), a strong argument against the possibility that immune-mediated changes caused the a-wave latency deficits observed in our study.

There are a number of possible explanations for the lack of functional recovery in our study. First, morphological and immunohistological data are highly suggestive that many of the progenitor cells differentiate into cells more characteristic of glia than neurons and this is further supported by lack of expression of the majority of the retinal neuronal markers and synaptic machinery proteins examined in our study. It is possible that differentiation into more neuronal elements may be accomplished in the future using specific growth factors and other signalling molecules that influence the path of cell differentiation. Second, non-modified neural progenitors release very little, if any, neurotrophic factors as previously reported for some other neural progenitors—(Ourednik et al., 2002), which could not only facilitate neuronal differentiation, but also survival and functional recovery of damaged host neurons. Third, the use of neural progenitors of non-retinal origin may contribute to the decreased integration and differentiation capacity in the retinal environment, however, remarkable examples of morphological integration and at least partial differentiation have been described with the use of neural progenitors of non-retinal origin (Guo et al., 2003; Van Hoffelen et al., 2003). Multiple studies which used retinal progenitor transplantation in different models of retinal diseases demonstrated only modest integration and differentiation of the transplanted cells within the host retina (Chacko et al., 2000, 2003; Coles et al., 2004; Klassen et al., 2004; Yang et al., 2002). While some studies have demonstrated the presence of some molecular markers specific for neurons of retinal lineage, the general morphological appearance of these cells was not consistent with a true retinal neuron morphology. Furthermore, the paucity of evidence of actual functional properties which could demonstrate that transplanted cells indeed fully differentiated into functional retinal neurons does not support an immediate advantage to restricting one’s use exclusively to retinal progenitors as the only source for retinal transplantation procedures at this time. Indeed, a recent study comparing mouse brain progenitors versus retinal progenitors found that the brain derived progenitors displayed a greater ability to morphologically integrate and respect the host architectural organization of the developing retina compared to the retinal progenitors (Sakaguchi et al., 2004). At this time it would seem vital to investigate the abilities of multiple stem and progenitor cell types for the purposes of cell based therapeutic approaches for the retina and CNS.

Until there is evidence to support the hypothesis that transplanted cells may integrate into the retinal circuitry to restore visual function, an alternative goal would be to use transplanted cells as a source for continued delivery of therapeutic substances, as previously demonstrated (Wang et al., 2002). A number of studies have demonstrated the ability of exogenously applied neurotrophic growth factors...
(BDNF, GDNF and CNTF) to facilitate survival of retinal neurons in degenerating or damaged retinas (Isenmann et al., 1998; Ko et al., 2001; Schmeer et al., 2002; Weise et al., 2000; Yan et al., 1999). We recently demonstrated that long-term delivery of BDNF and GDNF from slow releasing biodegradable polymer microspheres could significantly preserve optic nerve function in acute ocular ischemic rats (ARVO 2004, Abstract Program no. 906). Due to the affinity of the neural progenitors for migration into severely damaged regions, it is possible that grafted cells genetically modified to produce different neurotrophic growth factors may provide close trophic support that may stimulate survival and functional recovery of remaining cells after the injury much more effectively compared to simple intravitreal delivery. Future studies for the treatment of retinal and optic nerve degenerative diseases are likely to exploit these strategies by using genetically modified neural stem cells.

5. Conclusion

Although neural progenitor cells were transplanted into rat retina with relative success, their differentiation into neuronal elements was limited. Most integrated into the inner retina and expressed glial cell markers. Monitoring of the retina and optic nerve function of transplant recipient eyes over time by electroretinography and pupillometry did not reveal any significant functional deficit in normal retinas, nor did it reveal any improvement in damaged retinas over the course of this study. Since, the ultimate goal of cell transplant based therapies is the restoration of function, it seems that a functional analysis, and not just the morphological appearance of the tissue, is likely to provide a better representation of the actual condition of the retina and optic nerve after neural stem/progenitor cell transplantation and should be incorporated into future studies of progenitor transplantation.

Acknowledgements

The authors wish to thank Dr F. H. Gage (Laboratory of Genetics, The Salk Institute) who generously provided the AHPcs. This work has been supported by an InterInstitutional Grant from the College of Veterinary Medicine-Iowa State University and the College of Medicine-University of Iowa, the Iowa State University Biotechnology Foundation, the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, project number 3205, was supported by Hatch Act and State of Iowa Funds, The Glaucoma Foundation, NY, the National Institutes of Health (NINDS NS 44007), an unrestricted grant from Research to Prevent Blindness (Dept. of Ophthalmology, University of Iowa), NY and a Merit Review Grant from the Veterans Administration (R.H.K.). R.H.K. is also a Lew Wasserman Scholar (Research to Prevent Blindness). A.M.A. was funded by a summer internship from the Iowa State University Program for Women in Science and Engineering.

References


