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The Survival of Engrafted Neural Stem Cells Within Hyaluronic Acid Hydrogels

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Abstract

Successful cell-based therapy of neurological disorders is highly dependent on the survival of transplanted stem cells, with the overall graft survival of naked, unprotected cells in general remaining poor. We investigated the use of an injectable hyaluronic acid (HA) hydrogel for enhancement of survival of transplanted mouse C17.2 cells, human neural progenitor cells (ReNcells), and human glial-restricted precursors (GRPs). The gelation properties of the HA hydrogel were first characterized and optimized for intracerebral injection, resulting in a 25 min delayed-injection after mixing of the hydrogel components. Using bioluminescence imaging (BLI) as a non-invasive readout of cell survival, we found that the hydrogel can protect xenografted cells as evidenced by the prolonged survival of C17.2 cells implanted in immunocompetent rats ($p < 0.01$ at day 12). The survival of human ReNcells and human GRPs implanted in the brain of immunocompetent or immunodeficient mice was also significantly improved after hydrogel scaffolding (ReNcells, $p < 0.05$ at day 5; GRPs, $p < 0.05$ at day 7). However, an inflammatory response could be noted two weeks after injection of hydrogel into immunocompetent mice brains. We conclude that hydrogel scaffolding increases the survival of engrafted neural stem cells, justifying further optimization of hydrogel compositions.

Keywords

Hyaluronic acid; hydrogel; transplantation; neural stem cells; bioluminescence imaging

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1. Introduction

With the knowledge of stem cell biology increasing at a rapid pace, cell transplantation for therapeutic purposes has become a promising approach to treat a variety of diseases. Neurological disorders, for many of which there is no effective treatment, are considered prime applications for stem cell-based therapy and neurorepair. However, a major issue in stem cell transplantation is the substantial loss of transplanted cells following transplantation, which can be as much as 90 to 99% of the total number of grafted cells. Such dramatic cell loss has been reported for transplants into animal models of various neurological disorders, including stroke [1, 2] and Parkinson's disease [3, 4]. This problem is not unique to cells of the neural lineage, as substantial cell death has also been reported in cell therapy of myocardial infarction [5], muscular dystrophy [6, 7], and diabetes [8, 9].

During the last decade, various biomaterials have been used to improve cell engraftment by providing a unique, three-dimensional microenvironment. Among them, hydrogels hold great promise, as their unique physical properties are especially advantageous for cell scaffolding. Hydrogels are hydrated, water-insoluble polymeric networks that are cross-linked by water-soluble precursors [10]. A large number of *in vitro* and *in vivo* trials have demonstrated the feasibility of hydrogel-enhanced cell therapy for the regeneration of cartilage, cornea, liver, pancreatic islet cells, and nerves [11]. Hydrogels fabricated from extracellular matrix (ECM) components represent a natural *in vivo* milieu. Hyaluronic acid (HA), a major component of ECM, is a linear polysaccharide that consists of alternating units of a repeating disaccharide, β -1,4-D-glucuronic acid- β -1,3-N-acetyl-D-glucosamine. HA has become an important building block for the creation of new biomaterials, and has been modified in many ways to meet the needs of different applications in tissue engineering and regenerative medicine [12]. *In vitro*, it has been shown that the proliferation and differentiation of HA hydrogel-embedded neural stem cells (NSCs) can be manipulated by modifying the mechanical properties of the hydrogel [13]. Hydrogels can also be used as effective carriers of growth factors, which can support the survival of scaffolded NSCs *in vitro* [14].

Transplantation of cells into the CNS must be pursued with special precaution, as the outcome is determined by biophysical processes including bleeding, backflow, and perfusion of the graft. To minimize the injury associated with CNS implantation of hydrogel-embedded cells, we assessed the pro-survival effects of an injectable HA hydrogel. The hydrogel comes in liquid form and solidifies quickly after mixing with a cross-linker. It has been demonstrated that, upon injection into the infarct cavity of stroked rats, the gel forms a well-organized and uniform scaffold [15], which supports the survival of neural stem cells following transplantation [16]. In this study, we designed a simple method to determine the solidification time of hydrogel after mixing of its components in order to optimize the scaffolded cell/hydrogel preparation. We then evaluated the pro-survival effect of hydrogel on several stem cell lines *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Materials

HA hydrogels (HystemC, Glycosan, Salt Lake City, UT) were prepared by cross-linking thiol-modified sodium HA and gelatin with polyethylene glycol diacrylate (PEGDA). Alexa 488-conjugated C5 maleimide was purchased from Invitrogen (Carlsbad, CA). Cresyl fast violet (CV) was purchased from Waldeck GmbH (Munster, Germany). Hematoxylin and eosin (H&E) solutions were from Sigma (St. Louis, MO). Luciferin was obtained from Gold Biotechnology (St. Louis, MO).

2.2. Measurement of the gelation time of hydrogels

Immediately after mixing the three hydrogel components in different ratios, glass capillaries (Fisher Scientific, Pittsburgh, PA) were immersed in a polymerase chain reaction (PCR) tube containing 50 μ l of hydrogel solution, and the liquid was allowed to flow inside against gravity through the capillary force. The height of liquid in the capillary was measured repeatedly at different time points after the gel components were mixed. The difference between the starting height (H_0) and the height at a given time point (H_t) was used to calculate the gelation index (GI), which was defined as $GI=(H_0-H_t)/H_0$. As such, the GI reflects the degree of hydrogel solidification. Measurements were ended when the hydrogel could no longer enter the capillary. To exclude variations in diameter among capillaries, the same capillary was used repeatedly for one particular tube of the hydrogel composition after rinsing with 10 mM phosphate-buffered saline (PBS, pH=7.4) and fast drying using pressured air.

2.3. Cell cultures

C17.2 NSCs stably expressing LacZ (courtesy of Dr. Evan Snyder) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), 5% horse serum (Gibco), 2 mM L-glutamine (Gibco), and 1% penicillin/streptomycin (Sigma) at 37°C in a humidified 5% CO₂ atmosphere. ReNcell CX human neural progenitor cells were cultured in ReNcell NSC maintenance media (Millipore, Billerica, MA) supplemented with 20 ng/ml basic fibroblast growth factor (bFGF, Invitrogen) and 20 ng/ml epidermal growth factor (EGF, Invitrogen). Human glial-restricted precursor (GRP) cells (Q Therapeutics Inc.) were cultured in DMEM/F12 supplemented with N2, bovine serum albumin (BSA, 1 mg/ml), 10 ng/ml bFGF (Invitrogen), and 10 ng/ml platelet-derived growth factor (PDGF, Peprotech, Rocky Hill, NJ). Preparation of lentivector construct FU-luc2-IRES-Venus and viral packaging were performed as described previously [17][18]. C17.2 and the ReNcell CX NSC line were transduced with the viral vector, and positive cells were sorted and enriched by flow cytometry (FACS Aria cell sorter, Becton Dickinson, Franklin Lakes, NJ). For human GRP cells, pLenti4-CMV-luc2 (Invitrogen) was used to transduce the cells and positive clones were selected by hygromycin (50 μ g/ml) to make stable cell lines.

2.4. Stem cell survival assay

For assessment of the effects of the individual hydrogel components on cell proliferation, HA, gelatin, or PEGDA (5 μ l each) was added to a 96-well plate containing 1×10^4 cells in 100 μ l medium. Cell viability was determined prior to and 24 hours after adding the hydrogel component. A Cell Titer96 one-solution cell proliferation assay (Promega, Mannheim, Germany) was used to quantify the amount of viable cells. Briefly, 10 μ l of assay solution was added to each well containing 100 μ l medium at 37°C. One hour later, the absorbance at 490 nm was measured using a plate reader (Multilabel reader, Perkin Elmer, Waltham, MA). For cell quantification, a standard curve was first established for each cell type. For evaluation of cell survival in mixed hydrogel, stem cells (1×10^5 cells) were embedded in 50 μ l of hydrogel and loaded as drops on the surface of a low-adherence culture dish. Thirty minutes later, with the hydrogel fully solidified, the culture medium was added to the dish. The survival of HA gel-embedded cells was monitored using bioluminescence imaging (BLI). Luciferin (15 μ g/ml) in 10 mM PBS, (pH=7.4) was added to the wells, and the luminescent signal was collected 10 minutes later using an IVIS Spectrum optical imaging device (Caliper Life Sciences, Hopkinton, MA) equipped with a high sensitivity, cryogenically cooled, charge-coupled device detection system. The photon signal was integrated over one second. The BLI signal intensity was expressed as maximum photons per second per cm square per steradian (photons/sec/cm²/sr, abbreviated as p/s).

2.5. Cell transplantation studies

All animal procedures were approved and conducted in accordance with our institutional guidelines for the use and care of laboratory animals. The injectable hydrogel was prepared by mixing the hydrogel components at a ratio of HA:gelatin:PEGDA=2:2:1. The injection was initiated 25 minutes after mixing of the components, which was five minutes before complete gelation occurs, according to a predetermined gelation curve (Fig. 1). Sprague-Dawley (SD) rats (female, 180–220 g, Harlan laboratories, Walkersville, MD, n=8) were anesthetized by intraperitoneal (i.p.) injection of ketamine/xylazine (80/8 mg/kg, Vedco, St. Joseph, MO) for subcutaneous injection of luciferase-expressing 5×10^5 C17.2 cells, suspended in 50 μ l of hydrogel or PBS (control group). For intraspinal cord cell transplantation, rats as described above (n=5) were anesthetized with ketamine/xylazine, and the thoracic/lumbar area was shaved and prepped with betadine. Following a T13 laminectomy, the spine was stabilized in a stereotaxic frame (Stoelting Co. Wood Dale, IL) for precise injection. Immediately before injection, cells were pelleted and suspended in either 10 μ l of hydrogel or 10 μ l PBS (control group) with a final cell density of 5×10^4 cells/ μ l. 1.5 μ l of cell suspension was injected into the spinal cord gray matter (1 mm from midline, 1.5 mm deep from dura) at 0.5 μ l/min using a 31G microinjection needle attached to a 10 μ l Hamilton syringe (Hamilton, Reno, NV). The needle was withdrawn slowly two minutes after the injection was complete. For cell transplantation into the brain, immunodeficient, Rag2^{-/-} mice (male, 8–12 weeks old, n=18, Taconic Farms, Germantown, NY) or BALB/c mice (male, 8–12 weeks old, n=10, Jackson Laboratories, Bar Harbor, ME) were anesthetized with 2% isoflurane, shaved, and placed in a stereotaxic device (Stoelting). Cells were prepared as described above and a 3 μ l cell suspension was injected into the right striatum (AP=0; ML=2.0; DV=3.0) at a rate of 0.5 μ l/min. For the injection of hydrogel-scaffolded cells, two sets of experiments were performed: 1) immediate injection, where the hydrogel was injected immediately after preparation; and 2) delayed injection, where the hydrogel was injected 25 minutes after preparation, which was five minutes before complete gelation, according to the predetermined gelation curve (Fig. 1C).

2.6. BLI of transplanted cell survival

In vivo BLI was performed using the imaging system described above. Before imaging, each animal (mouse or rat) was anesthetized with 1–2% isoflurane and intraperitoneally injected with 150 mg/kg of luciferin in PBS. For mice, imaging was performed at 10, 20 and 30 minutes after luciferin injection. For rats, images were acquired at 20, 30 and 40 minutes after luciferin injection due to the delayed peak time of luminescent signal. The exposure time was one minute for each animal. Peak emission values were recorded for viable cell quantification using LIVINGIMAGE® software (version 2.50, Caliper Life Sciences). For signal quantification, the photon signal are expressed in units of maximum photons per second per cm square per steradian (photons/sec/cm²/sr, abbreviated as p/s), measured from a region of interest, which was kept constant in area and positioning for all experiments.

2.7. Histology and immunofluorescent staining

Following sacrifice, animals were perfused with 4% paraformaldehyde (PFA). Spinal cords or brains were dissected, cryopreserved with 30% sucrose in PBS, and cut into 25 μ m sections. For hydrogel-treated tissues, sections with graft inside were mounted onto slides and stained with 0.1% CV solution for 10 minutes. Routine histomorphological staining was performed on using H&E staining. For immunohistochemistry, sections were blocked with 10% goat serum prior to sequential incubation with primary (mouse anti-human nuclear antigen, 1:500, Millipore; rabbit anti-Iba-1, 1:1000, Wako, Japan; rat anti-CD45 1:500, Serotec, UK; rabbit anti-GFAP, 1:1000, Dako, USA; rabbit anti-CD3, 1:500, Abcam, UK) and secondary antibodies (anti-mouse Alexa-fluor 594, 1:2000; anti-rabbit Alexa-fluor 594, 1:2000; and anti rat Alexa fluor 594, all from Invitrogen). Histochemical and

immunofluorescent images were acquired using an Olympus BX51 microscope equipped with an Olympus DP70 camera.

2.8 Statistical analysis

Statistical analysis was performed using prism 4.03 software (GraphPad Software, San Diego, CA). One-way analyses of variance (ANOVA) were used to compare group differences with more than two groups, and Bonferroni's post-hoc tests were applied to compare specific group difference if the ANOVA test revealed a significant difference. Non-parametric grading of graft survival was performed using a Mann–Whitney test for comparisons between two groups. All data are expressed as means $\pm$ standard error of means (SEM). For all analysis, values of $p < 0.05$ were considered to be significant.

3. Results

3.1. Optimization of hydrogel solidification using different component ratios

To simplify the process of viscosity measurements, we designed a straightforward method to determine the gelation time of the hydrogel. This method uses the height of the liquid that is pushed by capillary force into a glass capillary tube as a measurement of viscosity (Fig. 1A). A complete gelation of hydrogel at standard composition (H:G:P=2:2:1) prevents flow into the capillary, and was considered baseline value). By defining the gelation index (GI) = $(H_0 - H_t)/H_0$, the dynamic changes in the degree of solidification can be determined for each hydrogel component was determined (Fig. 1B). We found that, using the standard composition suggested by the manufacturer (volume ratio of HA:gelatin:PEGDA=2:2:1), full gelation occurs in 30 min after mixing. An increase in the ratio of HA (H:P=2:1 or 4:1) was found to shortened the gelation time. Although the gelatin itself is not necessary for solidification, its presence effects the gelation process as evidenced by the prolonged gelation time in the group where gelatin was replaced by PBS (H:PBS:P=2:2:1). The failure of G+P and H+G groups to form a rigid hydrogel indicates that combination of HA and PEGDA is essential for the cross-linking process.

3.2. Optimization of hydrogel injection for efficient scaffolding

The cell injection procedure was then optimized to ensure the successful formation of a 3D hydrogel scaffold. We first found that cresyl violet (CV) can be used to stain the HA component in hydrogel with high affinity and specificity (Fig. 2A), allowing for visualization of the hydrogel scaffold after transplantation (Fig. 2B). Since we established that full gelation occurs approximately 30 minutes after hydrogel-mixing, we injected either a freshly prepared hydrogel (immediate-injection group), or a hydrogel that was prepared 25 minutes before injection (delayed-injection group). In both groups, C17.2 cells were encapsulated immediately after hydrogel mixing. The morphology of the hydrogel scaffold in each group was compared after histological staining. Notably, we found a well-defined scaffold shape in the delayed-injection group (Fig. 2B, right panel), in contrast to an irregular shape in the immediate-injection group (Fig. 2B, left panel). We hypothesize that gelatin may leak out of the scaffold during gel solidification in the immediate-injection group, given its weak binding to HA. To observe the distribution of gelatin, Alexa488 was used to label C5 maleimide present in gelatin, before injection as described above. A combination of CV and H&E staining was used to delineate the shape of the hydrogel scaffold in each group. We found that, in the immediate-injection group, gelatin diffused to a large area surrounding the needle track, and even to the corpus callosum (Fig. 2C). However, in the delayed-injection group, a well-defined, circular shaped scaffold was formed, with little outwards leakage of gelatin (Fig. 2D), consistent with Fig. 2B. Thus, immediate injection causes leakage of the hydrogel components and was not further pursued.

3.3. Effect of scaffolding on proliferation and migration of C17.2 cells *in vitro*

Next we evaluated the effect of the hydrogel scaffold on the survival of C17.2 cells *in vitro*. First, the influence of each individual component of hydrogel at low concentration (5%) was analyzed by quantifying the number of viable cells (Fig. 3A). In the control group, where cells were resuspended in PBS for injection, the number of cells almost doubled during the first 24 hours (1.97 ± 0.03 fold of change relative to starting cell number). Among the three components, HA significantly promoted cell proliferation (2.96 ± 0.12 , $p < 0.01$). No significant toxic effects from the hydrogel components were found. C17.2 cells were then scaffolded within hydrogels with different compositions and monitored for their survival using BLI. We found that, in the standard composition hydrogel group, C17.2 cells proliferated quickly. When the gelatin was absent in the hydrogel, a striking decrease in the intensity of BLI signal was observed (Fig. 3B). Quantification of BLI signal indicated that there were significantly less cells ($p < 0.001$) in the group without gelatin (Fig. 3C). In addition, a striking difference was found in the morphology of cells in the hydrogel with or without the presence of gelatin. Cells in the complete hydrogel composition grew in a densely packed manner while retaining their normal shuttle shape, while those in the hydrogel without gelatin tended to form clusters with a much lower cell density (Fig. 3D). Furthermore, in complete hydrogel cultured on gelatin-coated dishes, cells migrated out from scaffold without restriction (Fig. 3E). These data establish the importance of the presence of gelatin for the proliferation of C17.2 NSCs cells.

3.4. Protective effects of scaffolding for xenografted mouse C17.2 cells

To test the protective effect of hydrogel scaffolding against a xenogeneic rat host immune response, C17.2 cells were first scaffolded using the standard composition (H:G:P=2:2:1 unless noted otherwise), and injected (delayed-injection) subcutaneously in the dorsal area (Fig. 4A). In the control (PBS) groups without encapsulation of hydrogel, the BLI signal completely disappeared at 12 days post-injection (Fig. 4A, red arrows). This was in stark contrast to the scaffold group (Fig. 4A, green arrows), still displaying BLI signal at day 12. Quantification of BLI signal revealed a significant difference between scaffolded cells and non-scaffolded cells at days 8, 12, and 15 post-injection (Fig. 4B, $p < 0.05$). Notably, if cells were embedded in non-crosslinked hydrogel (no PEGDA addition), their survival was similar to the control groups (no gel) (Fig. 4A, white arrow, 4B), implicating that successful crosslinking of hydrogel is essential for its beneficial effect.

We then investigated the protective effect of scaffolded cells implanted in the lumbar spinal cords of rats. Similarly, non-scaffolded cells completely lost BLI signal at 12 days after transplantation (Fig. 4C, lower panel), while those that were scaffolded retained their signal at this time point (Fig. 4C, upper panel). Quantification of BLI signal demonstrated a large variation between the different animals (Fig. 4D). While the scaffolded group exhibited a higher BLI signal at later time points, the difference was not statistically significant ($p = 0.0556$). Despite the statistical analysis, histological examination provided convincing evidence that the scaffold protects the implanted cells. Numerous C17.2 cells expressing GFP were preserved in the center and periphery of the graft (Fig 4E), while only cell debris with a strong autofluorescence was left in the non-scaffolded group (Fig. 4F). CV staining revealed a well-formed scaffold in the hydrogel group (Fig 4G, arrows).

3.5. Protective effects of scaffolding for xenografted human ReNcells

We investigated the protective effect of hydrogel scaffolding for ReNcells, a human NSC line derived from an aborted human fetus and immortalized with the *c-myc* oncogene [18]. Since this is a sensitive cell line whose proliferation is highly dependent on growth factors, we chose this cell line for our studies. First, the effect of the individual hydrogel components on the proliferation of ReNcells was evaluated by incubating cells with diluted HA, gelatin,

or PEGDA (5%). Although PEGDA had no significant effects on the proliferation of ReNcells, HA (0.83 ± 0.04) and gelatin (1.06 ± 0.03) were found to be beneficial ($p<0.001$, Fig. 5A) compared to the PBS control group (0.68 ± 0.04). And Gelatin was more exerting more protective effect than HA ($p<0.001$). Furthermore, when scaffolded ReNcells were cultured *in vitro* for 1 week, gelatin was found to significantly enhance cell survival (gelatin vs non-gelatin, $p<0.001$ on day 2 and day 6 after plating) (Fig. 5B).

Scaffolded ReNcells (H:G:P=2:2:1) were xenografted in the striatum of immunodeficient mice. For the PBS control group and immediate-injection scaffold group, serial BLI demonstrated that cells died quickly within 8 days after transplantation (Fig. 5C). For the delayed-injection scaffold group, there was a significant improvement in survival on day 5 ($p<0.05$), but the BLI values at day 8, although higher, were no longer significant (Fig. 5D). CV staining of brain sections showed a well-defined hydrogel scaffold (Fig. 5D). Immunostaining of sections of mice sacrificed at day 8 post-injection demonstrated more viable ReNcells in the hydrogel group ($p<0.01$, Fig. 5E) relative to the PBS control group, although the overall total cell number was low, consistent with the BLI findings.

3.6. Protective effects of scaffolding for xenografted human GRPs

To demonstrate that the potential benefit from hydrogel scaffolds is not restricted to specific cell lines, we further evaluated the pro-survival effects for human GRPs *in vitro* and *in vivo*. We first again assessed the effects of the individual hydrogel components separately. Among the three components, only HA was found to significantly promote cell proliferation (1.6 ± 0.03 vs 1.3 ± 0.03 for control, $p<0.001$, Fig. 6A). Scaffolded cells were then cultured for 1 week. In contrast to C17.2 or ReNcells, we did not see any pro-survival effects from gelatin (Fig. 6B). The BLI signals in all groups decreased over time, indicating that the hydrogel does not support long-term survival of human GRPs. When human GRPs were transplanted into immunocompetent BALB/C mice, the signal decayed over time for both the PBS control group and the scaffolded group (Fig. 6C). Although BLI signal in both groups decay over two weeks to background level, there was significantly more BLI signal from the scaffold group at day 7 post-injection ($p<0.05$, Fig. 6D).

3.7. Biocompatibility and host response towards implanted hydrogel scaffolds

Lastly, we investigated if the implantation of hydrogel scaffolds could lead to neuroinflammation or a foreign body reaction. At day 12 post-injection of C17.2 cells into the immunocompetent rat spinal cord, there was a substantial macrophage/microglia infiltration around the injection site for both the hydrogel (Fig. 7A) and PBS control group (Fig. 7B) as evidenced by ant-Iba-1 staining. The inflammatory cells co-localized with the transplanted C17.2 cells within the hydrogel scaffold (Fig. 7A'). For the PBS control group, no grafted surviving cells could be detected (Fig. 7B'). At day 8 post-injection of ReNcells into immunodeficient Rag2^{-/-} mice brain, the intensity of Iba-1 staining in the brain area with the graft (dashed lines) was higher in the PBS control group (Fig. 7D, see needle track) compared to the scaffolded hydrogel group (Fig. 7C). This suggests that hydrogel may serve to mitigate the innate immune reaction against grafted cells. This suggests that hydrogel may serve to mitigate the innate immune reaction against grafted cells. We then investigated whether the hydrogel alone is able to induce an immune reaction in immunocompetent animals. Hydrogel or PBS was injected into the striatum of immunocompetent BALB/c mice, which were sacrificed two weeks after injection. We found that, based on the Iba-1 staining of brain sections, the hydrogel (Fig. 7F) induced a more pronounced microglial activation compared to the PBS group (Fig. 7F'). Moreover, the activated cells surrounding the hydrogel were positive for CD45 (Fig. 7G) and negative for CD3 (data not shown), indicating that those are most likely infiltrating monocytes. GFAP staining indicated that the glial reaction is restricted to microglial cells (Fig. 7G').

4. Discussion

A large number of studies have demonstrated the feasibility of hydrogel scaffolding of transplanted cells for the regeneration of cartilage, cornea, liver, islets, and nerves [11]. For treatment of neurological disorders, most studies have focused on *in vitro* studies [12]. A recent study reported a positive effect of an HA hydrogel on the survival of NSCs transplanted into the cavity of stroked rat brain, although the protective effect was modest (the average number of surviving cells increased to 8000 from 4000 after scaffolding), especially considering the large quantity of cells initially injected (1×10^5 , survival rate was then 8% vs. 4%, hydrogel vs. cells-only) [16]. The goal of our study was to optimize the hydrogel injection procedure and to perform a systematic serial study of its pro-survival effects for three different neural stem cell types *in vivo*.

An HA hydrogel is prepared in soluble form, allowing its scaffolding with cells and subsequent injection, after which it polymerizes *in vivo*. The polymerization of the HA hydrogel used in this study occurs through cross-linking of thiol-modified HA and gelatin with PEGDA. This reaction starts quickly upon mixing the hydrogel components and varies in time depending on the composition and molar ratio of the hydrogel components [15]. The time window of gelation ideally should be long enough for mixing and injecting of cells without needle clogging of the microsyringe or micropipette, but cannot be too long to prevent leakage of fluids into the host tissue parenchyma. Common methods for measuring the gelation time include mechanical measurements with atomic force microscopy or a rheometer [19, 20], as well as ultrasound and magnetic resonance imaging (MRI)-based methods [21]. We developed a simple measurement procedure for the determination of gelation time without the need of using specific instruments. For a standard molar ratio of the different hydrogel components as recommended by the manufacturer (H:G:P=2:2:1), we measured a gelation times of 30 minutes, which differs from the product information as provided by the manufacturer (20 minutes), underscoring the importance of gelation time measurements for each batch of injectable hydrogel. HA is the main substrate of PEGDA for cross-linking, with gelatin being a less effective substrate for PEGDA. The gelation time can be manipulated by the amount of gelatin: inclusion of gelatin extends the gelation time.

Although injectable hydrogels have been commercially available for several years, there are no systematic reports on reproducible hydrogel delivery into the central nervous system. Brain and spinal cord are well-perfused tissues, making microbleeding an unavoidable event during hydrogel injection. As a result, the gelation time of hydrogel may be different *in vivo* as compared to *in vitro* due to mixing with host fluid. To determine the optimal time for hydrogel delivery into the brain, we compared the resulting morphology of the hydrogel scaffold *in vivo* following an immediate- or delayed injection. We found that delayed injection produced optimal results, without leakage of gelatin into the brain tissue. We demonstrated here the importance of the formation of a well-defined 3D hydrogel scaffold in two cell transplantation models. In the first model using C17.2 cells, non-cross-linked hydrogel failed to protect xenografts in an immunocompetent host, in contrast to implanted cells in cross-linked hydrogel. In the second model using ReNcells, improvement in survival was only observed in the delayed-injection group, in which a 3D hydrogel scaffold was allowed to form, in contrast to the immediate-injection group.

HA has been found to play a key role in stabilizing and organizing the ECM, regulating cell adhesion and motility, and mediating cell proliferation and differentiation [22]. The biological functions of HA between its fragmented form (low-molecular weight polymer or oligosaccharides) and the native polymer form (high molecular weight polymer) are different [23]. In many inflammatory diseases, fragmented hyaluronan stimulates the expression of inflammatory genes by a variety of immune cells through binding to cell

surface proteins, such as Toll-like receptor (TLR)4 and TLR2; whereas high molecular weight HA is found to be supportive of cell survival against apoptosis [24]. The HA used in this study is equivalent to the polymer form of native HA. Consistent with the support of the survival function of HA in polymer form, we found that HA can significantly promote survival of ReNcells (1.2 fold), and the proliferation of C17.2 (1.5 fold) and human GRPs (1.2 fold).

The inclusion of gelatin (type 1 collagen from bovine) has been proposed to provide adhesion sites for cells embedded in hydrogel [15]. This is to prevent anoikis which is due to the need to detach these anchorage-dependent cells from their substrate for injection [25]. Our findings support the pro-survival or pro-proliferation effects of collagen. We found that collagen in the hydrogel kit alone is able to increase the proliferation of ReNcells, and, to a lesser extent, C17.2 cells. Although we did not observe the support effect of gelatin addition for GRP cells, this might be due to the difference in its properties as a glial precursor, compared to less lineage-committed neural stem cells (C17.2 and ReNcells).

The foremost mechanism underlying the protective effect of the hydrogel in immunocompetent animals is the shielding effect of the polymer. In all hydrogels that were well-defined in terms of shape, we could clearly distinguish a boundary between the host tissue and the graft, with a rigid matrix constituting a barrier between the implant and the host microenvironment. This strategy is similar to that used with cell microencapsulation, where cells are surrounded with a semi-permeable alginate membrane that permits the entry of nutrients while blocking penetration by antibodies and immune cells [26, 27]. The second potential mechanism of improved survival of scaffolded cells may be a better perfusion of cells with tissue fluids within the 3D scaffold, acting as a “sponge”. The 3D scaffold also provides more space between cells for the exchange of oxygen and nutrients, without scaffolding cells appear densely packed in and around the needle track.

The ReNcell CX (ReNcell) NSC line was derived from the cortical region of the human fetal brain and immortalized by retroviral transduction with the c-myc oncogene. The proliferation of the ReNcell CX cell line is highly dependent on growth factors [18]. It has been recently demonstrated that transplantation of ReNcells can improve the behavioral outcome in a rat stroke model [28]. Clinical trials in stroke patients are currently being performed although poor cell survival after transplantation remains an issue. Our BLI data showed that ReNcells die within a few days after intracerebral transplantation. However, it is known that neurodegeneration may promote survival and integration of exogenous NSCs compared to non-degenerated tissues [29]. Thus, it is conceivable that the survival of scaffolded ReNcells may be further improved by implantation in the diseased brain.

Human GRPs are defined by the marker A2B5+E-NCAM in the fetal spinal cord, with the ability to differentiate into oligodendrocytes and two distinct type of astrocytes (type 1 and type 2) when exposed to appropriate signals *in vitro* [30]. GRPs can migrate and differentiate in the neonatal and adult brain [31], in injured spinal cord [32], and in the inflamed spinal cord of a rodent model of ALS [33]. GRPs can migrate extensively, expand within inflammatory spinal cord lesions, do not form tumors, and adopt a mature glial phenotype that preserves electrophysiological functions [34]. In this study, we only observed a short-lasting of scaffolding for transplanted GRPs. Our data suggest that the protective effects of hydrogel scaffolding may not be equal for all cell types, and further refinement of the individual hydrogel compositions may be needed.

We also found that hydrogel implantation can evoke a hitherto unreported immune response in the brain of immunocompetent animals. The Rag2^{-/-} mouse strain was developed by combining the recombinase activating gene-2 (RAG2) and common cytokine receptor

gamma chain (gamma c) mutations, generating a completely a lymphoid (T-, B-, NK-) mouse line [35]. Since its innate immune system is still intact, activation of microglia and infiltrating leukocytes into the needle track may still be found in the control animals. In contrast, only a mild response occurred in the hydrogel scaffold groups. These findings suggest that the hydrogel suppresses the innate immune response induced by implanted human cells. However, for the immunocompetent mouse brains (BALB/c), we observed a microglial response two weeks after the injection of hydrogel without infiltrating cells. Immunostaining of brain sections confirmed the absence of an adaptive immune response. This activation of the innate immune system in the brain may be important for the long-term outcome of transplanted stem cell therapy, and requires further detailed studies. Nevertheless, the benefits of hydrogel scaffolding of implanted cells outweigh its potential adverse effects, which should encourage further development of cell type-specific protocols for treatment of neurodegenerative diseases.

5. Conclusions

We have demonstrated that HA hydrogel scaffolding can improve the survival and proliferation of three different cell lines in immunocompetent and immunodeficient animals. For a standard hydrogel composition, a 25 min delayed-injection after mixing was found to provide optimal results. Although a mild inflammatory response towards the implanted hydrogel was observed in immunocompetent mice, requiring further optimization, hydrogel scaffolding should be considered as a possible means to enhance the engraftment of otherwise poorly surviving cell types.

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Figure 1.

Determination of HA hydrogel gelation time. (A) For non-gelated hydrogels, the hydrogel is rising from the tube level (arrow) into the capillary by capillary force with a maximum height= H_0 (arrow). (B) The gelation index (GI) is defined as the ratio of $(H_0-H_t)/H_0$ during the gelation process, with a value of zero when complete gelation occurs. (C) Gelation curve of hydrogels prepared with different amounts of HA (H), gelatin (G), and PEGDA (P).

Figure 2.

Optimization of scaffolding following intracerebral hydrogel injection. **(A)** CV staining of drops the individual HA, gelatin, and PEGDA components. **(B)** CV-stained brain sections of a mouse showing the scaffold morphology using the immediate- or delayed-injection technique. **(C)** Fluorescence microscopy of Alexa 488 C5 maleimide-prelabeled gelatin showing the scaffold morphology using the immediate- or delayed injection technique. **(D)** CV and H&E staining of the same brain sections as in C. Insets are magnified images. Scale bar is 200 μm for insets, and is 1 mm for flat images in B, C and D.

Figure 3.

Effect of hydrogel on proliferation and migration of C17.2 cells *in vitro*. **(A)** Effect of soluble HA, gelatin, PEGDA, and PBS (control) on the proliferation rate of C17.2 cells after 24-hour incubation. A significant difference was found between the HA-treated group and control group (** $p < 0.01$, $n = 4$). **(B)** BLI of C17.2 cells at 1 and 8 days post-scaffolding in complete hydrogel or hydrogel without gelatin. **(C)** Quantitative analysis of BLI signal of C17.2 in different hydrogel compositions over time: HA:Gelatin:PEGDA=2:2:1 (closed triangles), HA:Gelatin:PEGDA=4:1:1 (open triangles), and HA:PEGDA=2:1 (open circles). Significantly less BLI signal was present in the non-gelatin group compared to the other two groups from day 6 onward (** $p < 0.001$, $n = 3$). On day 9, more BLI signal was found for the 2:2:1 group than for the 4:1:1 group (* $p < 0.05$, $n = 3$). **(D)** Morphology of C17.2 cells scaffolded in hydrogel with or without gelatin after 7 days in culture in low-adherence culture dishes. **(E)** Migration of C17.2 cells scaffolded in complete hydrogel after 1 and 6 days in culture on normal plasma-coated culture dishes. Scale bars = 200 μm .

Figure 4.

Protective effects of hydrogel scaffolding for xenografted C17.2 cells. **(A)** BLI of C17.2 cells grafted subcutaneously in the back of immunocompetent rats. Cells were embedded in complete cross-linked hydrogel (green arrows), non-crosslinked (-PEGDA) hydrogel, (white arrows), or PBS (red arrows). Cells were injected in duplicate symmetrical locations. **(B)** Quantification of BLI signal in **(A)** reveals a significantly higher signal from cells in the complete crosslinked hydrogel group (closed circles) as compared to non-crosslinked gel (open circles) or PBS control (triangles) onward from day 8 (* $p < 0.05$, ** $p < 0.01$, $n = 8$). **(C)** BLI of scaffolded C17.2 cells (top row) or cells in PBS (bottom row) transplanted into the spinal cord of immunocompetent rats twelve days after surgery. **(D)** Quantification of BLI signal of from experimental animals in **(C)** ($n = 5$). **(E)** Spinal cord sections of scaffolded C17.2 cells (green, GFP-positive). **(F)** Spinal cord sections of cells suspended in PBS, showing autofluorescence only. **(G)** CV staining of spinal cord sections visualizing the location of the scaffold. Scale bars: 200 μm (E,F) or 1 mm (G).

Figure 5.

Protective effects of hydrogel scaffolding for xenografted ReNells. **(A)** Effects of soluble HA, gelatin, and PEGDA, respectively, on the proliferation of ReNcells in normal culture medium. Survival of ReNcells was significantly improved in groups treated with HA or gelatin compared to control ($***p < 0.001$, $n=4$). **(B)** BLI signal of ReNcells scaffolded in different hydrogel compositions and cultured in normal medium. Hydrogel compositions were HA:Gelatin:PEGDA=2:2:1 (closed triangles), HA:Gelatin:PEGDA=4:1:1 (open triangles) and HA:PEGDA=2:1 (open circles). Asterisks indicate statistical significance ($***p < 0.001$, $n=4$). **(C)** Representative BLI of ReNcells xenografted into Rag2^{-/-} mice brains, without (PBS control, left) and with hydrogel scaffolding following the delayed-injection technique (right). **(C')** BLI signal quantification of cells without (open circles) and with hydrogel scaffolding using the immediate (closed squares) or delayed (closed triangles) -injection technique. Asterisk indicates statistical significance ($*p < 0.05$, $n=6$). **(D)** CV staining of brain sections showing the well-defined shape of the scaffold using the delayed-injection technique. Scale bar is 1 mm. **(E)** Quantification of surviving ReNcells immunostained by anti-human nuclear antigen immunostaining, showing significantly more surviving ReNcells in the scaffolded hydrogel group ($**p < 0.01$, $n=3$).

Figure 6.

Protective effects of hydrogel scaffolding for xenografted human GRPs. **(A)** Effects of soluble HA, gelatin, and PEGDA, on the proliferation of human GRPs in normal culture medium. Asterisk indicates statistical significance ($p < 0.05$, $n=4$). **(B)** Quantification of BLI signal of human GRP scaffolded in different hydrogel compositions and cultured in normal medium ($n=4$): HA:Gelatin:PEGDA=2:2:1 (closed triangles), HA:Gelatin:PEGDA=4:1:1 (open triangles), and HA:PEGDA=2:1 (open circles). **(C)** Representative BLI of human GRPs on day 7 post-transplantation in BALB/C mouse brain without (top row) or with (bottom row) hydrogel scaffolding using the delayed injection-technique. **(D)** Quantification of BLI signal on day 7 for animals receiving cells without (open triangles) or with hydrogel scaffolding using the delayed-injection technique. Asterisk indicates statistical significance ($p < 0.05$, $n=5$).

Figure 7.

Host response to implanted hydrogel. (A) Anti-Iba-1 immunostaining (red) of rat spinal cord 12 days after transplantation of hydrogel-scaffolded C17.2 cells. (A') Overlay of Anti-Iba-1 staining (red) and hydrogel-scaffolded C17.2 cells (GFP, green channel). (B) Anti-Iba-1 immunostaining (red) of rat spinal cord 12 days after transplantation of non-scaffolded C17.2 cells (in PBS). (B') Overlay of Iba-1 staining (red) and non-scaffolded C17.2 cells (green). (C) Anti-Iba-1 immunostaining (red) of Rag2^{-/-} brains 8 days after transplantation of hydrogel-scaffolded ReNcells. (C') Hoechst staining (blue) of same section in (C), showing cell nuclei. (D) Anti-Iba-1 immunostaining (red) of Rag2^{-/-} 8 days after transplantation of non-scaffolded ReNcells. (E) Contralateral (non-transplanted) side of the same section in (D). (F) Anti-Iba-1 immunostaining (green) of BALB/c brain two weeks after transplantation of hydrogel only (no cells). (F') Contralateral side of section in (F), injected with PBS in the striatum. (G) Anti-CD45 immunostaining (red) of a BALB/c brain with hydrogel injection 2 weeks after transplantation. (G') The same section as in (G) stained with anti-GFAP antibody (red). Scale bars: A,B,C,D,C', and E share the same scale bar of 200 μ m. F,F',G, and G' share the same scale bar of 200 μ m, and A' and B' share the same scale bar of 200 μ m.