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Clusters of neural stem/progenitor cells cultured on a soft poly(vinyl alcohol) hydrogel crosslinked by gamma irradiation

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Neural stem/progenitor cells (NSPCs) in the central nervous system (CNS) have the capacity to self-renew by proliferation and are multipotent, giving rise to neurons, astrocytes, and oligodendrocytes. NSPCs can be amplified in neurosphere suspension cultures for cell transplantation therapy to treat CNS diseases as well as for *in vitro* pharmacological/toxicological assays; however, these suspension cultures have certain limitations, including the inconvenience of changing the culture medium as well as difficulty of live imaging. In the present study, we prepared a gamma-crosslinked poly(vinyl alcohol) (PVA) hydrogel and assessed its suitability as a substrate for adherent NSPC cultures. Differentiation was determined by evaluating the expression of the markers nestin (progenitors), βIII tubulin (neurons), and glial fibrillary acidic protein and S100β (glia) by immunocytochemistry and quantitative reverse transcriptase PCR. The levels of the marker genes were similar between the two types of culture; although some variability was observed, there were no fold differences in expression. NSPCs adhered to the PVA gel as clusters and grew without differentiating into neurons and glia. The proliferation rate of cells grown on the soft PVA gel [3.75–7.5% (w/v) PVA] was approximately 70% of that of neurospheres in suspension. We conclude that gamma-crosslinked PVA hydrogels can function as a novel scaffold for maintaining adherent NSPCs in an undifferentiated state.

[**Key words:** Poly(vinyl alcohol); Neural stem cell; Neurosphere; Cell adhesion; Gamma ray; Hydrogel]

Poly(vinyl alcohol) (PVA) is a biologically inert, biocompatible, hydrophilic polymer (1–9) that can be made into a hydrogel by physically crosslinking molecules via a hydrogen bond (5) or by chemical crosslinking via a covalent bond. An elastic, transparent PVA hydrogel can be formed by inducing the covalent crosslinking of PVA molecules in aqueous solution via a radical reaction with a high dose of gamma rays (2–4,6). The PVA hydrogel is hydrophilic, nontoxic to many cell types, and is non-adhesive to cells except when conjugated with peptides mimicking integrin-binding sites in the extracellular matrix (ECM) (10).

Neural stem cells (NSCs) derived from central nervous system (CNS) are a promising tool for transplantation therapy and assays used to evaluate drug efficacy and toxicity (11), since neurons are terminally differentiated and therefore do not proliferate. NSCs can normally be amplified in neurosphere cultures (12,13) in which undifferentiated immature cells adhere to each other and proliferate, forming spherical, heterogeneous cell clusters suspended in serum-free medium. Cells generated in neurosphere cultures are referred to as neural stem/progenitor cells (NSPCs) owing to the difficulty in distinguishing neuronal and glial progenitor cells from the original stem cells. Neurosphere cultures are considered as the gold standard method for amplifying NSPCs despite limitations such as the time required to obtained high cell densities and the inconvenience of changing the medium in which cells are always suspended.

Another method for culturing NSPCs is adherent monolayer culture on a laminin-coated substrate at low cell densities (14,15). NSPCs derived from fetuses (16,17) and adults (18) have been established using this method, which is more convenient for continuous monitoring and cell manipulation.

Monolayer and neurosphere cultures fundamentally differ in terms of the microenvironment to which NSPCs are exposed. NSCs originate from ectodermal neuroepithelial cells present in the neural tube at early stages of CNS development and remain only in a restricted region of the adult brain (19). Neurospheres cultured *in vitro* partially mimic fetal brain tissue as organoids consisting of closely packed NSPCs (20,21) that maintain their undifferentiated state via Notch signaling while possessing a capacity for motility and active migration within the neurosphere (20). Thus, the neurosphere microenvironment is suitable for NSPC proliferation, much like a stem cell niche (19–21).

Monitoring the location of a single cell in a neurosphere and tracing its motility can provide important information on cell–cell interactions and morphogenesis during neural development. Neurosphere cultures were recently used as a three-dimensional (3D) model for developmental neurotoxicity screening (22–24), which would not be possible with a monolayer NSPC culture. On the other hand, adherent cultures are more suitable for stable and reliable cell monitoring, imaging, and controlled amplification of NSPCs and are also more amenable to automation for large scale NSPC generation.

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Artificial CNS tissue has potential applications for cell transplantation therapy in the treatment of intractable CNS diseases (13,25) as well as pharmacological/toxicological assays to evaluate the efficacy and toxicity of chemical substances (11). The objective of the present study was to establish a technique for culturing NSPC clusters attached to a soft substrate that combines the advantages of the neurosphere stem cell niche and the ease of handling of adherent cultures. After testing various substrate materials, we found that NSPCs could be amplified and maintained in an undifferentiated state on soft gamma ray-crosslinked PVA hydrogels. The characterization of the PVA hydrogel as a scaffold for amplifying NSPCs is described in this report.

MATERIALS AND METHODS

Materials The following reagents were used: PVA (Nacalai Tesque, Kyoto, Japan), recombinant human epidermal growth factor (EGF) (Pepro Tech, Rocky Hill, NJ, USA), recombinant human basic fibroblast growth factor (b-FGF) (Pepro Tech), B27 supplement (Life Technologies, Grand Island, NY, USA), Dulbecco's modified Eagle's medium nutrient mixture (DMEM/F-12), antibiotic-antimycotic solution (Nacalai Tesque), 0.05% (w/v) trypsin/EDTA solution (Nacalai Tesque), O.C.T. compound (Sakura Finetek, Tokyo, Japan), normal goat serum (Vector Laboratories, Burlingame, CA, USA), Sepasol-RNA I Super G (Nacalai Tesque), SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan), Prime Script RT reagent kit (Takara Bio), mouse monoclonal anti-tubulin βIII antibody (MMS-435P; Covance, Emeryville, CA, USA), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antibody (G9269; Sigma–Aldrich, St. Louis, MO, USA), mouse monoclonal anti-nestin antibody (MAB353; Merck Millipore, Darmstadt, Germany), rabbit monoclonal anti-Ki67 antibody (RM-9106-S0; Thermo Fisher Scientific, Fremont, CA, USA), Alexa Fluor 488-conjugated goat anti-mouse antibody (Life Technologies), Alexa Fluor 568-conjugated goat anti-rabbit antibody (Life Technologies), and 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Dojindo, Mashikimachi, Japan).

The following materials and instruments were also used: cell culture dishes with diameters of 6 and 9 cm (Nunc, Roskilde, Denmark), 24-well microplates (Nunc), glass slides (S9443; Matsunami, Kishiwada, Japan), an epifluorescence microscope (Optiphoto-2; Nikon, Tokyo, Japan) equipped with a digital camera (DS-5Mc; Nikon), a cryostat microtome (CM1850; Leica Microsystems, Wetzlar, Germany), a compressive testing machine (FGP-FGS; Nidec-Shimpo, Kyoto, Japan), and a Fourier transform infrared (FTIR) spectrometer (IRAffinity-1; Shimadzu, Kyoto, Japan).

Preparation of the PVA hydrogel An aqueous solution of PVA [1.5 ml at concentrations of 3.75% (w/v), 7.50 % (w/v), or 15.0 % (w/v)] in a 6-ml sample tube (0203-22; Maruemu, Osaka, Japan) was irradiated with various doses of gamma ray (10, 20, or 40 kGy) at a dose rate of 4.08–4.45 kGy/h at room temperature (25°C) at the cobalt 60 (^{60}Co) gamma ray facility of the Radiation Research Center of Osaka Prefecture University. Samples of 3.75% (w/v), 7.50% (w/v), and 15.0% PVA irradiated at 10, 20, and 40 kGy, respectively, were used for rheological measurements in the swelling test.

Measurement of FTIR spectra PVA gels impregnated with distilled water for 15 days were dried in a vacuum, crushed into powder, and placed on the sample folder for the measurement of FTIR spectra in attenuated total reflection mode. Lyophilized PVA solution without gamma irradiation was used as a control sample. The scan was repeated 40 times to obtain average data at 700–4000 cm^{-1} with a resolution of 2.0 cm^{-1}.

Swelling test The wet weight of the PVA hydrogel prepared as described above was measured under sterile conditions before swelling in solution. Gels were then incubated for the swelling test in either distilled water or culture medium (DMEM/F-12 with antibiotic-antimycotic solution) at room temperature (25°C). The wet weight of the gels was measured again at 1, 2, 6, and 12 h, and 1, 2, 4, 6, 8, 10, 12, and 14 days.

The swelling ratio, Q_w, of the gel was calculated from the wet weight at n days after swelling (W_{wn}) and before swelling (W_{w0}), according to the following formula.

$$Q_w = W_{wn}/W_{w0} \quad (1)$$

Rheological measurements The compression test was carried out to determine the Young's modulus, E, of PVA gels. The surface of the PVA gel was compressed using the flat circular disk plate (12 mm in diameter) of the compressive testing machine at a speed of 20 mm/min, and the load value and thickness of the gel were recorded. E was obtained by calculating the relationship (from a stress–strain graph) between stress (σ) and strain (ε) converted from the load value P and the thickness deformation of PVA gels, as described by Eq. 2:

$$E = \sigma/\varepsilon = (P/A)/(dL/L) \quad (2)$$

where A, L, and dL are the area of the compressing disk plate, original thickness of the gel, and the thickness of the gel under compression subtracted from L, respectively. The stress value at 10% strain was used to calculate E.

NSPC neurosphere cultures NSPCs were isolated from the fetal brain of an imprinting control region mouse (embryonic day 14) and resuspended in DMEM/F-12 medium supplemented with EGF (20 ng/ml), b-FGF (20 ng/ml), and B-27 supplement, as previously described (25,26). Cells (1.0×10^5/ml in 10 ml medium) were cultured in dishes at 37°C and 5% (v/v) CO_2 to induce neurosphere formation and were expanded by dissociation with trypsin and reseeding every 7 days. All animal experiments were conducted in accordance with institutional guidelines and national standards with approval from the Animal Experiment Committee of Osaka Prefecture University. NSPCs amplified for several passages as described above were used for analysis on a PVA gel.

NSPC culture on PVA hydrogels Three different types of PVA gel [3.75%, 7.50%, and 15.0% (w/v) PVA irradiated at 10, 20, and 40 kGy, respectively] in a 24-well plate (0.5 ml/well) were incubated with 0.5 ml of sterile distilled water for 30 min, washed twice to removed unreacted polymer, and incubated with 0.5 ml DMEM/F-12 medium for 2 days. Samples were incubated for an additional 5 days before cell culture in order to completely equilibrate the culture medium. NSPCs resuspended in medium (0.5 ml of 1.0×10^5 cells/ml) were seeded on PVA gels placed in each well of the 24-well plate and cultured at 37°C and 5% (v/v) CO_2. After 3 days of culture, 0.5 ml of medium was added to each well. Cells were analyzed after 7 days of culture. To evaluate growth, cells were dissociated with trypsin/EDTA, stained with Trypan Blue dye to distinguish live from dead cells, and counted under the microscope.

Immunocytochemistry and imaging Frozen sections of NSPCs grown on PVA gels were prepared for immunocytochemistry. Cells were not detached from the surface of the PVA hydrogel, which was removed from the culture medium and fixed with 4% (w/v) paraformaldehyde in Dulbecco's phosphate buffered saline (PBS) for 30 min at 4°C. Samples were rinsed three times with PBS for 10 min each, then incubated in PBS containing 20% (w/v) sucrose overnight at 4°C. The samples were transferred to a mold and mounted in O.C.T. compound, flash-frozen on dry ice, and stored at −80°C. The frozen sample was sectioned at a thickness of 12 μm on a cryostat microtome; sections were collected on glass slides, which were washed twice in PBS for 5 min. Samples were permeabilized by incubating for 5 min in PBS containing 0.3% (w/v) Triton X-100. After blocking for 1 h in PBS containing 10% (v/v) normal goat serum and 0.01% (w/v) Triton X-100, samples were incubated overnight at 4°C with primary antibodies against the following proteins diluted in PBS containing 10% normal serum and 0.01% (w/v) Triton X-100: tubulin βIII (1:500), GFAP (1:100), nestin (1:500), and Ki67 (1:500). After washing three times for 5 min with PBS, samples were incubated with Alexa Fluor 488-labeled goat anti-mouse (1:500) or Alexa 568-labeled goat anti-rabbit (1:500) IgG diluted in PBS containing 10% normal serum and 0.01% (w/v) Triton X-100 for 1 h at room temperature in a humid chamber. Nuclei were labeled by staining for 5 min with DAPI (1 μg/ml) diluted in the same solution. After washing three times with PBS, samples were mounted with PermaFluor mounting medium (Thermo Fisher Scientific, Waltham, MA, USA) and protected with a coverslip in the dark.

Quantitative reverse transcriptase-PCR The mRNA expression of five marker genes (tubulin βIII, *Tubb3*; GFAP, *Gfap*; S100β, *S100b*; nestin, *Nes*; sex-determining region Y-box 2, *Sox2*) in NSPCs cultured for 7 days was analyzed by quantitative reverse transcriptase-PCR (qRT-PCR) according to a previously described protocol (27). Total RNA was extracted from NSPCs with Sepasol-RNA I Super G solution and complementary DNA was synthesized with the Prime Script RT reagent kit; qRT-PCR was performed with SYBR Premix Ex Taq II on an Opticon real-time PCR thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following gene-specific forward and reverse primer sets: mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5′-CAT GGC CTT CCG TGT TCC T-3′ and 5′-TGC CTG CTT CAC CAC CTT CT-3′; mouse *Gfap*, 5′-GAG AAA GGT TGA ATC GCT GGA-3′ and 5′-GCT TGG CCA CAT CCA TCT C-3′; mouse *Nes*, 5′-TGG AAG GTG GGC AGC AA-3′ and 5′-GGG GTC AGG AAA GCC AAG A-3′; mouse *Tubb3*, 5′-CCC CAG CGG CAA CAT GTA-3′ and 5′-AAC CTG GGC TGT CTC TCT GG-3′; mouse *S100b*, 5′-ACT GAA GGA GCT TAT CAA CAA CGA-3′ and 5′-ACA CTC CCC ATC CCC ATC T-3′; and mouse *Sox2*, 5′-ACA TGG CCC AGC ACT ACC A-3′ and 5′-AAA ATC TCT CCC CTT CTC CAG TTC-3′. Relative mRNA expression levels were normalized to that of Gapdh by calculating the comparative cycle time relative to the control group and is expressed as a fold change ($2^{-\Delta\Delta CT}$).

Statistical analysis Data are represented as mean ± standard deviation. Statistical analysis was performed by statistical software (Excel and JSTAT) using one-way ANOVA with multiple comparison post hoc tests (William's multiple comparison test was used for statistical significance of concentration-dependent increasing of Young's modulus of PVA gels in Fig. 3B, and Dunnett's multiple comparison test for comparisons of cell number and the expression levels of marker genes in NSPCs on each PVA gel with control groups in Figs. 5 and 7). N.S. was presented if significance was not obtained by one-way ANOVA. The data in Tables 1 and 2 were tested by Kruskal–Wallis test with Tukey's multiple comparison test, because the homoscedasticity were rejected by Bartlett's test.