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Development of an *in situ* evaluation system for neural cells using extracellular matrix-modeled gel culture

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Two-dimensional monolayer culture is the most popular cell culture method. However, the cells may not respond as they do *in vivo* because the culture conditions are different from *in vivo* conditions. However, hydrogel-embedding culture, which cultures cells in a biocompatible culture substrate, can produce *in vivo*-like cell responses, but *in situ* evaluation of cells in a gel is difficult. In this study, we realized an *in vivo*-like environment *in vitro* to produce cell responses similar to those *in vivo* and established an *in situ* evaluation system for hydrogel-embedded cell responses. The extracellular matrix (ECM)-modeled gel consisted of collagen and heparin (Hep-col) to mimic an *in vivo*-like environment. The Hep-col gel could immobilize growth factors, which is important for ECM functions. Neural stem/progenitor cells cultured in the Hep-col gel grew and differentiated more actively than in collagen, indicating an *in vivo*-like environment in the Hep-col gel. Second, a thin-layered gel culture system was developed to realize *in situ* evaluation of the gel-embedded cells. Cells in a 200-μm-thick gel could be evaluated clearly by a phase-contrast microscope and immunofluorescence staining through reduced optical and diffusional effects. Finally, we found that the neural cells cultured in this system had synaptic connections and neuronal action potentials by immunofluorescence staining and Ca^{2+} imaging. In conclusion, this culture method may be a valuable evaluation system for neurotoxicity testing.

[**Key words:** Extracellular matrix; Collagen; Heparin; *In situ* evaluation; Neural stem cells; Gel-embedded culture]

In vitro cell culture technologies are used for the production of useful biological products as well as safety and toxicity testing of new cosmetics and drugs. Two-dimensional (2D) monolayer culture is the most popular cell culture method because it is appropriate for high throughput evaluations. However, the cells cultured in monolayer conditions may not respond as those *in vivo* because the culture conditions are different from *in vivo* conditions surrounded by an extracellular matrix (ECM) (1). Therefore, monolayer culture is insufficient for use in neurotoxicity testing because reliable results are difficult to obtain.

To produce *in vivo*-like cell responses, 3D structures have been constructed. Spherical multicellular aggregates with cell-cell interactions similar to those *in vivo* promote organ-specific functions and the differentiation of cells (2,3). However, there is the severe problem of the mass transfer limitation including O_2 and substrate delivery by diffusion. In addition, the inner structure of a spheroid can be hardly observed by microscopes. Therefore, spheroids are difficult to apply as an evaluation system, although an *in vivo*-like environment can be constructed. Moreover, spheroids are not ideal to create *in vivo*-like microenvironments around cells because they cannot mimic cell-ECM interactions.

Conversely, hydrogel-embedding culture has been studied widely, which cultures cells in a biocompatible culture substrate such as collagen. This culture method can provide *in vivo*-like 3D conditions mimicking cell-cell interactions (4,5). Furthermore, it is possible to realize cell-ECM interactions, and this culture method is considered as the most *in vivo*-like culture system available. However, general hydrogel culture of 2 mm in thickness prevents optical penetration and substance diffusion (6–8). For these reasons, *in situ* evaluations are difficult, such as phase contrast microscopic and immunohistochemical evaluations. As a result, high costs or a long period of time are required for confocal laser microscopy or preparation of gel slices, respectively. Thus, an *in vitro* cell culture system requires: (i) realizing *in vivo*-like environments at the *in vitro* level to produce cell responses similar to those *in vivo*, and (ii) high throughput *in situ* evaluation of cell responses.

In our previous study, we developed heparin-immobilized collagen (Hep-col). This substrate can electrostatically bind to growth factors such as hepatocyte growth factor and vascular endothelial growth factor (9–11). Additionally, Hep-col promotes albumin synthesis of hepatocytes and angiogenesis in transplanted conditions. Therefore, Hep-col is considered to be an ECM-modeled material. Proteoglycans, which are one of the main components of nerve ECMs, consist of core proteins and a number of glycosaminoglycans such as heparan sulfate (12). Heparan sulfate has the ability to combine with certain functional molecules such as growth factors, netrin, semaphorin, and slit protein (13). It is considered that heparan sulfate plays important roles by interacting with heparin-binding molecules (14–17).

For these reasons, Hep-col may be useful as a nerve ECM-modeled material for gel culture. The Hep-col substrate may be able to create 3D culture conditions with interactions among cell-ECM functional molecules to produce *in vivo*-like cell responses.

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In addition, we proposed a thin-layered gel culture system to reduce gel thickness. It is expected to decrease optical effects in the gel, such as refraction and scattering, and avoid substance diffusion by reducing the gel thickness.

In this study, we developed the thin-layered gel culture system using a heparin-immobilized collagen substrate. We expect that this technology will realize *in situ* evaluation of *in vivo*-like neuronal cell behaviors.

MATERIALS AND METHODS

Preparation of ECM-modeled gels (Hep-col gels) Heparin sodium (Wako Pure Chemicals, Osaka, Japan) was immobilized in collagen (Cellmatrix Type 1-A; Nitta Gelatin, Osaka, Japan) based on the methods of Yao et al. (18) and Steffens et al. (19). Heparin, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, Peptide Institute Inc., Osaka, Japan), and *N*-hydroxysuccinimide (NHS, Wako Pure Chemicals) were dissolved in 50 mM MES buffer (pH 5.6) at 10, 10, and 6 mg/ml, respectively. After 1 h of incubation, the EDC/NHS-activated heparin solution was dialyzed through a Spectra/Por dialysis membrane (molecular weight cut-off:1000, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) with a dialysate of 500 ml MES buffer at 4°C for 6 h to remove excess inactivated EDC/NHS molecules that are cytotoxic in gel cultures. Then inactivated EDC/NHS molecules were removed by dialysis (9). After the EDC/NHS-activated heparin solution was added to 10 × concentrated Dulbecco's modified Eagle's medium Ham's nutrient mixture F-12 (DMEM/F12, Sigma, St. Louis, MO, USA), 3 mg/ml collagen, the EDC/NHS-activated heparin in 10 × DMEM/F12, and reconstituting buffer were mixed (final concentrations in the gel: collagen, 2.4 mg/ml; heparin, 10–30 μg/ml). Then, the sol was gelated at 37°C.

Measurement of heparin in Hep-col gels After preparation of the Hep-col gels, the gels were washed with 5 M NaCl to remove unconjugated heparin. Heparin-mixed collagen gels, which were not cross-linked by EDC/NHS, were prepared and washed in the same manner. The gels were freeze dried after any remaining NaCl in the gels was washed out by deionized water. The freeze-dried gels were dissolved by 20 μl of a collagenase solution at 37°C for 30 min. The amount of heparin was measured by a Wieslab sGAG quantitative kit (Euro Diagnosica, Malmö, Sweden).

Evaluation of growth factor immobilization in Hep-col gels A Hep-col gel and collagen gel without heparin (50 μl each) were prepared in a 24-well plate. The gels were washed with 5 M NaCl and PBS. A 1% bovine serum albumin (BSA) solution (2 ml) was added in each well, followed by 12 h of incubation to block non-specific protein adsorption to the gel. Empty wells were also washed and blocked as a blank. Then, 400 μl of 0, 10, and 100 ng/ml recombinant human basic fibroblast growth factor (bFGF, R&D Systems Inc., Minneapolis, MN, USA) in 1% BSA was added to the wells. bFGF was immobilized in the gel by shaking at 70 rpm. After immobilization of bFGF, the bFGF concentration in the supernatants was measured by a Duo Set ELISA Development Kit (R&D Systems Inc.). Immobilized bFGF densities were calculated from the change of bFGF concentrations in supernatants. Next, the amount of immobilized bFGF was measured by a direct detection method. The bFGF-immobilized gels were washed with 1 ml wash buffer for 3 days with daily buffer changes. After 400 μl of detection antibody solution was added to each well, the plate was shaken for 24 h. The gels were blocked with 1% BSA after washing with wash buffer. Streptavidin-HRP (1/200 dilution; R&D Systems Inc.) was added at 400 μl/well. The plate was then shaken overnight. The gels were washed with wash buffer until the buffer did not react with the chromogenic substrate. The chromogenic substrate was incubated with the gels for 12 min at room temperature. Then, 200 μl of 2 N H_2SO_4 was added to stop the chromogenic reaction. Absorbances were then measured at 450 and 540 nm.

Preparation of thin-layered gels A coverslip (18 × 18 mm) was soaked in 1% BSA for 10 min to block the surface, washed with deionized water twice, and then dried. Two Polydimethylsiloxane (PDMS) spacers were placed 6 mm apart and parallel to each other on the bottom surface of a 6-well plate. The BSA-blocked coverslip was placed on the spacers. Collagen sol flowed into the gap between the bottom and coverslip by capillary force. After the sol had gelated by incubation for 15 min at 37°C, the coverslip and spacers were removed. The gel thicknesses were measured by the following method. Coverslips of a known thickness were observed under a phase-contrast microscope. The rotation angles were measured focusing on the upper surface to lower surface. Then, the migration distance of the focus per rotation angle was calculated. The thickness of thin-layered gels was measured by this relationship (Fig. S1).

Observation of cells in thin-layered gels using a phase contrast microscope PC12 cells were purchased from RIKEN (Saitama, Japan). The cells were embedded in thin-layered gels (gel thicknesses: 100, 200 and 300 μm) and a 2-mm-thick gel at 5×10^5 cells/ml of gel. The cells were cultured in DMEM (Sigma) containing 0–10 ng/ml recombinant rat β-neuron growth factor (NGF, R&D Systems Inc.) and N2 supplement (N2, Life Technologies, Carlsbad, CA, USA) for 7 days. In addition, PC12 cells were cultured on poly-L-lysine coated 48-well plates at 5×10^3 cells/cm^2 in the same culture medium. After 7 days, the differentiated cells were photographed using a phase-contrast microscope (ECLIPSE TE 300; Nikon, Tokyo, Japan). Then, neurite lengths were measured. Then, the projected lengths of neurite in the images of phase contrast were measured by using an image analysis software (ImageJ).

Evaluation of substrate diffusion in collagen gels Fluorescein-5-isothiocyanate (FITC)-conjugated goat anti-mouse IgG was mixed with 2.4 mg/ml collagen sol at 100 μg/ml of sol. Fluorescent antibody-mixed, thin-layered gels were prepared using 100-, 200-, and 300-μm-thick spacers (gel volumes: 10, 20, and 30 μl). In addition, 2-mm-thick, fluorescent antibody-mixed gels were prepared in a 48-well plate (gel volume: 200 μl). The gels were washed in a 10 × volume of PBS and then fluorescence micrographs were obtained using an IX71 inverted microscope (OLYMPUS, Tokyo, Japan) over time. ISO speed was 1600, exposure time was 1 s, and the black balance was 300. Before obtaining fluorescence micrographs, gels were placed in fresh PBS. Green intensities of the fluorescence micrograph were measured by Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA).

Immunofluorescence staining of neural stem cells in thin-layered gels ED14 Wistar rats were purchased from Kyudo (Tosu, Japan). Neural stem cells were harvested by the primary neurosphere method with minor modifications (20). ED14 embryonic rat brain was treated with Accumax (Innovation cell technologies, Inc., San Diego, CA, USA) for 15 min. After isolation with CellTrics Filter (pore size: 30 μm, SYSMEX, Hyogo, Japan), supernatant were removed by centrifugation for 5 min at 150 ×g. Cells were suspended at 5×10^5 cells/ml of DMEM/F-12 containing 20 ng/ml EGF, bFGF, 1% N2 supplement, 10 μg/ml heparin and seeded in LIPIDURE-COAT 6-well plate (NOF, Tokyo, Japan). Primary neurospheres were obtained by culturing for 7 days and treated with Accumax. Then primary neural stem/progenitor cells were isolated by filtration with CellTrics Filter (pore size: 20 μm, SYSMEX, Hyogo, Japan). The neural stem cells were embedded in a 2-mm-thick collagen gel and thin-layered gel. Cell density was 2×10^6 cells/ml of gel. The cells were cultured in Neurobasal Medium (Life Technologies) containing 10 ng/ml bFGF, 2% B27 supplement (Life Technologies), and 2 mM GlutaMAX (Life Technologies) for 7 days. After formalin treatment, the gels were blocked with 1% BSA for 2 h. Cells were dyed by immunofluorescent staining for nerve cells marker protein (β-tubulin III), astrocytic marker protein (GFAP), and nucleus. Chicken anti-rat β-tubulin III IgY (Aves Labs Inc., Tigard, OR, USA) and Mouse anti-rat glial fibrillary acidic protein (GFAP) IgG (Biosensis Pty Ltd., Temecula, CA, USA) in 1% BSA were added to the gels, followed by incubation for 2 h. The gels were washed with PBS for 2 h. The gels were stained with Goat anti-chicken IgY Alexa Fluor 488 conjugated (10 μg/ml; Abcam, Cambridge, UK), goat anti-mouse IgG Alexa Fluor 546 conjugated (10 μg/ml; Life Technologies), and Hoechst 33,342 (5 μg/ml; Dojindo Molecular Technologies Inc., Kumamoto, Japan) in 1% BSA for 2 h. After washing with PBS for 2 h, the gels were observed under the IX71 inverted microscope. The experimental protocol was reviewed and approved by the Ethics Committee on Animal Experiments of Kyushu University.

Culture of neural stem cells in a brain-mimicking cell culture system Neural stem cells were suspended in sols at 2×10^6 cells/ml of sol. Collagen sol, Hep-col sol, 200 ng/ml bFGF-mixed collagen sol, and 200 ng/ml bFGF-mixed Hep-col sol were used. The sols were placed in a 96-well plate at 50 μl/well. The sols were incubated for 30 min at 37°C. After gelation of the sols, media were added to the wells at 80 μl/well. Neurobasal Medium containing 2% B27 supplement and 2 mM GlutaMAX (NB/B27), 10 ng/ml bFGF-supplemented NB/B27, or 10 ng/ml bFGF and 10 μg/ml heparin-supplemented NB/B27 were used. The media were changed every day. After 7 days, cell viabilities were measured by a Cell Counting Kit 8 (Dojindo Molecular Technologies Inc.). In addition, neural stem cells were cultured in thin-layered gels under the same culture conditions by gel culture in a 96-well plate. β-Tubulin III, GFAP, and nuclei in the cells cultured in thin-layered gels were stained as described above at days 7 and 14 after formalin treatment.

Immunofluorescence staining of a synapse marker Neural stem cells were embedded in a thin-layered Hep-col gel at 2×10^6 cells/ml of sol. The cells were cultured in Neurobasal Medium containing 10 ng/ml bFGF, 2% B27 supplement, and 2 mM GlutaMAX for 7 days. After formalin treatment, the gels were blocked with 1% BSA for 2 h. A presynaptic terminal marker (synaptophysin), spine marker (PSD95), and neuronal marker (β-tubulin III) were stained as follows. Chicken anti-rat β-tubulin III IgY (0.6 μg/ml), mouse anti-rat PSD95 IgG (2.5 μg/ml; Abcam), and rabbit anti-rat synaptophysin IgG (2.5 μg/ml; Abcam) in 1% BSA were added to the gel. After washing the gel with PBS, 10 μg/ml goat anti-chicken IgY Alexa Fluor 488 conjugated, 10 μg/ml goat anti-mouse IgG Alexa Fluor 546 conjugated, and 10 μg/ml goat anti-rabbit IgG Alexa Fluor 350 conjugated (Life Technologies) in 1% BSA were added to the gel. After washing with PBS, cells were observed under an All-in-One Fluorescence Microscope (BZ-X700; Keyence, Osaka, Japan).

Ca^{2+} imaging Neural stem cells were embedded in thin-layered Hep-col gels at 2×10^6 cells/ml of sol. The cells were cultured in Neurobasal Medium containing 10 ng/ml bFGF, 2% B27 supplement, and 2 mM GlutaMAX for 7 days. Loading Buffer was prepared using Calcium Kit-Fluo4 (Dojindo Molecular Technologies Inc.). After washing the gel with PBS, the Loading Buffer was added to the gels at 200 μl/well. Fluo4 was absorbed into the cells by incubation for 1 h at 37°C with 5% CO_2. After washing the gel with PBS, 100 or 500 mM NMDA

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