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Enhancing the neuronal interaction on fluoropolymer surfaces with mixed peptides or spacer group linkers

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Abstract

Embryonic hippocampal neurons cultured on surface modified fluoropolymers showed enhanced interaction and neurite extension. Poly(tetrafluoroethylene-*co*-hexafluoropropylene) (FEP) film surfaces were aminated by reaction with a UV-activated mercury ammonia system yielding FEP-[N/O]. Laminin-derived cell-adhesive peptides (YIGSR and IKVAV) were coupled to FEP surface functional groups using tresyl chloride activation. Embryonic (E18) hippocampal neurons were cultured in serum-free medium for up to 1 week on FEP film surfaces that were modified with either one or both of GYIGSR and SIKVAV or GGGGGGYIGSR and compared to control surfaces of FEP-[N/O] and poly(L-lysine)/laminin-coated tissue culture polystyrene. Neuron-surface interactions were analyzed over time in terms of neurite outgrowth (number and length of neurites), cell adhesion and viability. Neurite outgrowth and adhesion were significantly better on peptide-modified surfaces than on either FEP or FEP-[N/O]. Cells on the mixed peptide (GYIGSR/SIKVAV) and the spacer group peptide (GGGGGGYIGSR) surfaces demonstrated similar behavior to those on the positive PLL/laminin control. The specificity of the cell–peptide interaction was demonstrated with a competitive assay where dissociated neurons were incubated in media containing peptides prior to plating. Cell adhesion and neurite outgrowth diminished on all surfaces when hippocampal neurons were pre-incubated with dissolved peptides prior to plating.

Keywords: Laminin; Peptides; Hippocampal neurons; Regeneration; Central nervous system; Poly(tetrafluoroethylene-*co*-hexafluoropropylene)

1. Introduction

Central nervous system (CNS) neurons, unlike those of the peripheral nervous system, do not regenerate spontaneously following injury; however, CNS neurons can regenerate in a peripheral nerve graft [1,2] or in an environment that mimics that of the peripheral nerve [3]. In an attempt to mimic the contact-mediated attractive cues to which axons respond, we have prepared surfaces with well-defined chemistry and topography using poly(tetrafluoroethylene-*co*-hexafluoropropylene) (FEP) [4–6]. Because FEP is chemically inert, functional groups can be introduced to defined depths with minimal or no effect on surface topography [7]. We previously demonstrated that FEP films modified with a UV-activated mercury ammonia system (the mercat reaction) resulted in functionalized surfaces [4] having amine, hydroxyl and carboxylic acid groups available for peptide coupling. Furthermore, we demonstrated that hippocampal neurons respond to surfaces modified with either YIGSR or IKVAV in a similar manner to positive controls — glass-coverslips coated with poly(L-lysine) (PLL) and laminin [5]. Of the two laminin-derived peptides, YIGSR has been shown to enhance cell adhesion [8] and IKVAV to enhance neurite outgrowth [9].

We hypothesize herein that surfaces modified with both YIGSR and IKVAV will yield a better cellular response than surfaces containing a single peptide type. By combining on a surface the cell adhesive and neurite promoting sequences of laminin, a better biomimetic surface will be created. To determine the effect of peptide mobility on cellular interactions, a hexaglycine spacer group was introduced to YIGSR [i.e. GGGGGGYIGSR or G_6YIGSR] prior to coupling it to FEP. The neuronal response to G_6YIGSR was compared to that of

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G_1YIGSR. By increasing the spacer group length between the active YIGSR peptide and the FEP surface, the peptide would have increased conformational degrees of freedom that may enhance cellular interaction. Thus, the focus herein is the cellular response to the surfaces: cell viability, neurite number and length, and specificity of interaction.

2. Materials and methods

All chemicals were purchased from Aldrich (Milwaukee, WI) and used as received unless otherwise indicated. Tetrahydrofuran (THF, Fisher, Nepean, ON) was distilled from sodium benzophenone dianion and stored under nitrogen in Schlenk flasks. Neurobasal medium, B27 supplement and phosphate buffered saline (PBS) were sterile-filtered with 0.22 μm cellulose acetate filters (all from Gibco BRL, Burlington, ON). All peptides were purchased from Vetrogen (London, ON) and used as received. FEP films (5 mil thickness, received from DuPont and cut into 2 cm × 2 cm samples) were Soxhlet-extracted in THF for 24 h prior to use. All reactions were done under inert nitrogen atmosphere unless otherwise indicated. Deioinized distilled water was obtained from a Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) and used at 18 MΩ resistance.

Embryonic rat hippocampal neurons (BrainBits, Springfield, IL) were obtained as undissociated brain tissue. Cultures on all surfaces were photographed under normal light or filters for fluorescently-stained cells at 20 × magnification under an Axiovert S-100 inverted microscope (Zeiss, Germany) using a digital camera DC-130 (Sony, Japan) connected to a computer. Digital images were then analyzed using the Empix Imaging Northern Eclipse software (version 5.0, Toronto, ON) for neurite length, number and cell count.

2.1. Introduction of nitrogen functionality by the Mercat method, FEP-[N/O]

FEP-[N/O] film samples were prepared as previously described [4,6]. Briefly, a quartz Schlenk tube, containing FEP film samples and a drop of mercury, was evacuated ($P < 0.01$ mm Hg) and purged with argon (3 times). After the fourth evacuation, the tube was re-filled with gaseous ammonia (99.99% purity, BOC, Toronto, ON) to 1 atm pressure and the films were irradiated with eight 15 W mercury lamps (254 nm) in a UV photoreactor (Rayonet, Branford, CT) for 24 h. The samples were then washed three times each with THF, methanol and dichloromethane.

2.2. Peptide coupling to functionalized FEP, FEP-[N/O]-peptide

The peptides (GYIGSR, GGGGGGYIGSR and SIKVAV) were coupled to FEP-[N/O] film surfaces using the trifluoroethanesulfonyl chloride (tresyl chloride) coupling agent as previously described [5,10]. Briefly, FEP-[N/O] film samples were immersed in a solution containing 200 μl of tresyl chloride, 1 ml pyridine and 19 ml THF for 20 min at RT. Activated films were then transferred to a beaker containing 10 ml of a 0.2 M pH 10 sodium carbonate-buffered solution and 0.1 μg/ml of peptide for 24 h: either 100% GYIGSR (FEP-[N/O]-GYIGSR), 100% GGGGGGYIGSR (FEP-[N/O]-G_6YIGSR), 100% SIKVAV (FEP-[N/O]-SIKVAV), or 50% GYIGSR and 50% SIKVAV (FEP-[N/O]-Mix). The films samples were sequentially rinsed five times each with the pH 10 buffer solution, dilute hydrochloric acid, water and THF prior to drying under vacuum.

2.3. Hippocampal neuron–fluoropolymer interaction

FEP-[N/O]-peptide films and controls (FEP and FEP-[N/O]) were immersed in 70% ethanol for 1 h and then rinsed four times with sterile water before air-drying. Positive control surfaces were prepared by coating 24-well tissue culture polystyrene plates with 1 ml of an aqueous 1 mg/ml solution of poly(L-lysine) (PLL, Sigma, MW = 37,000 g/mol) for 24 h at 37°C and then with 10 μl of a 1 mg/ml aqueous solution of laminin (Gibco) for 2 h at 37°C. The PLL/laminin-coated wells were rinsed with sterile water and air-dried prior to plating the hippocampal neurons. Embryonic day 18 (E18) mouse hippocampal neurons were dissociated by incubating with papain (Sigma) for 30 min and mechanically triturated in serum-free media (SFM). One ml of the dissociated hippocampal neurons was then plated at 10^5 cells/ml in SFM on each film sample. After 3 h, the media was replaced with fresh SFM. The SFM was made with 2 ml B27 supplement, 100 mg chicken egg albumin (Sigma), 10 mg pyruvic acid (Sigma), 1 ml glutamine (Sigma) and 1 ml penicillin/streptomycin (Gibco, 10,000 unit/ml and 10,000 μg/ml, respectively) in 100 ml neurobasal medium. The anti-mitotic agent, fluorodeoxyuridine/uridine (Sigma), was added after 24 h. The cells were incubated at 37°C in 5% CO_2 for up to 1 week.

To assess cell viability, samples were incubated for 30 min at 37°C with 150 μl of stock viability assay solution (20 μl ethidium homodimer and 5 μl calcein AM in 10 ml PBS, Molecular Probes, Eugene, OR) in fresh SFM. The cell–material interaction was assessed each day from days 1 to 4 in terms of the number and length of extended neurites. At each time point, the number and length of neurites/cell of 50 cells that were chosen at random were quantified: only neurites longer than the cell body length were quantified.

2.4. Competitive assay

To assess for the specificity of the cell–peptide interaction, dissociated neurons were incubated with 0.1 mg/ml

of dissolved GYIGSR and SIKVAV in SFM for 30 min prior to plating on the surfaces. After 3 h, the media was replaced with fresh SFM (without peptides) and the cultures analyzed as described above.

2.5. Statistics

For every surface, on which 50 cells were evaluated, triplicate experimental data sets were subjected to statistical analysis using SigmaStat (Chicago, IL). One-way ANOVA analysis, assuming a constant variance and a 95% confidence interval, was used to determine the statistical differences of various data sets. Results are reported as the mean ± the standard error of the mean.

3. Results

FEP film samples were surface modified by the mercat reaction to yield hydrophilic surfaces, composed of nitrogen and oxygen functional groups [4]. These functional groups served as reactive handles to couple GYIGSR and SIKVAV peptides individually or together (1 : 1, w/w) yielding 3–6 fmol/cm^2 of peptide, as previously determined by radioactive labeling [6]. The neuronal response to the modified surfaces was investigated by the number and length of neurites on a given surface.

3.1. Length of neurites

The average length of the longest neurite for 50 randomly chosen cells was measured by tracing the distance along the neurite using a calibrated image analysis program. As shown in Fig. 1, neurite length increased steadily over time for all surfaces. While single peptide-modified surfaces of either YIGSR or IKVAV alone had similar neurite lengths — from 40 μm on day 0 to 87 μm on day 3, mixed peptide surfaces, containing both YIGSR and IKVAV, had significantly longer neurites — from 35 μm on day 0 to 124 μm on day 3. Notwithstanding that a difference in neurite extension between YIGSR and IKVAV-modified surfaces was not observed, a synergistic effect for neurite extension seemed to be observed on surfaces containing both YIGSR and IKVAV. Furthermore, spacer-group-modified peptide surfaces of G_6YIGSR had significantly longer neurites than G_1YIGSR modified surfaces — from 38 μm on day 0 to 142 μm on day 3 — demonstrating the importance of distance between surface and active group. The shortest neurites were observed on FEP-[N/O] control surfaces and the longest on PLL/laminin positive controls, where neurites increased from 61 μm on day 0 to 190 μm on day 3. Interestingly, the rate of neurite extension over the three day incubation period was greatest on both mixed peptide (YIGSR/IKVAV) and spacer group peptide (G_6YIGSR) surfaces: for both samples, neurite length increased by 3.5 times from day 0 to day 3 whereas it increased by only 3.1 times over the same time period for positive controls of PLL/laminin.

To test the specificity of the peptide on neurite length, a competitive assay was done where cells were pre-incubated with peptides for all surfaces prior to plating. As shown in Fig. 2, after pre-incubation, neurite length decreased on all surfaces except on control FEP-[N/O] film samples, where the cell–surface interactions are non-specific. Neurite length decreased most significantly on IKVAV surfaces, as was expected given that IKVAV is known to promote neurite outgrowth [9]. It is worth noting that the cells were able to extend long neurites over time (data not shown); this result was expected

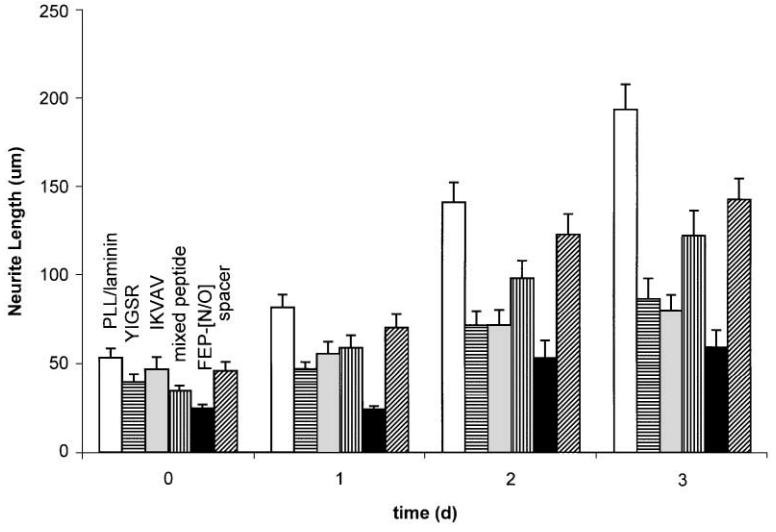

Fig. 1. The length of the longest neurites extended per hippocampal neuron (E18) cultured in serum-free medium was averaged over 50 cells per sample ($n = 3$ samples) over 3 days for the following surfaces: (□) PLL/laminin coated tissue culture dish, (▤) FEP-[N/O]-YIGSR; (▢) FEP-[N/O]-IKVAV; (▥) FEP-[N/O]-Mix; (■) FEP-[N/O]; (▨) FEP-[N/O]-spacer.

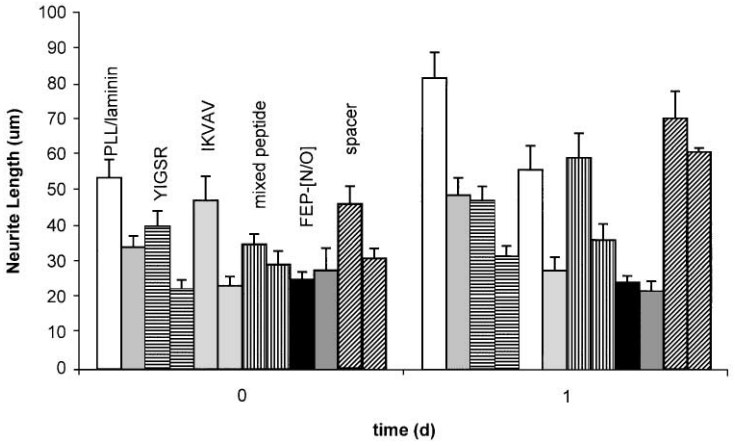

Fig. 2. A competitive assay was done where cells were pre-incubated with peptides prior to being cultured on the surfaces. The length of the longest neurites extended per hippocampal neuron (E18) cultured in serum free medium was averaged over 50 cells per sample ($n = 3$ samples) for the following surfaces: (□) PLL/laminin-coated tissue culture dish, (▤) FEP-[N/O]-YIGSR; (▢) FEP-[N/O]-IKVAV; (▥) FEP-[N/O]-Mix; (■) FEP-[N/O]; (▨)FEP-[N/O]-spacer. The gray-shadowed bars (to the right of each sample) are the competitive data for each surface.

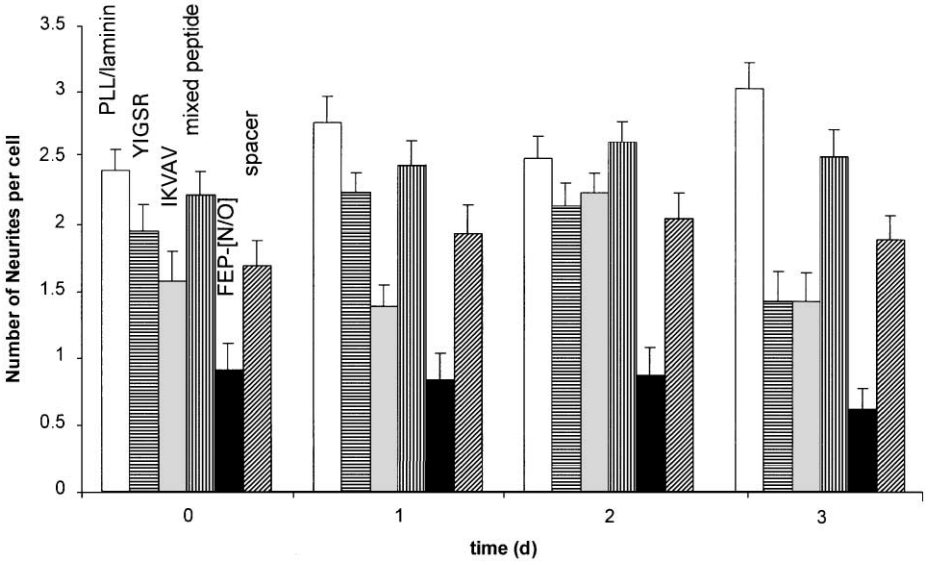

Fig. 3. The number of neurites extended per hippocampal neuron (E18) cultured in serum-free medium was averaged over 50 cells per sample ($n = 3$ samples) over 3 days for the following surfaces: (□) PLL/laminin-coated tissue culture dish, (▤) FEP-[N/O]-YIGSR; (▢) FEP-[N/O]-IKVAV; (▥) FEP-[N/O]-Mix; (■) FEP-[N/O]; (▨)FEP-[N/O]-spacer.

because the cells were only briefly (3.5 h) pre-incubated with peptides after which the serum-free medium was changed and not supplemented with peptides.

3.2. Number of neurites

The number of neurites per cell was quantified for each surface, the averages of which are summarized in Fig. 3. As shown, all peptide-modified surfaces supported a greater number of neurites per cell than the aminated FEP (FEP-[N/O]) controls, yet fewer neurites per cell than PLL/laminin positive control surfaces. The most significant differences between the surfaces were observed on day 3. The differences between YIGSR and IKVAV surfaces were again insignificant; however, mixed peptide surfaces of YIGSR and IKVAV had significantly more neurites per cell than single-peptide-modified films on day 3. The spacer group did not significantly influence the number of neurites extended per cell relative to G_1YIGSR until day 3, where G_6YIGSR surfaces had a significantly greater number of neurites per cell than GYIGSR surfaces.

In the competitive assay study of hippocampal neurons incubated with dissolved GYIGSR and SIKVAV peptides prior to plating, the number of neurites per cell decreased on all surfaces, as shown in Fig. 4. As expected

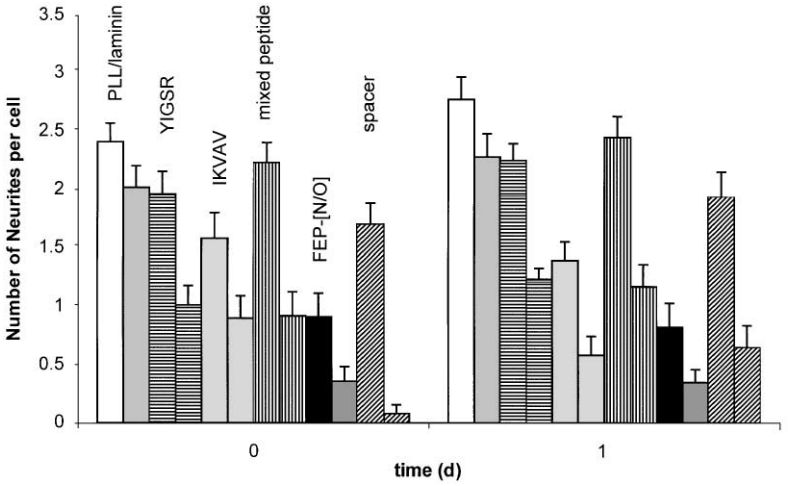

Fig. 4. A competitive assay was done where cells were pre-incubated with peptides prior to being cultured on the surfaces. The number of neurites extended per hippocampal neuron (E18) cultured in serum free medium was averaged over 50 cells per sample ($n = 3$ samples) for the following surfaces: (□) PLL/laminin-coated tissue culture dish, (▤) FEP-[N/O]-YIGSR; (▭) FEP-[N/O]-IKVAV; (▥) FEP-[N/O]-Mix; (■) FEP-[N/O]; (▨)FEP-[N/O]-spacer. The gray-shadowed bars (to the right of each sample) are the competitive data for each surface.

and as was shown for neurite length, the greatest percent decrease was observed on the IKVAV surfaces. A small decrease in the number of neurites per cell was observed for PLL/laminin and FEP-[N/O] control surfaces, indicating that merely saturating cellular receptors influenced outgrowth. The neurons extended more neurites per cell from day 2 onwards (data not shown), reflecting the continued replenishment of surface receptors.

4. Discussion

The goal of peptide modification is to create analogs of protein modified surfaces to obviate the use of full proteins. This allows control of concentration and orientation of the peptides on the surfaces and overcomes the use of natural proteins derived from animal tissues, which may cause an inflammatory response when implanted. Peptide modification also allows for a greater concentration of the active group to be incorporated on a surface than protein adsorption. YIGSR and IKVAV were studied because they promote adhesion [8] and outgrowth [9], respectively, and are derived from laminin, which is an important extracellular matrix protein for neurons [11].

Previous research in our [4–6] and other laboratories [12] have demonstrated the utility of single peptide-modified surfaces, yet few, if any, have studied the additional, synergistic effect of mixed peptide surfaces. Our results demonstrate that the mixed peptide surface is a better analog for laminin than single peptide surfaces, as shown by both neurite length and number. While the longest neurite was not as long as that observed on PLL/laminin positive control surfaces, the percent increase in neurite length on the mixed peptide and spacer group surfaces were the greatest of all surfaces studied. This emphasizes (1) the greater biomimetic effect of having two active moieties immobilized on a surface and (2) the importance of peptide mobility/conformation for cellular interaction.

It is important to note that positive controls consisted of laminin/poly(lysine)-modified tissue culture polystyrene dishes whereas peptide-modified films were prepared on FEP. The underlying surface chemistry/hydrophilicity may have impacted our results. Notwithstanding these differences, the data indicate that neurite extension requires stimulation of multiple receptor types and/or greater mobility of the peptide. Therefore, in order to fully mimic the neuron–laminin response, other peptide sequences of laminin such as RGD [13] or RKRLQVQLSIRT [14], and extension of the peptides from the surface using spacer groups, may be required.

5. Conclusions

Peptide-modified FEP film surfaces enhanced cellular interaction of hippocampal neurons. FEP surfaces modified with a 1 : 1 mixture of YIGSR and IKVAV peptides promoted a greater number of neurites per cell and longer neurites than surfaces modified with one peptide type. YIGSR coupled to FEP surfaces through a spacer group promoted longer neurites than YIGSR coupled directly to the surface. The interaction of the neurites with the surface was blocked by competitive binding of soluble peptides in the media, indicating that cell–surface interactions are peptide-receptor specific. The mixed peptide-coupled FEP surface served as the best analog for poly(L-lysine)/laminin-coated tissue culture polystyrene surfaces, yet was still lacking, especially in terms of

number of neurites per cell. In order to match positive control results, two additional studies will be investigated: (i) several relative concentrations of YIGSR and IKVAV will be studied (herein we investigated only 1 : 1 w/w ratios); and (ii) longer amino acid sequences to accommodate the three-dimensional conformation observed in laminin will be studied.

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