Functional immobilization of interferon-gamma induces neuronal differentiation of neural stem cells

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Abstract: Stem cell transplantation provides significant promise to regenerative strategies after injury in the central nervous system. Neural stem/progenitor cells (NSPCs) have been studied in terms of their regenerative capacity and their ability to differentiate into neurons when exposed to various soluble factors. In this study, interferon-γ (IFN-γ) was compared with brain-derived neurotrophic factor (BDNF) and erythropoietin and was shown to be the best single growth factor for inducing neuronal differentiation from adult rat brain-derived NSPCs. Next, IFN-γ was surface immobilized to a methacrylamide chitosan (MAC) scaffold that was specifically designed to match the modulus of brain tissue and neuronal differentiation of NSPCs was examined *in vitro* by immunohistochemistry. Bioactive IFN-γ was successfully immobilized and quantified by ELISA. Both soluble and immobilized IFN-γ on MAC surfaces showed dose dependent neuronal differentiation with soluble saturation occurring at 100 ng/mL and the most effective immobilized IFN-γ dose at 37.5 ng/cm^2, where significantly more neurons resulted compared with controls including soluble IFN-γ. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 93A: 625–633, 2010

Key words: neural stem cells; interferon-gamma; immobilized factors; chitosan; neurons

INTRODUCTION

Neural stem/progenitor cells (NSPCs) present a valuable cell source for tissue engineered treatments of central nervous system (CNS) disorders such as stroke and spinal cord injury. Recent work has demonstrated that NSPCs can be transplanted into injured tissues to promote regeneration and/or replace dead or damaged cells with limited success.[1–3] To promote regeneration after traumatic CNS injury, our group has investigated cell delivery strategies after complete spinal cord injury utilizing tailored biomaterials and exogenous cells.[3,4] Previous studies have demonstrated that NSPC lineage specification can be guided by biochemical factors and neuronal differentiation can be induced by the presence of soluble cytokines, such as brain-derived neurotrophic factor (BDNF),[5–7] erythropoietin (EPO)[8,9] and IFN-γ.[10–12] The actions of pro-neuronal factors on stem cells in their soluble form are well documented, but exposure to pro-neuronal factors immobilized on a soft hydrogel biomaterial substrate has not yet been studied.

Adult stem cells are defined by their ability to self-renew through mitotic cell division and differentiate into a diverse range of specialized cell types. NSPCs are able to differentiate into astrocytes, oligodendrocytes and, neurons.[13–15] During developmental mammalian neurogenesis, the number of NSPCs declines rapidly and is further diluted by the production of restricted progenitors and terminally differentiated cells; however, a small population of uncommitted NSPCs remain in specialized niches of the adult brain.[16] Glia (astrocytes and oligodendrocytes) are often considered to be the default cell type into which brain subventricular zone-derived NSPCs differentiate.[17] Consequentially, previous research has found it difficult to attain high percentages of neurons differentiated from NSPCs after exposure to exogenous factors[18] or implantation.[1,19] Neurons are central to processing and transmitting electro-chemical signals throughout the body via the nervous system in a process known as synaptic transmission.[18] Neurons play a central role in the nervous system and their loss after injury or disease results in neurodegeneration.

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A number of growth factors that induce neuronal differentiation have been identified,[20,21] including, but not limited to, the secreted cytokines EPO, BDNF, and interferon-γ (IFN-γ). Generally, these factors activate receptor tyrosine kinases, leading to the recruitment of pro-neuronal signaling pathways. EPO is present in the adult CNS and is known to play an important part in hematopoietic, neural, and cardiac development.[22] EPO acts by binding to its membrane-bound receptor EpoR and enhances neurogenesis by activating intracellular signaling intermediates, including phosphoinositide 3-kinase (PI3K) and serine-threonine kinase Akt.[22] Previous work has demonstrated that soluble EPO promotes neuronal differentiation of NSPCs and neurite outgrowth.[8,9,23–26] BDNF is primarily localized in the brain and has been demonstrated to activate neurogenesis through both TrkB and p75 mediated pathways.[27] *In vitro* studies with mouse NSPCs have shown that BDNF preferentially promotes neuronal differentiation over other lineages.[5–7] IFN-γ ligates its membrane-bound receptor in a dimeric fashion, leading to the activation of the janus kinase-signal transducer and activator of transcription pathway followed by further downstream transcriptional modification.[11] IFN-γ has been used for a number of medical treatments because it has demonstrated antiviral, immunoregulatory and antitumor properties.[28] Treatment of NSPCs with IFN-γ has been shown to facilitate neuronal differentiation.[10–13]

The role of soluble factors in guiding stem cell functions, such as self-renewal and fate decisions, has been widely studied.[5–13,20,21] As a result, we have a nascent understanding of the ligands and signal transduction pathways that are used for self-renewal and differentiation. Studies have begun to reveal how covalent immobilization of growth factors/cytokines modifies cell and stem cell function.[29–36] Chemical immobilization of cytokines to biomaterial substrates allows for spatial control of differentiation with sustained dosing, which is not possible with soluble factors.[29,33] At the same time, cytokine immobilization allows for the study of the dynamics and the necessity of cellular internalization for activation of signal transduction.[36]

The main objectives of this study were to select a single growth factor for differentiating adult rat NSPCs into neurons, and then to compare the action of this growth factor when immobilized to a methacrylamide chitosan (MAC) substrate. Immobilized growth factors are advantageous for implantation strategies because sustained and local differentiation are promoted on the biomaterial scaffold. MAC is an attractive biomaterial because it can be designed to match the modulus of CNS tissue. Moreover, it is water soluble, photochemically crosslinkable, biocompatible, cell adhesive, and possesses free amine groups for facilitated chemical conjugation.[37] As MAC is degradable, which is important for tissue engineered scaffolds, it does not degrade in the timeframe of the *in vitro* studies described herein.[37] We hypothesized that immobilization of a previously identified soluble pro-neuronal growth factor to MAC scaffold surfaces would preferentially stimulate neuronal differentiation of NSPCs and that this differentiation would be similar to that obtained with the soluble growth factor.

MATERIALS AND METHODS

Preparation of MAC and formation of hydrogel scaffolds

Chitosan (Protosan UP B 80/20, NovaMatrix, Drammen, Norway) was modified with methacrylic anhydride (Sigma-Aldrich, St. Louis, MO) to yield MAC containing 23% methacryl groups, as previously described.[37] Freeze-dried MAC was dissolved in distilled and dionized H_2O as a 2 wt % solution and sterilized by autoclaving. For photo-crosslinking, the photo-initiator 2,2-dimethoxy-2-phenylacetophenone (DMAP, Sigma-Aldrich) was dissolved in 1-vinyl-2-pyrrolidone (NVP, Sigma-Aldrich) at a concentration of 300 mg/mL, sterilized by 0.2 μm filtration and mixed into MAC solution at 1.5 μL/mL. The mixture was thoroughly mixed and degassed (SpeedMixer DAC 150 FVZ, Hauschild Engineering, Hamm, Germany). Afterward, 50 μL of the MAC solution was loaded into chamberslide wells (0.4 cm^2/well area; Lab-Tek 178599, Nunc, Rochester, NY) so that a thin layer fully covered the bottom. The well plates were placed under UV light (λ = 365 nm) for 2 min to allow for UV-crosslinking. Mechanical testing of the resulting hydrogels (Mach-1 micromechanical testing system, BioSyntech, Laval, QC, Canada) found a Young's modulus (E_Y) of 0.8 ± 0.2 kPa, which is similar to native brain tissue.[38,39] After UV crosslinking, gels were thoroughly washed in PBS (pH 7.4) before cell culture or biomolecule immobilization.

Differentiation of NSPCs caused by soluble BDNF, EPO, and IFN-γ on glass and MAC hydrogels

The dose response of NSPCs to soluble recombinant human BDNF (Peprotech, Rocky Hill, NJ), recombinant rat EPO (R&D Systems, Minneapolis, MN) and recombinant rat IFN-γ (Peprotech) was conducted on laminin coated glass coverslips and MAC hydrogels. Laminin was included in all experimental groups to maximize cell adhesion and to better mimic the ECM found in the native brain where laminin is known to be a key component of the *in vivo* NSPC microenvironment.[40] Sterile glass coverslips (12 mm in diameter) were coated first with poly-D-lysine (50 μg/mL in sterile distilled H_2O, Sigma-Aldrich,) for 4 h, washed three times in sterile distilled H_2O, then coated with laminin (5 μg/mL in PBS, Invitrogen, Carlsbad, CA) overnight.

Before experimentation, NSPCs were isolated from the subventricular zone of the lateral ventricles of 6–8 wk old Wistar male rat brains and expanded in neurosphere culture as described previously.[4] Single NSPCs (passage 3–5) were obtained by dissociation, counted using trypan blue exclusion (Sigma-Aldrich) and seeded onto culture surfaces at a density of 40,000 cells/cm^2 in proliferation neurobasal media (NBM, Invitrogen) containing B27 neural supplement (Invitrogen), 2 m*M* L-glutamine (Sigma-Aldrich), 100 μg/mL penicillin-streptomycin (Sigma-Aldrich), 20 ng/mL epidermal growth factor (EGF-recombinant human, Invitrogen), 20 ng/mL basic fibroblast growth factor (bFGF-recombinant human, Invitrogen), and 2 μg/mL heparin (Sigma-Aldrich). Cells were cultured in a humidified incubator at 37°C and 5% CO_2. After an overnight incubation period, the proliferation medium was removed and replaced with media containing either a growth factor or 1% fetal bovine serum (FBS). The growth factor concentrations used in this study were selected based on previous work with BDNF,[5,41,42] EPO[8,25,43] and IFN-γ.[10,11,44,45] Initial screening was performed separately for each growth factor on laminin coated glass and compared with 1% FBS. Serum was used as a positive control because it offers favorable conditions for NSPC differentiation into neurons, astrocytes, and oligodendrocytes.[5,46] The following growth factors concentrations were studied: 10, 20, 50 ng/mL of BDNF; 40, 80, 120 ng/mL of EPO; and 10, 50, 100, and 150 ng/mL of IFN-γ. Four concentrations of IFN-γ (10, 50, 100, and 150 ng/mL) were also studied on MAC hydrogels. After the overnight attachment in proliferation medium, NSPCs were cultured for an additional 7 d in growth factor or 1% FBS with a half media change at day 4 of culture. On day 8, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for immunohistochemical (IHC) analysis.

Immunohistochemistry and fluorescent microscopy

After paraformaldehyde fixation, samples were washed three times with PBS and incubated in blocking/permeabilization solution (1.5% BSA and 0.2% Triton-X100, Sigma-Aldrich) for 1 h. Next, samples were incubated for 1 h with the following mouse primary antibodies: anti-β-III tubulin (1:1000, Abcam, Cambridge, MA) for neurons; anti-RIP (1:5, Developmental Studies Hybridoma Bank, Iowa City, IA) for oligodendrocytes; anti-glial fibrillary acidic protein (GFAP, 1:100, Cell Sciences, Canton, MA) for astrocytes; and anti-Ki-67 (1:100, Nova Castra Laboratories, Newcastle upon Tyne, UK) for proliferating cells. These differentiation markers have been confirmed with RT-PCR to be suitable markers for adult rat-derived NSPCs.[12] After three PBS washes, the samples were incubated in the secondary antibody Alexa Fluor 546 goat antimouse IgG (1:400, Invitrogen) at room temperature (RT) for 1 h, followed by three additional washes in PBS. Cell nuclei were counterstained with Hoechst 33342 (Invitrogen) for 10 min, washed three times in PBS, then mounted and coverslipped using ProLong Gold antifade reagent (Invitrogen). Samples were imaged using a fluorescent microscope (DP70, Olympus, Tokyo, Japan). For each scaffold, images of six different regions were obtained at 20X magnification. The total cell number was determined by counting intact Hoechst 33342 stained cell nuclei in comparison to the number of cells staining positive for the markers of interest.

Immobilization of IFN-γ on MAC hydrogel surfaces

1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Sigma-Aldrich) *N*-hydroxysulfosuccinimide (sulfo-NHS, Pierce Biotechnology, Rockford, IL) chemistry[47] was used to immobilize IFN-γ carboxylic acids to MAC surface amine groups (Fig. 1). Specifically, the carboxylic groups of IFN-γ were activated with sterile filtered EDC (5 m*M*) and sulfo-NHS (5 m*M*) in PBS (pH 7.4) with 100, 500, 1000, and 1500 ng/mL IFN-γ for 15 min. Activated IFN-γ solution was loaded into each well with cross-linked MAC so that the following initial surface concentrations were achieved: 25, 125, 250, and 375 ng/cm^2. After 1 h of reaction at RT, the solution was removed from each well and the hydrogel was washed with PBS for 2 d with PBS changed twice a day at 4°C, before cell culture or quantification.

Quantification of immobilized proteins

The quantification of IFN-γ immobilized onto MAC surfaces was performed after overnight lysozyme (2 mg/mL)

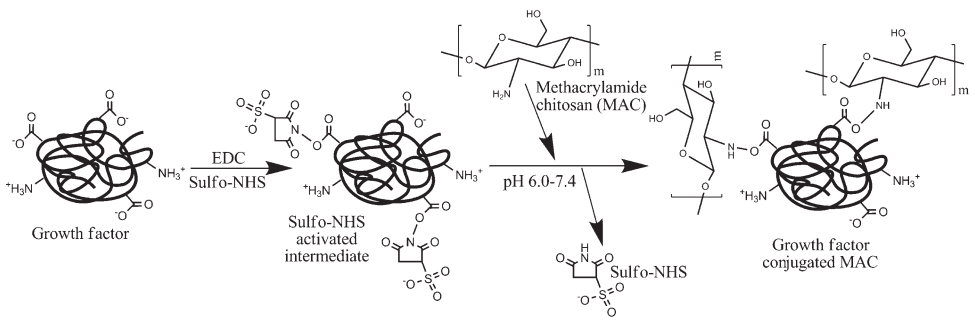

Figure 1. Use of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC)/*N*-hydroxysulfosuccinimide (sulfo-NHS) chemistry to form growth factor conjugated MAC. The sulfo-NHS ester intermediate survives longer than the active intermediate formed by EDC and carboxyl groups. This linkage can be carried out in a single reaction and results in a high yield of amide bond formation.

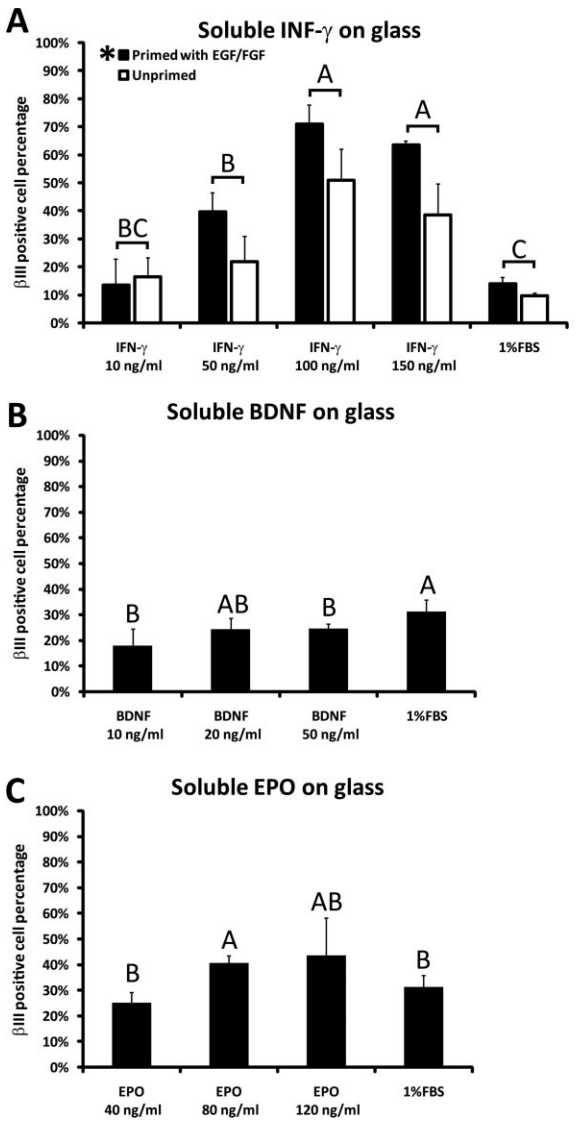

Figure 2. βIII tubulin (neuron)-positive cell percentages on laminin coated glass after treatment with IFN-γ, BDNF, EPO, and 1% FBS (positive control). (A) Two-factor ANOVA with *post-hoc* analysis (groups not sharing the same letter are significantly different) revealed that IFN-γ treatment resulted in a dose-dependent increase in βIII-positive cells compared with 1% FBS ($p < 0.0001$) and EGF/FGF priming (1 d followed by 7 d in differentiation medium) yielded significantly more neurons than not priming (8 d in differentiation medium, $^*p < 0.0001$). (B) BDNF and (C) EPO did not result in more βIII-positive cells when compared with 1% FBS ($p > 0.05$). (Data are presented as mean ± SD, $n = 3$.)

digestion of chitosan at 37°C. Enzyme-linked immunosorbent assay (ELISA) was utilized to quantify the amount of rat IFN-γ (eBioscience, cat# 88-7315-22, San Diego, CA) in each digest following standard ELISA protocols. Two negative control groups were included: MAC hydrogels with physically adsorbed IFN-γ (375 ng/cm^2 for 1 h) and MAC hydrogels with no IFN-γ. ELISA standard curves were prepared using lysozyme digested MAC as the diluting solvent.

Cell culture on growth factor immobilized MAC

MAC hydrogels were formed in chamberslide wells and IFN-γ was immobilized at final (after washing) surface concentrations of 12.5, 25, and 37.5 ng/cm^2 (equivalent to 50, 100, and 150 ng/mL). Immobilized samples were compared with two controls: (1) 6.8 ng/cm^2 adsorbed and (2) 150 ng/mL soluble IFN-γ. Samples were cultured as described earlier.

Statistical analysis

Statistics were performed using JMP IN 7 (SAS Institute, Cary, NC). ANOVA with Tukey's *post hoc* analysis was performed to detect significant differences between groups. An alpha level of 0.05 was used to determine significance between groups. Data are reported as mean ± standard deviation (SD).

RESULTS

NSPC dose response to soluble BDNF, EPO, and IFN-γ on glass

Initial dose response tests with soluble BDNF, EPO, and IFN-γ were used to compare their efficacy in inducing NSPC neuronal differentiation and to determine saturation concentrations. An overnight incubation in EGF/FGF proliferation medium (priming) was applied in all experiments based on a preliminary study comparing primed versus unprimed NSPC differentiation to neurons [Fig. 2(A)]. Two-factor ANOVA revealed that EGF/FGF priming induced higher neuronal differentiation of NSPCs as determined by number of cells staining positive for βIII tubulin ($p < 0.0001$). As a result, all remaining experimentation involved overnight incubation in EGF/FGF proliferation medium followed by 7 d in differentiation medium.

The comparison of neuronal differentiation induced by soluble BDNF, EPO, and IFN-γ on glass is presented in Figure 2. Soluble IFN-γ induced significantly more neuronal differentiation than 1% FBS at 50, 100, and 150 ng/mL [$p < 0.0001$, Fig. 2(A)]. The highest number of βIII tubulin-positive cells was seen after treatment with 100 ng/mL (71 ± 7% positive). Figure 2(A) shows a dosage response to IFN-γ in the range of 10–100 ng/mL with saturation occurring by 150 ng/mL. Neither BDNF (25 ± 2% βIII tubulin-positive at 50 ng/mL) nor EPO (44 ± 15% βIII tubulin-positive at 120 ng/mL) treatment resulted in significantly higher neuron differentiation than the 1% FBS control group [$p > 0.05$, 31 ± 4% positive, Fig. 2(B,C)].

Images of βIII tubulin staining (Fig. 3) of NSPCs differentiated with IFN-γ, BDNF, EPO, and 1% FBS controls show distinct morphological differences

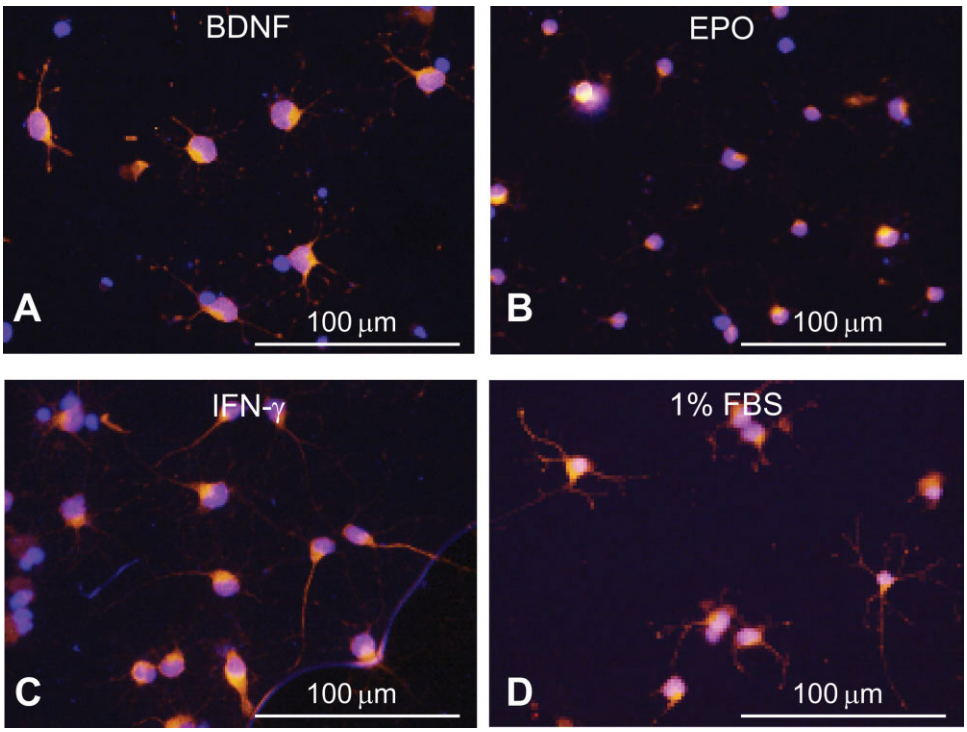

Figure 3. Morphologic variation of βIII tubulin-positive cells. A–D: are representative IHC images of cells cultured in the presence of 50 ng/mL BDNF, 120 ng/mL EPO, 100 ng/mL IFN-γ, and 1% FBS after 8 d. Red fluorescence marks βIII tubulin (neurons and neurites) and blue fluorescence indicates Hoechst 33342 stained nuclei. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

between the treatments. BDNF treated cells [Fig. 3(A)] have short processes whereas EPO treated cells [Fig. 3(B)] show almost no neurite outgrowth. IFN-γ [Fig. 3(C)] shows the most neurite outgrowth, even greater than that of 1% FBS controls [Fig. 3(D)]

These results demonstrate that IFN-γ induced the most neuronal differentiation and neurite outgrowth compared with BDNF and EPO. On the basis of these positive findings, the neuronal differentiation capacity of NSPCs grown on MAC hydrogels in the presence of either soluble or immobilized IFN-γ was further investigated.

Dose response to soluble IFN-γ on MAC

The dose response of NSPCs cultured on MAC hydrogels to soluble IFN-γ (10–150 ng/mL) was conducted to determine the resulting neuronal differentiation. The percentage of βIII tubulin-positive cells on MAC hydrogels are given in Figure 4. The trends on MAC were similar to those observed on glass [Fig. 2(A)] with a dose response observed from 10 to 100 ng/mL and saturation at 150 ng/mL. About 100 ng/mL of soluble IFN-γ yielded the most neurons (54 ± 6% positive) on MAC hydrogels and was significantly higher than 1% FBS treatment ($p < 0.0001$, 6 ± 2% positive); however, fewer βIII tubulin-positive cells were observed on MAC compared with glass surfaces for both IFN-γ and FBS treatment groups.

Immobilization and quantification of surface immobilized IFN-γ

IFN-γ was immobilized to MAC hydrogels using EDC/sulfo-NHS chemistry. ELISA for IFN-γ (Fig. 5) showed that immobilization to MAC was successful and that the concentration of immobilized protein increased nearly linearly with the initial amount of

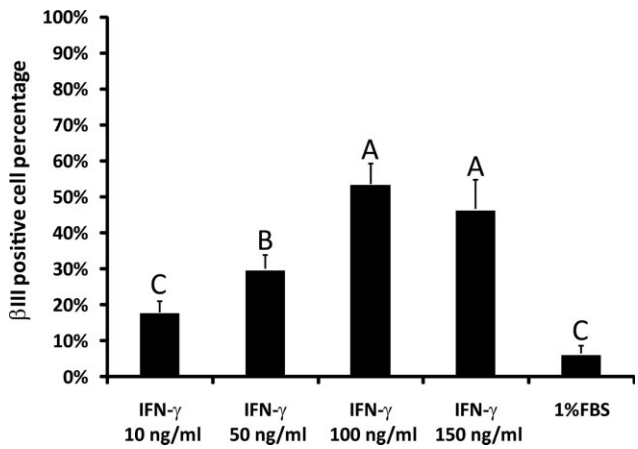

Figure 4. βIII tubulin (neuron)-positive cell percentages after soluble IFN-γ treatment on MAC hydrogel surfaces and 8 d in culture. Results on MAC are similar to glass [Fig. 2(C)] and show a dose dependent increase in the number of βIII tubulin-positive cells. Different letters indicate significant differences by one-factor ANOVA ($p < 0.0001$). (Data are presented as mean ± SD, $n = 4$.)

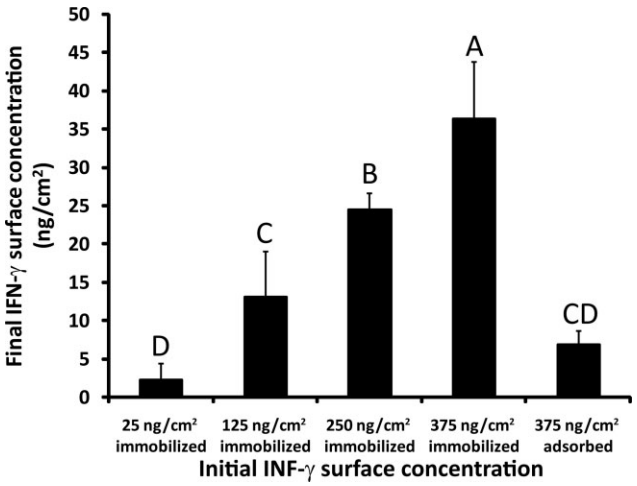

Figure 5. Concentrations of IFN-γ immobilized to MAC as measured by ELISA. Immobilized groups used 25, 125, 250, and 375 ng/cm^2 of IFN-γ as the initial concentration for the EDC/sulfo-NHS reaction, whereas the adsorbed group used 375 ng/cm^2 IFN-γ without EDC/sulfo-NHS. The amount of IFN-γ immobilized was linearly proportional to the starting concentration. Different letters indicate significance by one-way ANOVA ($p < 0.05$). (Data are presented as mean ± SD, $n = 6$.)

soluble IFN-γ. ELISA performed on the adsorption control (375 ng/cm^2 IFN-γ for 1 h) showed that 1.8 ± 0.5% of IFN-γ from the solution adsorbed to the MAC surface. It was determined that the immobilization efficiency averaged 8.0 ± 0.6% for all treatments after subtracting away adsorbed protein. This immobilization efficiency was taken into account when preparing IFN-γ immobilized surfaces for NSPC culture.

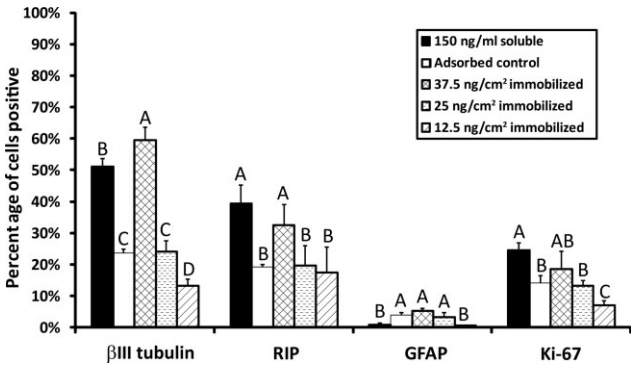

Figure 6. Percentages of cells staining positive for βIII tubulin, RIP, GFAP, and Ki-67 on IFN-γ immobilized and control MAC surfaces after 8 d in culture. Statistics were performed separately for each marker using one-way ANOVA with Tukey's *post-hoc* analysis, letters indicate significance ($p < 0.05$). Neurons primarily resulted from the highest soluble and immobilized concentrations of IFN-γ ($p < 0.0001$). Similar trends with less total positive cells were seen for RIP (oligodendrocytes, $p < 0.01$) and Ki-67 (proliferating cells, $p < 0.05$). As there were significant differences between the groups ($p < 0.01$), very little astrocyte differentiation was observed. (Data are presented as mean ± SD, $n = 3$.)

Differentiation of NSPCs on IFN-γ immobilized MAC surfaces

NSPCs primarily differentiated into neurons on IFN-γ immobilized MAC surfaces (Figs. 6 and 7). The percentage of βIII tubulin-positive cells increased as the surface concentration of immobilized IFN-γ increased from 12.5 to 37.5 ng/cm^2, demonstrating a dose-dependent response of NSPC neuronal differentiation to immobilized IFN-γ.

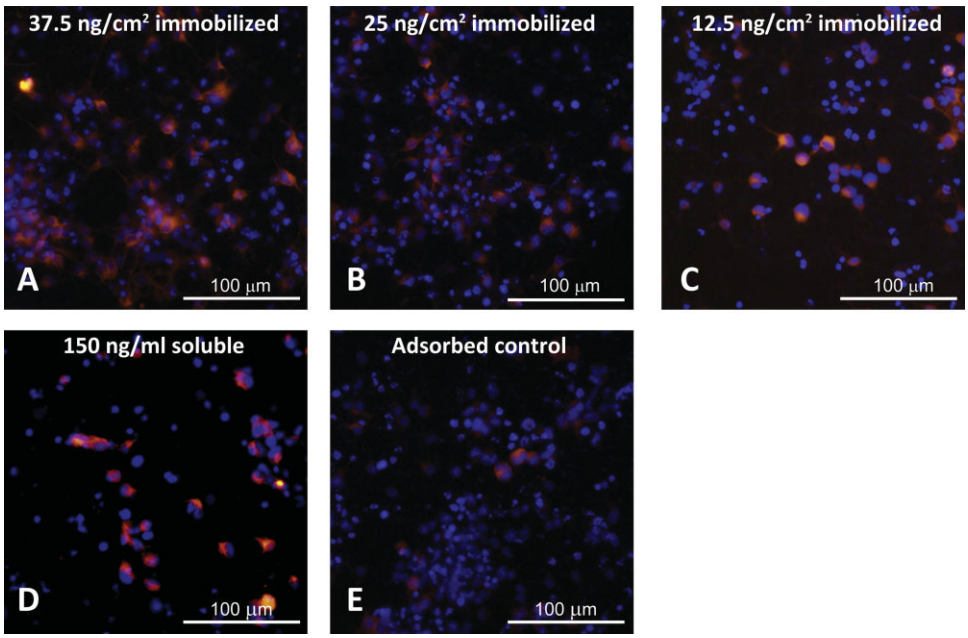

Figure 7. Images of βIII IHC from IFN-γ immobilized (A–C), soluble IFN-γ treated (D) and physically adsorbed IFN-γ MAC scaffolds (E) after 8 d in culture. βIII tubulin (neurons) appears in red whereas the nucleus (Hoechst 33342) appears in blue. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

About 60 ± 4% of NSPCs differentiated into neurons on 37.5 ng/cm^2 IFN-γ immobilized MAC. The number of neurons was significantly greater ($p < 0.0001$) than all other immobilized groups including the equivalent soluble 150 ng/mL IFN-γ (with 51 ± 2% βIII tubulin-positive) and adsorbed IFN-γ controls where 24 ± 1% of NSPCs differentiated into neurons. Both RIP staining (for oligodendrocytes) and Ki-67 staining (for proliferation) showed significantly higher positive expression for the 150 ng/mL soluble and 37.5 ng/cm^2 immobilized treatments when compared with the other groups ($p < 0.01$ and $p < 0.05$, respectively). GFAP-positive staining showed that on average less than 3% of the cells were positive for the astrocyte marker. As there were statistical differences between the immobilized and soluble groups ($p < 0.01$), the total percentage of astroctyes was less than 5% for all groups. Figure 7 presents images of βIII tubulin staining for all treatment groups.

DISCUSSION

Soluble growth factors have been shown to influence neuronal lineage specification of NSPCs; however, the action of immobilized factors on the pro-neuronal differentiation of adult NSPCs has not yet been studied. Immobilization offers a number of advantages over soluble dosing such as maintenance of constant local growth factor concentrations, constant differentiation stimulus, and requiring smaller total amounts of growth factor over the course of the experiment.

Before the immobilization of a single pro-neuronal factor could be performed, a bioactive ligand that induced neuronal differentiation of NSPCs through membrane bound receptors first needed to be identified. Preliminary dose response tests to BDNF, EPO, and IFN-γ were completed and compared with the 1% FBS control, because FBS treatment has been previously shown to induce neuronal differentiation and maintain cell survival.[5,46] The results of these studies (Fig. 2) identified IFN-γ as the only factor that yielded significantly greater neuronal differentiation than 1% FBS controls. BDNF and EPO did not show high neuronal expression as has been previously shown in literature.[5–9] however, these previous studies were completed with mouse-derived NSPCs that exhibit some differences in lineage commitment when compared with rat-derived NSPCs.[4,34,48,49]

The observed morphologic differences (Fig. 3) were especially interesting for the EPO treated groups. Treatment with EPO resulted in cells with very few and extremely short cellular extensions when compared with the other two factors and 1% FBS. This finding is in contrast to a previous study where NSPCs differentiated into neurons with significant neurite outgrowth using recombinant human EPO.[26] However, these studies used passage 1 cells cultured for 10 d, whereas, we used passage 3 cells cultured for 7 d in the presence of recombinant rat EPO. In our studies, EPO at 120 ng/mL did produce higher percentages of βIII-positive cells than BDNF at 50 ng/mL (43 ± 15% vs. 25 ± 2%, Fig. 2), but were not significantly greater than 1% FBS controls (31 ± 4%, $p > 0.05$). It is possible that longer culture periods or combinatorial treatments with other factors would encourage neurite outgrowth.[43] Of the three soluble factors studied, IFN-γ treatment yielded the greatest neuronal differentiation and neurite outgrowth and was selected for immobilization to MAC.

Experimentation with soluble IFN-γ on MAC hydrogels showed similar neuronal differentiation trends and dose responses compared with glass [Figs. 2(A) and 4], indicating that MAC did not impact lineage commitment of IFN-γ. These studies also suggest that a dosing saturation point for IFN-γ begins at ~100 ng/mL at which both surfaces exhibited the highest number of βIII-positive cells. Interestingly, fewer βIII tubulin-positive cells were observed on MAC surfaces when compared with glass [Figs. 2(A) and 4]. This small reduction is likely caused by proliferation and colony formation on MAC (Fig. 7), which may delay neuronal differentiation.

EDC/sulfo-NHS allowed activated IFN-γ carboxyl groups to be covalently bound to reactive amines on MAC in a one step reaction. The sequence of Rattus Norvegicus IFN-γ (NCBI accession number NP620235) shows that each IFN-γ protein (of 134 amino acids) contains five aspartic acids and nine glutamic acid residues. EDC/sulfo-NHS coupling chemistry resulted in a yield of 8.0 ± 0.6% which is similar to what we have previously reported with vascular endothelial growth factor immobilized onto collagen,[35] and is approximately eight times greater than what others have achieved with leukemia inhibitory factor immobilized on several polymer surfaces.[33]

Covalently immobilized IFN-γ stimulated neuronal differentiation, confirming that the IFN-γ receptor is membrane bound[11] and demonstrating that activation does not require protein-receptor complex internalization. IFN-γ immobilized MAC maintained its bioactivity, as demonstrated by the differentiation profile of NSPCs (Fig. 6); however, some activity was likely lost as the number of βIII tubulin-positive cells on adsorbed controls (6.8 ± 1.7 ng/cm^2 by ELISA) was similar to that with 25 ng/cm^2 immobilized IFN-γ and better than 12.5 ng/cm^2 immobilized. Notwithstanding some loss in bioactivity after immobilization, Figures 6 and 7 show that immobilized IFN-γ induced a dose response in terms of

neuronal expression of NSPCs. The 37.5 ng/cm^2 (~150 ng/mL) immobilized group yielded significantly more neurons than the 150 ng/mL soluble treatment group ($p < 0.0001$, 60 ± 4% vs. 51 ± 2%), demonstrating the merits of immobilization, because the total amount of IFN-γ in each treatment is approximately equal and the modulus of the MAC hydrogel, on which the cells were cultured, was constant. The results of RIP-positive quantification (Fig. 6), demonstrated that the highest concentrations of IFN-γ yielded more oligodendrocytes, yet, fewer NSPCs differentiated into oligodendrocytes than neurons for most treatments. This is a significant result because adult rat subventricular zone-derived NSPCs preferentially differentiate into oligodendrocytes,[4,34] thereby demonstrating IFN-γ's ability to override this preference.

To our knowledge, this is the first time a factor has been immobilized to a soft hydrogel, shown to retain its bioactivity and influence adult NSPC differentiation to neurons. The immobilization of IFN-γ may also be advantageous for hematopoetic progenitor cell development, activation of antigen-presenting cells (dendritic cells, macrophages, and B lymphocytes) and T cell differentiation[28] where immobilization could be utilized to define the cellular microenvironment for controlled spatial differentiation/activation with sustained dosing.

CONCLUSIONS

We have shown that IFN-γ can preferentially induce neuronal expression from NSPCs and that EDC/sulfo-NHS immobilization of IFN-γ to a MAC hydrogel surface provides a preferred environment for the differentiation of NSPCs toward neurons. This work begins to refine our understanding of the conditions that neural stem cells prefer for neuronal specification and provides an opportunity to define therapeutic strategies for treatment of diseases and disorders of the nervous system.

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