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SCIENCE DIRECT
Biomaterials 27 (2006) 3432–3440
Biomaterials
www.elsevier.com/locate/biomaterials

Combination of engineered neural cell adhesion molecules and GDF-5 for improved neurite extension in nerve guide concepts

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Received 11 October 2005; accepted 24 January 2006
Available online 23 February 2006

Abstract

Current therapeutical approaches for the treatment of severe lesions in the peripheral nervous system rely on the use of autologous tissue or the body's own Schwann cells. However, these approaches are limited and alternative strategies for peripheral nerve regeneration are required. Here we evaluate combinations of a variety of neuronal regeneration factors including engineered cell adhesion molecules and growth factors in embryonic model neurons to test the possible improvement of artificial nerve guides by cooperative mechanisms. Cell adhesion molecules L1 and neurofascin synergistically promote neurite elongation. The outgrowth promoting properties of both proteins can be combined and further increased within one chimeric protein. Addition of growth and differentiation factor 5 (GDF-5) further enhances neurite outgrowth in a substrate-independent manner. This effect is not due to a protective mode of action of GDF-5 against pro-apoptotic stimuli. Consequently, the study supports the idea that different modes of action of pro-regenerative factors may contribute synergistically to neurite outgrowth and emphasizes the applicability of combinations of proteins specifically involved in development of the nervous system for therapeutical approaches.
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Keywords: Cell adhesion; Nerve guide; Nerve tissue engineering; Recombinant protein

1. Introduction

In contrast to the situation in the central nervous system, neurons of the peripheral nervous system have an intrinsic potential to regenerate. Axons can regenerate across smaller lesions after suturing the ends of the proximal and the distal stump [1]. However, after severe lesion of the peripheral nervous system other therapeutical approaches to promote regeneration are required [1]. A well-established method to bridge larger gaps is the use of nerve conduits composed of autologous living transplants preferentially isolated from sensory nerves like the *Nervus suralis* [2]. But suchlike autologous transplants require a second lesion to obtain the required material and the availability is limited [1,3].

Therefore, strategies are to be developed to replace autologous transplants by implantable nerve guides. Cell adhesion molecules (CAMs) and/or neurotrophic factors embedded in biocompatible scaffolds are currently discussed as promising concepts [4–6]. CAMs of the immunoglobulin superfamily (IgSF) including L1/NgCAM, neurofascin, axonin-1/TAG1 and F11/contactin are interesting candidates. These proteins are expressed in the course of the development of the nervous system and are found on neurons or Schwann cells [7–10]. During embryogenesis they serve as guidance cues for outgrowing neurons, mediate neuronal differentiation, and promote neuronal survival. Their ectodomains, which can readily be expressed as secreted IgFc-fusion proteins in eukaryotic cells, act as permissive substrates for neurite outgrowth [11]. Fc-fusion protein of L1 has already been evaluated in vitro [12,13]. In an animal model, L1-Fc enhanced axonal regeneration and myelination of transected optic nerve [4].

Amongst soluble recombinant factors, nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), fibroblast growth factor 1 (FGF-1), ciliary neurotrophic factor (CNTF) and growth and differentiation factor 5

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0142-9612/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2006.01.037

(GDF-5) have already been successfully applied for regeneration in vivo [14–16]. Within the superfamily of transforming growth factor β (TGF-β)-like molecules, GDF-5 is a member of the subfamily of bone morphogenetic proteins (BMP) [17,18]. GDF-5 was characterized in several reports as neurotrophic factor, which promotes survival of dopaminergic neurons and astroglial cells of the central as well as dorsal root ganglia (DRG) cells of the peripheral nerve system [19–21]. Furthermore, administration of GDF-5 was shown to rescue dopaminergic neurons in a rat lesion model for Parkinson's disease [22].

Although recombinant factors have yet been successfully employed for peripheral nerve regeneration, several problems are still prevailing, which require conceptual improvements. For example, the simulation of a complex interaction of factors inducing neurite outgrowth as it is found in a natural environment might be an important step towards the replacement of grafted nerve tissue. To establish such a strategy, optimal mixtures of different recombinant neural factors need to be evaluated.

In this study we have determined the efficiency of combinations of neural CAMs along with GDF-5 to promote neurite outgrowth using NGF-dependent neurons of embryonal chick DRG as a model system. Adding soluble GDF-5 to neurons grown on immobilized CAMs as substrate enhances neurite elongation in a substrate-independent manner. This outgrowth-promoting effect is not due to a protective function of GDF-5 in response to apoptotic stimuli. A synergistic effect on neurite outgrowth is observed on a substrate mixture of L1 and neurofascin, whose outgrowth-promoting capabilities can be combined within one molecule and further enhanced by addition of GDF-5.

2. Material and methods

Vector construction: The generation of a vector encoding soluble neurofascin (isoform NF15) fused to IgFc is described elsewhere [11]. For expression of soluble axonin, L1 and chimeric L1/neurofascin proteins as IgFc-fusion proteins the cDNA sequences encoding the ectodomains of the proteins (Fig. 1A) were amplified and cloned into pIG2 vector.

Production and purification of recombinant proteins: Stably transfected, CAM expressing monoclonal HEK293 cell lines (293-axonin-1-Fc; 293-L1-Fc; 293-NF15-Fc) were generated for the production of Fc-fusion proteins in miniaturized bioreactors (CELLine CL 350, Integra Biosciences). Expression of the chimeric L1/neurofascin fusion molecules was done with the help of the FreeStyle™ 293 Expression System (Invitrogen). Proteins were purified as described previously [11].

Isolation of DRG neurons: DRG were isolated from embryonic chick (day 9) and collected in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) following incubation in 0.08% trypsin/PBS for 15 min. Cells were dissociated and washed once with HBSS and afterwards resuspended in culture medium (DMEM containing $1 \times$ N2 supplements [Invitrogen], 2 mM glutamine [PAA Laboratories], 100 U/ml penicillin and 100 μg/ml streptomycin [PAA Laboratories]). Neurite outgrowth in the presence of NGF (25 ng/ml) was quantified as described previously [11].

Cell viability assays: Dissociated DRG neurons were seeded on polyethyleneimine (PEI)-coated coverslips or 96 well plates in NGF (25 ng/ml, Roche Biochemicals) supplemented culture medium for 24 h before treatment with 400 nM staurosporine. For quantification of intact nuclei, neurons were stained with Hoechst 33342 (1 μg/ml) for microscopical evaluation of intact nuclei. DNA fragmentation after 8 h of staurosporine treatment was analysed by TUNEL-assay using In situ Cell Death Detection Kit, TMR red (Roche Biochemicals). Determination of cell viability was done after addition of alamar Blue™ (BioSource International). For quantification of caspase-3 activity in staurosporine-treated (5 h) DRG neurons, cells were permeabilized in 50 μl lysis buffer (20 mM Hepes pH 7.4; 84 mM KCl; 10 mM MgCl2; 0.2 mM EDTA; 0.2 mM EGTA; 0.5% Triton X-100; 100 M DTT; protease inhibitor cocktail [Roche Biochemicals]) following addition of 50 μM caspase substrate Ac-DEVD-amc (Biomol) dissolved in 150 μl reaction buffer (50 mM Hepes pH 7.4; 2 mM EDTA; 0.2 mM EGTA; 10% sucrose; 0.1% Triton X-100; 3 mM DTT). Increase in fluorescence intensity at 460 nm was measured over a period of 2 h using a microplate reader.

3. Results

3.1. Induction of neurite elongation by individual substrate CAMs

If applied as a neuronal substrate, CAMs including L1, neurofascin, axonin-1 and F11 were shown to promote neurite extension. While the extracellular domains of individual CAMs have already been tested, a systematic comparison of different CAMs for induction of neurite outgrowth remained elusive. We therefore examined the potency of a panel of CAMs to stimulate neurite outgrowth in vitro.

CAMs were expressed as recombinant IgFc-fusion molecules. Beyond correct protein folding, eukaryotic expression ensures the formation of the highly complex glycosylation pattern of the extracellular domains of neural CAMs. Proteins isolated from cell culture supernatants of stably transfected HEK293 cells were submitted to SDS–PAGE. Silver staining of the gels confirmed a high degree of purity and the correct molecular weight of the CAMs (Fig. 1B, lanes 1–3). In Western blot analyses the proteins were recognized by antibodies to the Fc fragment of human IgG1 and by CAM-specific antibodies, respectively (Fig. 1C, lanes 1, 2, 5, 8, and 9).

In a first set of experiments, CAMs L1, neurofascin, axonin-1, and F11 were supplied as immobilized substrates for outgrowing sensory neurons and compared with neurite outgrowth induced by laminin (Fig. 1D). With the exception of F11 (data not shown) all substrates induced neurite outgrowth. Inspection of neurite elongation induced by the CAMs revealed comparable outgrowth promoting capabilities of L1 and neurofascin (median neurite lengths: 123.1 and 136.2 μm, respectively) when coated at a concentration of 25 μg/ml, while of axonin-1-induced neurite outgrowth to a lesser extent even at higher concentrations of this protein (median neurite length: 95.7 μm after coating of 50 μg/ml). Therefore, out of the neural CAMs tested so far, L1 and neurofascin were the most potent promoters of neurite elongation.

3.2. Neurite induction by combinations of different CAMs

Neurite outgrowth presumably relies on the parallel action of many different environmental factors encountered

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