Expression of interleukin-6 and its receptor in the sciatic nerve and cultured Schwann cells: relation to 18-kD fibroblast growth factor-2

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Abstract

Expression of interleukin-6 (IL-6) and fibroblast growth factor-2 (FGF-2) in Schwann cells is modulated by external stimuli. To study possible interactions of both factors we have analyzed mutual effects of exogenous IL-6 and FGF-2 on the expression of each other and the corresponding receptor (R) molecules IL-6R and FGFR1 after peripheral nerve lesion in vivo and in vitro using cultured Schwann cells. Using rat Schwann cells we found that IL-6 did not exert any effects on the expression of FGF-2 and FGFR1 whereas exogenously applied 18-kD FGF-2 strongly increased the expression of the mRNAs of IL-6 and its receptor. In addition, immortalized Schwann cells over-expressing the 18-kD FGF-2 isoform showed elevated levels of IL-6 and IL-6R whereas immortalized Schwann cells over-expressing the high-molecular-weight isoforms (21 kD and 23 kD) displayed unaltered IL-6 and IL-6R expression levels. According to in situ hybridization studies of intact and crushed sciatic nerves in vivo, Schwann cells seems to be the main source of IL-6 and IL-6R. Following sciatic nerve crush, the FGF-2 and the IL-6 system are upregulated after the first hours. Furthermore, we showed that the early increase of the FGF-2 protein is mainly confined to the 18-kD isoform. These results are consistent with the idea of a functional coupling of FGF-2 and the IL-6 system in the early reaction of Schwann cells to nerve injury.

Keywords: IL-6; Schwann cell; Sciatic nerve; FGF-2; Rat

1. Introduction

The expression of interleukin (IL)-6 and fibroblast growth factor (FGF)-2 in cultured Schwann cells and Schwann cell lines is modulated by external stimuli [2,19]. Since both molecules are upregulated after peripheral nerve injury [2,19,27], the question arises whether the expression of both molecules is causally linked to each other. IL-6 belongs to a family of neuropoietic cytokines which also includes ciliary neurotrophic factor (CNTF) leukemia inhibitory factor (LIF), oncostatin M, cardiotrophin-1, growth promoting activity, and IL-11 [23,25]. The fact that the IL-6 and IL-6 receptor mRNAs are developmentally regulated in the brain is evidence for a physiological role of IL-6 in the nervous system [12,13]. Central nervous system-derived IL-6 might be involved in neuronal survival, differentiation, and transmitter metabolism [15]. Sympathetic and sensory ganglia also show a development-dependent expression [14]. IL-6 appears to mediate effects on survival and neuropeptide expression of sensory and sympathetic neurons [15]. Other cytokines, such as IL-1β and tumor necrosis factor (TNF)-α are known to modulate IL-6 expression in fibroblasts and astrocytes [15,43]. However, nothing is known about the signals triggering the up-regulation of IL-6 in the injured nerve.

FGF-2 is a member of the FGF family which comprises heparin-binding proteins that promote mitogenesis of mesoderm- and neuroectoderm-derived cells [4]. FGF-2 expression has been reported in distinct areas of the developing and mature nervous system [1,21,41]. In vitro studies have revealed stimulating effects of FGF-2 on

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proliferation of astrocytes, oligodendrocytes, and Schwann cells [7,8,37] and on survival, neurite outgrowth, transmitter metabolism, and synapse formation of various neuron populations [18,42]. With regard to the functional role of FGF-2 after peripheral nerve injury, evidence from in vivo studies suggests that the molecule could mediate neurotrophic effects on axotomized motor and sensory neurons. This notion is supported by the finding that nerve lesion results in an up-regulation of FGF-2 expression in motoneurons, dorsal root ganglia, and sympathetic ganglia [19,24,26] and that exogenously applied FGF-2 can prevent the lesion-induced neuron death [17,36]. At the lesion site itself FGF-2 might be involved in the myelination process [30]. The molecular signals which regulate FGF-2 expression after lesion, as well as the events that are triggered by the increased expression of FGF-2 are not known.

In the present study we have investigated mutual effects of IL-6 and FGF-2 on the expression of the respective endogenous molecules in immortalized Schwann cells which were used as a model system. In immortalized Schwann cells and immortalized Schwann cells over-expressing the 18-kD FGF-2 or the high-molecular-weight FGF-2 isoforms, 18-kD FGF-2 stimulated the expression of the IL-6 and IL-6R mRNAs. Furthermore, physiological (secondary) Schwann cells of the newborn rat also displayed an upregulation of IL-6 and IL-6R mRNAs in response to FGF-2. In addition, in situ hybridization of the intact and injured sciatic nerve revealed Schwann cells as the main source of IL-6 and IL-6R. Finally, FGF-2 and the IL-6 system are upregulated within the first hours after sciatic nerve injury. These data may reflect a functional coupling of Schwann cell-derived FGF-2 and IL-6 system in the injured peripheral nervous system.

2. Materials and methods

2.1. Schwann cell cultures

Immortalized Schwann cells (15–20 passages; [40]) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 10% horse serum. At a density of 25 000 cells/cm² medium was changed and cells received serum-containing medium or serum-free medium supplemented with N1 additives [3] and 0.25% bovine serum albumin overnight. Physiological Schwann cells were produced from spinal nerves of 1-day-old rats essentially as described by Brockes et al. [5]. After plating on poly-L-lysine (0.5 mg/ml) cells were expanded in DMEM supplemented with 10% FCS containing forskolin (2 μM) and stored in masterstocks. After removal of forskolin cells stopped proliferating and returned to their spindle-shaped phenotype, expressing S100- and p75 nerve growth factor receptor-immunoreactivity. Such cells were used for the experiments. Schwann cells were treated with IL-6 (100 units/ml; 10 ng/ml) for 1, 2, 6 and 24 h or with FGF-2 (50 ng/ml) for 5, 10 and 24 h. Recombinant IL-6 and recombinant 18-kD FGF-2 were purchased from Preprotec. Controls were cultured in the absence of cytokines in the respective medium for 24 h. In a second series of experiments, transfected clonal Schwann cells over-expressing the 18-kD or 21/23-kD FGF-2 isoforms [20] were analyzed for IL-6 and IL-6 receptor expression and compared with Schwann cells transfected with a control vector, non-transfected Schwann cells, and non-transfected Schwann cells treated with 18-kD FGF-2. Dislodging of cells for RNA or protein isolation was performed by trypsinization (0.125% trypsin/20 mM EDTA). Pellets were stored at −80°C.

2.2. Sciatic nerve crush

Hanover Wistar rats (Charles River Wiga, Sulzfeld, Germany) were anaesthetized using sodium pentobarbital (50 mg/kg, i.p.). After exposing the left sciatic nerve, the crush was performed with a fine forceps at the mid-thigh level. For quantitative studies, 5 h and 2 days after the operation, rats were killed by decapitation and 8-mm nerve segments proximal and distal to the crush site were dissected; identical segments of the contralateral nerve were isolated in the same way. For each time point three animals were used.

Experimental protocols were approved by Regierungspräsidium Freiburg (AZ 37/9185.813/784) and Bezirksregierung Hannover (AZ 509i-42502-98/47) and meet the guidelines of the Tierschutzgesetz (i.d.F.v. 17.02.93).

2.3. RNA preparation

For total cellular RNA isolation frozen tissue samples were thoroughly homogenized in lysis buffer and RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction [6] and quantified spectrophotometrically by absorbance at 260 nm. The quality of RNA was checked by formaldehyde agarose gel electrophoresis.

2.4. IL-6 and IL-6 receptor mRNA determination

Transcripts of IL-1 and IL-6 receptor were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was reverse-transcribed into cDNA (Superscript™ II, Gibco-BRL) and then subjected to PCR amplification using primers specific for IL-6 (sense: 5’-GACTGATGTGTGACGCCACTG-3’; antisense: 5’-TAGCCACTCTCTGTGACTCTAA-3’) and IL-6 receptor (sense: 5’-TGGTGCAGCCGGATCCACCTGCCA-3’; anti: 5’-CTGGGGCGAGGACACTCGTTGCTT-3’).
The number of cycles used to amplify each cDNA was chosen to allow the PCR to proceed in a linear range according to the Elongase™ enzyme mix-protocol (Gibco-BRL). PCR amplification of the constitutively expressed ribosomal protein S12 cDNA was used as a measure of input RNA (S12: sense: 5'-GGAAGGCATTGCTGCTGG-3'; anti: 5'-CTTCAATGACATCCTTGG-3'). Controls using RNA samples without reverse transcription or controls without RNA were used to demonstrate absence of contaminating DNA. The amplification steps involved denaturation at 94°C for 1 min, annealing for 50 s at 55°C with specific primers and extension for 1 min at 68°C. The PCR products (5 ml) were analyzed by electrophoresis using 1.5% agarose gels followed by alkaline blotting of the fragments onto nylon membranes and subsequent hybridization with specific digoxigenin-labeled DNA probes. Detection was performed using CDP-Star™ (Tropix) as chemoluminescence substrate for alkaline phosphatase conjugated to anti-digoxigenin-antibodies (Boehringer Mannheim) as indicated by the manufacturers. Appropriate exposures of Kodak X-OMat films were quantified using a video densitometer (Model 620, Bio-Rad).

2.5. FGF-2 and FGFR1 mRNA determination

Transcripts of FGF-2 and FGFR1 were analyzed by ribonuclease protection assay performed as described previously [32]. Hybridization was performed overnight at 49°C after total RNA was dissolved and heated to 95°C for 10 min in hybridization solution [80% formamide, 40 mM PIPES (pH 6.4), 400 mM NaCl, and 1 mM EDTA] containing 100 000 cpm of 32P-labeled cRNA probe. After addition of RNase digestion buffer [10 mM Tris–HCl (pH 7.4), 300 mM NaCl, and 5 mM EDTA] containing RNases A and T1 incubated for 1 h at 30°C, proteinase K and sodiumdodecyl sulfate were added. After an incubation for 25 min at 35°C phenol–chloroform extraction and ethanol–glycogen precipitation were performed. Pellets were suspended in loading buffer (0% formamide, 10 mM EDTA, and 0.1% bromphenol blue), heated for 5 min at 85°C, and separated on a 4% polyacrylamide–urea sequencing gel. Following fixation and drying, gels were exposed to Kodak bioMax film. tRNA was used as negative control.

2.6. Western blot analysis

Lysis and homogenization of sciatic nerves was performed in distilled water. Protein concentration of the crude cytoplasmic fraction (received after centrifugation for 45 min at 14 000 g) was determined using the Bio-Rad protein assay. Following sodiumdodecyl sulfate–polyacrylamide gel electrophoresis and semidy blotting onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) immunological detection was performed using a monoclonal anti-FGF-2 antibody (Transduction Laboratories, USA) and the ECL system (Amersham, Braunschweig, Germany).

2.7. In situ hybridization

At 5 h after sciatic nerve crush, ipsi- and contralateral nerves were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Sections of 8 µm thickness were placed on coated slides (aminoalkyl silane-prepared slides, Sigma). After proteinase K (2 µg/ml) treatment, prehybridization was performed in 5× standard sodium citrate (SSC; SSC×1=0.15 M NaCl and 0.015 M sodium citrate, pH 7.2) containing 50% formamide, 5× Dehnardt’s solution, tRNA (250 µg/ml) and salmon sperm DNA (500 µg/ml) for 2 h at 55°C. Hybridization was done in the same solution containing digoxigenin-labelled cRNAs (2.5 µg/200 µl/slide; labelling kit, Boehringer, Mannheim, Germany) for 24 h at 55°C. Washing procedure was performed according to the supplier’s information (Boehringer). Both, antisense and sense (control) sections were used in all experiments. Five independent experiments were performed.

2.8. Immunocytochemistry

Consecutive sections were used for in situ hybridization and S100 immunocytochemistry. Binding of S100 antibody (Dako, Hamburg, Germany) was visualized using peroxidase antiperoxidase system and dianaminobenzidine. For Ox-42 immunocytochemistry native sections were fixed in ethanol or acetone, respectively. Binding of Ox-42 antibody (Serotec, UK) was visualized by Cy3-labelled second antibodies.

3. Results

3.1. Effects of IL-6 on immortalized Schwann cells

It has been previously demonstrated that immortalized Schwann cells and physiological (secondary) Schwann cells constitutively express FGF-2 and FGFR1 [19]. Exogenous stimuli like FGF-2 itself and glucocorticoids modulate the expression of FGF-2; the FGFR1 transcript is up-regulated by increasing the intracellular cAMP levels [19,33]. Using serum-free culture conditions, the effects of IL-6 on FGF-2 and FGFR1 expression were analyzed. FGF-2 mRNA and protein were detectable in immortalized Schwann cells and showed no alterations of the expression
level 1, 2, 6, and 24 h after administration of IL-6 (not shown). In addition, the level of FGFR1 transcript was not altered after IL-6 treatment for 2, 6 and 24 h (not shown).

3.2. Effects of FGF-2 on immortalized and physiological Schwann cells

Recently, it was shown that IL-6 expression in Schwann cells is modulated by various cytokines [2]. To determine whether FGF-2 can influence IL-6 and IL-6 receptor expression, immortalized Schwann cells were treated with FGF-2 for 5, 10 and 24 h under serum-containing and serum-free culture conditions. Both IL-6 and IL-6 receptor transcript levels were elevated after FGF-2 treatment under both culture conditions. For example, IL-6 mRNA level showed a 2.5-fold increase 5 and 10 h after addition of FGF-2 which had returned to control values by 24 h (Fig. 1). Levels for the IL-6 receptor mRNA displayed a 2.2-fold increase 5 h after FGF-2 treatment and were slightly

![Graph showing time-dependent induction of IL-6 and IL-6 receptor mRNA transcription in immortalized rat Schwann cells stimulated with FGF-2.](image-url)

**Fig. 1.** Time-dependent induction of IL-6 and IL-6 receptor mRNA transcription in immortalized rat Schwann cells stimulated with FGF-2. Cells were incubated for 5, 10 and 24 h with 50 ng/ml FGF-2. After incubation RT/PCR, Southern blotting and chemoluminescent detection, and densitometric evaluation of the IL-6 and IL-6 receptor mRNA signals were performed. Top: Quantitation of IL-6/IL-6 receptor transcripts. Data are presented as ratio of densitometric scores for IL-6/IL-6 receptor and S12 PCR-products ± SEM (*, P<0.05; **, P<0.01 compared to unstimulated controls). Bottom: Southern blot analysis of PCR products.
reduced after 10 h; after 24 h the transcript level was still 1.5-fold elevated compared to untreated cultures (Fig. 1).

In a second set of experiments, transfected clonal immortalized Schwann cells over-expressing the 18-kD or the 21/23-kD FGF-2 isoforms [20] were analyzed for IL-6 and IL-6 receptor expression and compared with immortalized Schwann cells transfected with a control vector, non-transfected immortalized Schwann cells, and non-transfected immortalized Schwann cells treated with 18-kD FGF-2. The IL-6 and IL-6 receptor mRNA levels were found to be significantly increased only in those Schwann cell clones that were either over-expressing or receiving the 18-kD FGF-2 isoform (Fig. 2). Exogenous administration as well as endogenous over-expression of the 18-kD FGF-2 but not of the 21/23-kD isoforms led to an increased IL-6 and IL-6 receptor expression.

Physiological Schwann cells of the newborn rat displayed a 2.5- and 2.8-fold increase of IL-6 and IL-6 receptor mRNA in response to FGF-2 treatment as well, however, with a slightly different time pattern. The expression peak of the IL-6 transcript was found after a 10 h-treatment with FGF-2, the IL-6 receptor mRNA maximum was reached after 5 h (not shown).

3.3. Cellular localization of IL-6 and IL-6 receptor mRNAs in the sciatic nerve

Although immortalized physiological Schwann cells express IL-6 and IL-6 receptor mRNAs and up-regulate these transcripts in response to 18-kD FGF-2, the cellular source of these factors in the peripheral nerve is not yet known. Therefore, in situ hybridization was performed using sciatic nerve preparations 5 h after nerve crush in comparison to contralateral intact nerves. In the intact nerve, prominent staining for both transcripts (IL-6, IL-6 receptor) was found in vascular endothelial cells and in Schwann cells as revealed by S100 immunostaining (Fig. 3). Staining pattern refers to the cellular distribution which was unaltered 5 h after crush lesion (Fig. 4). Ox-42 immunoreactive cells were sporadically found 5 h after

![Graph](image-url)

Fig. 2. Induction of IL-6 and IL-6 receptor mRNA transcription in transfected clonal Schwann cells over-expressing the 18-kD (lane 5, 18 kD FGF-2) or 21/23-kD (lane 4, HMW=high-molecular-weight FGF-2) FGF-2 isoforms as well as non-transfected cells treated with 18-kD FGF-2 (lane 3). Non-transfected Schwann cells (lane 1, C=control) and Schwann cells transfected with a control vector (lane 2, C-Vect.=control-vector) were the appropriate controls. Top: Quantitation of IL-6/IL-6 receptor transcripts. Data are presented as ratio of densitometric scores for IL-6/IL-6 receptor and S12 PCR-products±SEM (**, P<0.01, compared to unstimulated controls; P<0.01, compared to control-vector-transfected cells). Bottom: Southern blot analysis of PCR products.
sciatic nerve crush. However, in order to verify Ox-42 immunostaining sciatic nerves 24 h after injury were used. Positive cells were exclusively localized around the lesion site (Fig. 5). The temporal and spatial pattern of the Ox-42 immunostaining was completely different to the IL-6 and IL-6 receptor mRNA staining. These results clearly demonstrate that Schwann cells and vascular endothelial cells but not macrophages represent the main sources for IL-6 and IL-6R, also after sciatic nerve crush with increased IL-6 and IL-6R in RNA levels.

3.4. Sciatic nerve crush

From previous studies it is known that peripheral nerve injury results in an elevated IL-6 mRNA expression level in the nerve stumps as early as 2 and 3 h after operation [2,38]. To investigate whether the IL-6 receptor transcript level is also increased after lesioning, the mRNA was analyzed 5 h after sciatic nerve crush. Nerve segments proximal and distal to the crush site showed a 2.3-fold increase of the IL-6 receptor mRNA level compared to the contralateral intact nerve or to untreated nerves (Fig. 6). FGF-2 is known to be up-regulated after peripheral nerve injury [19,30]. However, mRNA and protein values for early time points after lesion have not been published so far. Therefore, FGF-2 mRNA was analyzed 5 h and FGF-2 protein was studied 5 h and 2 days after crush lesion. Ribonuclease protection analysis revealed elevated FGF-2 mRNA levels in the proximal and distal nerve stump compared to the contralateral side 5 h after injury (Fig. 7). In addition, the FGF-2 protein displayed a significant increase after 5 h, at which the 18-kD FGF-2 isoform showed the strongest up-regulation compared to the 21- and 24-kD isoforms (Fig. 8). Two days after peripheral nerve crush the FGF-2 protein level was only slightly elevated (not shown).

4. Discussion

In this study we have shown that FGF-2 is able to modulate the expression of IL-6 and IL-6 receptor in immortalized and physiological (secondary) Schwann

Fig. 3. Cellular localization of IL-6 and IL-6 receptor mRNAs in Schwann cells and vascular endothelial cells. (A) Immunocytochemical staining for S100 localized in Schwann cells (short arrows); (B) non-radioactive in situ hybridization with digoxygenin-labelled IL-6 receptor (IL6R) riboprobes localized in Schwann cells (short arrows) and vascular endothelial cells (long arrow). Magnification ×390.
in vitro interaction of FGF-2 and IL-6, the early increase of both molecules after nerve injury suggests a functional coupling of FGF-2 and IL-6.

Previously, it was shown that in addition to various cytokines, co-culturing of Schwann cells with phaeochromocytoma (PC)12 cells or NIH 3T3 fibroblasts stimulate IL-6 synthesis in Schwann cells [2]. Since it is known that both cell types express FGF-2 constitutively [33,35], the present data suggest that this up-regulation of IL-6 might be mediated by FGF-2 released from PC12 cells and 3T3 fibroblasts.

Different FGF-2 isoforms between 18 and 24 kD have been described representing alternative translation products from a single mRNA which occur in a tissue- and species-specific manner [10,16,31]. Initiation at the AUG codon results in the 18-kD FGF-2, the higher-molecular-weight forms (21 kD, 23/24 kD) arise after initiation at CUG codons [9]. Our data indicate that it is the 18-kD FGF-2 isoform which is mainly responsible for the induction of IL-6 and IL-6 receptor. This is based on the following observations: (1) Exogenously applied 18-kD FGF-2 induced an up-regulation of both transcripts. (2) Over-expression of 18-kD but not of 21/24-kD FGF-2 resulted in increased IL-6 and IL-6 receptor transcript levels. Stable over-expression of the FGF-2 isoforms in transfected Schwann cells was documented previously [20]. (3) Peripheral nerve crush showed specific induction of the 18-kD FGF-2 isoform in the proximal and distal nerve stumps after 5 h. Further experiments, aiming to selectively eliminate the endogenous 18-kD FGF-2, e.g. by using specific antisense probes, should verify this hypothesis.

The present in situ hybridization data are the first demonstration of IL-6 and IL-6 receptor synthesis in Schwann cells and vascular endothelial cells. Macrophages did not seem to be a major source of IL-6 and IL-6 receptor mRNAs. This result correlates with studies in IL-6-deficient mice where the injury-induced invasion of macrophages was not altered compared to the wild-type animals [44]. These data suggest that the IL-6 and its receptor are synthesized in Schwann cells and possibly involved in the Schwann cell reaction to injury whereas the macrophage reaction takes place independently of IL-6.

We have shown previously that the FGF-2 transcript level is up-regulated over a period of 3 weeks after peripheral nerve lesion [19]. This, together with the observation that FGF-2 is capable of suppressing the forskolin-induced myelin protein Po [34] and stimulates the proliferation of Schwann cells in vitro [7] suggests that FGF-2 is involved in the initial steps of regeneration, e.g. by preventing myelination and inducing Schwann cell proliferation to form bands of Bungner. In addition, the present findings suggest that, as a short term effect, FGF-2 could promote IL-6 and IL-6 receptor expression. Physiological functions of IL-6 during early phases of regeneration could include stimulation of survival [22,28], regulation of growth factor expression such as NGF [11,29], and autocrine effects [15,39].
Fig. 5. Immunolocalization of Ox-42 in macrophages 24 h after sciatic nerve crush. Macrophages accumulate at the lesion site. Magnification ×273.

Fig. 6. Induction of IL-6 receptor mRNA transcription in the proximal and distal nerve stumps 5 h after sciatic nerve crush. RT/PCR, Southern blotting and chemoluminescent detection, and densitometric evaluation of the IL-6 receptor mRNA signals were performed. Top: Quantitation of IL-6 receptor transcripts. Data are presented as ratio of densitometric scores for IL-6 receptor and S12 PCR products±SEM. (**, P<0.01; compared to the intact control nerve or the intact contralateral nerve). Bottom: Southern blot analysis of PCR products. (N. isch. int. r.: right and left intact sciatic nerve; N. isch. int. cont.: intact contralateral sciatic nerve; OP prox./dist.: operated sciatic nerve, proximal/distal nerve stump).
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