Interleukin-12 promotes neurite outgrowth in mouse sympathetic superior cervical ganglion neurons

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Abstract

To determine the role of cytokines in the nervous system, we examined the effect of interleukin-12 (IL-12) on the nerve regeneration of mouse superior cervical ganglion cells (SCG). IL-12 enhanced the neurite outgrowth in a concentration-dependent manner. Immunocytochemical studies demonstrated the expression of IL-12 receptors in neuronal bodies and neurites. The mRNA expression of IL-12 receptors in SCG cells was confirmed by reverse transcription-polymerase chain reaction. Our data demonstrated the presence of IL-12 receptors in sympathetic neurons and suggest that IL-12 plays an important role in neuronal regeneration. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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Interleukin-12 (IL-12), a cytokine of a 40-kDa subunit (p40) disulfide-bonded to a 35-kDa subunit (p35), has multiple effects on both T and natural killer (NK) cells. Interleukin-12 is produced predominantly by macrophages and B lymphocytes. IL-12 plays important regulatory roles in the proliferation, cytotoxic ability, and lymphokine production in these two cell types [13,17]. The biological activities of IL-12 are mediated through binding of IL-12 to cell surface receptors on T and NK cells [2]. Both mouse and human IL-12 receptors have been cloned and characterized [10,16,19]. IL-12 receptors in the nervous systems, however, has been poorly characterized.

Damage to peripheral nerve results in the rapid degeneration of damaged axons and myelin sheath, which is known as Wallerian degeneration (WD) [14]. The importance of WD is to create an appropriate local environment for successful regeneration of damaged axons. The macrophage is an important cellular component in the process of peripheral degeneration and regeneration. During WD, macrophages secrete a series of growth-promoting factors and cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF-α), which may play a role in the process of axonal regeneration [4,7,8,15]. TNF-α and IL-6 have recently been shown to be produced in distal stumps during WD. IL-1 may be involved via stimulating nerve growth factor (NGF) synthesis in the process of axonal regeneration after nerve transaction. IL-2 increases rat sympathetic neurite outgrowth [11].

The expression of IL-12 mRNA in the distal stump of crushed sciatic nerves during WD is increased between days 7 and 14 in WD [9], when the infiltration of macrophages around the injured nerve is at maximal [18], suggesting that macrophages secrete IL-12 during WD. However, whether IL-12 plays any functional roles in the process of nerve regeneration during WD remains unknown. We thus examined the effects of IL-12 on the neurite outgrowth of sympathetic superior cervical ganglia (SCG) neurons. We here demonstrate that IL-12 stimulates the neurite outgrowth of sympathetic neurons.

Primary SCG cultures were prepared from C57BL/6 mice of 10–15 weeks old. Mice were placed in an airtight tin container infused with diethyl ether for anesthesia (Wako Pure Chemical Industry, Japan). SCG ganglia were dissected and then incubated sequentially in the following solutions: (1) Hank’s balanced salt solution (BSS) containing 0.25% trypsin (Sigma, St. Louis, MO, USA) for 15 min at 37°C; (2) Ham’s F12 solution (Gibco BRL, Tokyo, Japan) containing 0.25% collagenase (Worthington Biochem., Freehold, NJ, USA) for 90 min at 37°C; (3) F-12 solution containing both 0.25% collagenase and 0.25% trypsin for 15 min at 37°C. Soybean trypsin inhibitor was used at the end of incubation to inactivate trypsin. SCG were dissociated by gentle pipeting, layed on 30% Percoll solution (Pharmacia

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LKB., Uppsula, Sweden), and then centrifuged to remove non-neuronal cells. Cells were washed twice in a Ham’s F12 solution containing 10% heat-inactivated fetal calf serum (FCS) (Mitsubishi Kasei Co., Tokyo, Japan). Cells were dissociated and plated sparsely on poly-L-lysine coated dishes in a F12 solution containing 10% FCS at 37°C for 12 h in an atmosphere of 5% carbon dioxide. The culture medium was then changed to a serum-free medium, i.e., a F12 solution containing insulin (5 μg/ml), progesterone (20 nM), sodium selenite (30 nM), transferrin (5 μg/ml), and putrescine (100 μM) (Sigma, St. Louis, MO, USA).

Under a phase contrast microscope, a living SCG neuron was identified as cell with a bright and round cell body, Schwann cell with a bipolar spindle cell body, and fibroblast as a flat cell body with shorter processes. Less than 10% of cells were recognized as Schwann cells and fibroblasts. The data were excluded if the proportion of non-neuronal cells was increased over 10% at last day in culture because of possible proliferation of non-neuronal cells. Cells were allowed to extend neurites for 24 h prior to examining SCG neurons with neurites. Counts were made each day in culture to estimate the growth of SCG neurons. Neurite outgrowth was evaluated by dividing the number of surviving neurons with neurite length over 300 μm by the total number of surviving neurons observed in culture at each day. The observation of neurite outgrowth of SCG neurons had been observed for 3 days in culture. After 3 days in culture, the extended neurites of SCG neurons were twined together and so it was difficult to calculate the exact neurite lengths of SCG neurons. Statistical analysis was made by unpaired Wilcoxon–Mann–Whitney test. Values were expressed as means ± SEM.

The exposure of SCG neurons to either IL-12 (2 ng/ml) or NGF (200 ng/ml) for 3 days elicited a significant increase of neurite outgrowth of SCG neurons over that of control (P < 0.05) (Fig. 1a). IL-12 significantly enhanced the neurite outgrowth of SCG neurons over that of control cultures in a concentration-dependent manner (2–200 ng/ml) (P < 0.05) (Fig. 1b). Fig. 2 shows that cells incubated in a serum-free medium had short neurites whereas IL-12 treated cells had long branch-like neurites.

To examine whether IL-12 receptors are indeed present in sympathetic neurons, reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect the mRNA expression of IL-12 receptors (β1 and β2 chains). The SCG ganglia were dissociated as described above to remove non-neuronal cells and immediately used for RT-PCR. One-hundred nanograms of total RNA from the cells was reverse transcribed with 200 units of Superscript II-Reverse Transcriptase (Gibco BRL, Tokyo, Japan) in a solution containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl2, 0.5 mM dNTP, 10 mM DTT, 0.5 μg oligo (dT) primer, and 20 units of RNase Inhibitor (Promega, Madison, WI, USA) at 42°C for 60 min, and then at 51°C for 30 min. One microliter of this reaction mixture was used for subsequent PCR analysis in a 50 μl solution containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl2, 200 μM dNTP, AmpliTAQ DNA polymerase (PE Applied Biosystem, Foster City, CA, USA), and 0.25 μM of each primer at the following condition: (1) 12 min at 94°C; (2) 30 s at 94°C and 45 s at 60°C for 40 cycles; (3) 7 min at 72°C. The following IL-12 receptor and β-actin primers were used: IL-12 receptor β1 chain (accession No. U23922) sense primer 5′-AAAGTTCAAGTGCGGCGG-3′ (1670–1688); antisense primer 5′-CGGGAATTCTCAAGGGTTGC-3′ (1951–1932); β2 chain (accession No. U64199) sense primer 5′-CTGGGTGCTGGCTGGCTCCTAC-3′.
(1439–1458); antisense primer GCGTCG- GTACTG- AATTTCGC-3' (1917–1898); β-actin (accession No. X03672) sense primer 5'-CGTGGGCCGCCCCATAGGCA- CCA-3' (182–202); antisense primer 5'-TTGGCCCTTAGG- GGTTCAAGGGGG-3' (424–403). The length of PCR products of both subunits of IL-12 receptors (β1 and β2) were 282 and 479 bp, respectively, and that of β-actin was 243 bp. The amplified products were analyzed using agarose gel electrophoresis and stained with ethidium bromide. The PCR product of the IL-12 receptor β1 was 97 bp shorter than what expected for the IL-12 receptor β1, which is most likely due to a known splicing event within the TM region [3]. mRNA for the IL-12 receptor β2 and β actin were also detected in SCG cells (Fig. 3).

An immunocytochemical study to detect IL-12 receptors was performed as follows. SCG neurons were cultured either in Ham’s F12 containing 10% FCS without NGF for one day or in Ham’s F12 containing 10% FCS supplemented with 200 ng/ml of NGF in an atmosphere of 5% carbon dioxide for two days. NGF was used to provide a better condition for neurite growth of SCG neurons. Then the cells were treated with microwave (500 W, 10 s) and fixed for 30 min with 2% paraformaldehyde. The cells were incubated with a rabbit anti-IL-12 receptor antibody (5 μg/ml) (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) in PBS for 3 h at room temperature, followed by incubation with an FITC-conjugated goat anti-rabbit Ig G antibody (5 μg/ml) (Zymed, Camarillo, CA, USA) for 1 h in the dark at room temperature. The cells were washed three times with PBS for 5 min between each step. Controls were treated similarly, but in the absence of the primary antibody. As shown in Fig. 4, both SCG neurons cultured in the presence and absence of NGF were stained specifically by an anti-IL-12 receptor antibody. In contrast, non-neuronal cells, such as Schwann and fibroblast, were not stained (data not shown).

Thus, our data demonstrated the functional expression of IL-12 receptors and the role of IL-12 in regenerating SCG neurons. In Wallerian degeneration, the nerve regeneration of injured nerve have been thought to be regulated by cytokines secreted by macrophages. Schwann cells and fibroblasts also produce NGF and cytokines like IL-1 and IL-6 which may be involved in WD [1,12]. In order to examine the direct effect of IL-12 on the neurite outgrowth of SCG neurons, it is necessary to remove non-neuronal cells such as Schwann cells and fibroblasts to exclude the possible involvement of NGF and cytokines by non-neuronal cells in the neurite outgrowth of SCG neurons. In our experiments, cells were centrifuged to remove non-neuronal cells in a Percoll solution to eliminate the effect of other non-neuronal cells on SCG neurons. Furthermore, the cells were cultured in serum-free media to create an unfavorable condition for the growth of Schwann cells and fibroblasts. and thus the contamination of Schwann cells and fibroblasts were minimized in our SCG culture. The cells were also dispersed to avoid the cell-to-cell interaction. The detection of IL-12 receptors mRNA by RT-PCR did not exclude the possibility that Schwann cells and fibroblasts might also have IL-12 receptors. However, our immunocytochemical study with antibodies against IL-12 receptors demonstrated that only sympathetic neurons had IL-12 receptors. Thus IL-12 had a direct effect on SCG neurons, not on Schwann cells or fibroblasts, to promote their regeneration.

It is well known that the innervation of peripheral nerve into its target cells is stimulated by NGF. In the absence of NGF, the nerve cells may die and lose the ability of innervation. Lymphoid tissues, including spleen and thymus, are innervated by the sympathetic nervous system and the sympathetic nerve terminals form synaptic-like contacts with lymphocytes in the spleen [5,6]. IL-12 is predomi-

![Fig. 3. RT-PCR analysis of mRNA for IL-12 receptors in SCG neurons. Freshly dissociated SCG neurons were used for RT-PCR analysis. mRNA for IL-12 receptors (β1 and β2 subunits) and β-actin were detected.](image-url)
nantly produced by macrophages and B-lymphocytes in the lymphoid organs. Putting together with our findings, it may be implicated that the cells in the lymphoid organs release IL-12 and increase the sympathetic innervation via stimulating the sympathetic neurite outgrowth.

Although there is still no direct evidence to show that IL-12 secretion increases after the sympathetic injury in vivo, an increase of IL-12 mRNA expression has been reported in vivo in crushed sciatic nerve during WD [9,18]. Infiltrated macrophages around injured sympathetic nerves may secrete IL-12 to promote the regeneration of damaged sympathetic nerves in vivo during WD. It will be necessary to clarify the molecular role of IL-12 in regenerating neurons during WD in future studies.


