NGF-independent survival of postganglionic sympathetic neurons in neuronal-vascular smooth muscle cocultures

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Damon, Deborah H. NGF-independent survival of postganglionic sympathetic neurons in neuronal-vascular smooth muscle cocultures. Am J Physiol Heart Circ Physiol 280: H1722–H1728, 2001.—The present study tests the hypothesis that vascular cells promote the survival of postganglionic sympathetic neurons in the absence of nerve growth factor (NGF). To test this hypothesis, neurons isolated from superior cervical ganglia of 2- to 4-day-old rat pups were grown in the absence of NGF and in the absence and presence of vascular smooth muscle cells (VSM). Neuronal survival was assessed as a function of time in culture. At all time points studied, VSM promoted the survival of the neurons. After 5 days in the absence of NGF, 7 ± 2% of neurons survived in the absence and 28 ± 7% survived in the presence of VSM. An endothelin receptor antagonist reduced neuronal survival in cocultures grown in the absence of NGF. These data indicate that VSM produce factors other than NGF that promote the survival of cultured postganglionic sympathetic neurons. The data also indicate that endothelin contributes to this effect and suggest that endothelin as well as other VSM-derived factors may play a role in the development of sympathetic innervation to vascular smooth muscle. The present study tests the hypothesis that vascular cells produce factors other than NGF that promote the survival of postganglionic sympathetic neurons.

MATERIALS AND METHODS

The use of animals in the present study was in accordance with the National Institutes of Health guidelines for the humane care and use of animals in research and was approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Vascular cell cultures. Endothelial cells (ECs) isolated from adult (>90 days old) male Sprague-Dawley rats were a generous gift from Dr. Paula Grammas, University of Oklahoma. The EC identity of these cells was confirmed by distinct cobblestone morphology and by uptake of acetylated low-density lipoprotein (22). ECs were used from passages 11–20. Vascular smooth muscle cells (VSM) were isolated from explants of adult male Sprague-Dawley rat aortas (17). These cells exhibited characteristic “hill and valley” growth patterns and immunohistochemical labeling with a monoclonal antibody for smooth muscle-specific α-actin. VSM were used from passages 3 to 10. Vascular cells were grown in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, 100 units penicillin, and 100 units streptomycin. Cells were maintained at 37°C in a humidified 5% CO₂ environment.

Superior cervical ganglion cultures. Sympathetic superior cervical ganglia (SCG, left and right) were collected from 2- to 4-day-old rat pups and enzymatically dissociated for 20 min at 37°C in a collagenase-hyaluronidase solution (10 mg/ml bovine serum albumin, 4 mg/ml collagenase, and 1 mg/ml hyaluronidase) and then for 3 min in trypsin (3 mg/ml). Dissected cells were applied to collagen-coated dishes and grown in DMEM supplemented with 10% FBS, glutamine, penicillin, streptomycin, and 50 ng/ml NGF. One day after plating, SCG cultures were treated with an antimitotic agent (mitomycin C, 10 μg·ml⁻¹·h⁻¹) to prevent the growth of nonneuronal cells. After mitomycin C treatment, SCG cultures were washed extensively to remove the mitomycin C and were then grown in the absence or presence of vascular cells and indicated additions.

Cell survival. Two methods were used to assess neuronal survival. First, numbers of phase-bright neurons were counted in 10 microscope fields. The culture dish was evenly divided into 10 quadrants. One field from each quadrant was sampled. The 10 counts were averaged to obtain a representative number of neurons per field. Neuronal counts were determined.

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the day after plating (after mitomycin C treatment and washes) represented 100% survival.

Cell survival was also determined by counting the numbers of neurons electronically with a Coulter counter. For these experiments equal numbers of cells were plated in 24-well culture dishes. These cultures contained neurons as well as nonneuronal cells, and the cell count obtained with the Coulter counter would include both neuronal and nonneuronal cells. Thus the number of nonneuronal cells had to be determined and subtracted from the total number of cells to obtain the number of neurons in each culture. To do this, within each experiment, a subset of the culture wells were grown in the absence of NGF for 6 days. Preliminary studies indicated that this duration of NGF deprivation resulted in the death of 100% of the neurons. Nonneuronal cells are not dependent on NGF for survival. Coulter counts of cultures grown in the absence of NGF for 6 days thus represented the number of nonneuronal cells in the cultures. Each of these NGF-deprived cultures was thoroughly examined to verify that there were no neurons. The nonneuronal cells had been grown the day after plating, and thus this number was constant throughout the experiment. Thus this nonneuronal count could be subtracted from total counts obtained at any time during the experiment to obtain the number of neurons in a culture. As with the first method of assessing survival, neuron counts made the day after plating represented 100% survival.

**Immunohistochemistry.** SCG and SCG-VSM cultures were rinsed with 0.1 M PBS (19 mM sodium phosphate monobasic, 81 mM sodium phosphate dibasic, and 0.05 mM sodium chloride; pH 7.4) and fixed for 2 h in 4% paraformaldehyde in 0.1 M PBS. The cells were then permeabilized (1 h in 0.1 M PBS, 0.2% Triton X-100, and 0.9% hydrogen peroxide) and blocked (30 min in 5% normal goat serum). Cells were incubated with tyrosine hydroxylase (TH) primary antibody (1:500 rabbit) overnight at room temperature, washed with 0.1 M PBS, and incubated with a fluorescein-labeled secondary antibody [1:200 donkey anti-rabbit immunoglobulin G (IgG) fluorescein] overnight at 4°C.

**Experimental protocols.** The overall hypothesis of the present study is that vascular cells produce factors other than NGF that promote the survival of postganglionic sympathetic neurons. An assumption inherent in this hypothesis is that the neurons would not survive in the absence of NGF and/or the vascular cells. The first series of experiments was performed to verify that this was the case. Cultures of postganglionic sympathetic neurons were grown for 5 days in the absence of vascular cells and in the presence (+NGF) and absence (−NGF) of 50 ng/ml NGF, a concentration of NGF that is known to support the survival of these neurons. To ensure that no NGF was present in the cultures, an antibody that neutralized the activity of NGF was added to the cultures that did not contain NGF. Survival was then assessed by counting the numbers of phase-bright neurons as described above.

In the second set of experiments, neurons were grown in the absence of NGF and in the absence (−NGF) and presence (−NGF + VSM) of VSM. Survival was assessed by counting the numbers of phase-bright neurons present 1, 2, and 5 days after the removal of NGF and the addition of VSM.

In the third set of experiments, neurons were grown in the absence of NGF and in the absence (−NGF) and presence of ECs (−NGF + ECs). Survival was assessed by counting numbers of phase-bright neurons present 5 days after removal of NGF and the addition of ECs.

In a subset of experiments (+NGF, −NGF, and −NGF + VSM), the survival assays were immediately followed by immunohistochemistry for TH. TH is the rate-limiting enzyme in catecholamine synthesis and thus is a marker for postganglionic sympathetic neurons in the cultures used in the present study. Immunohistochemistry was performed to verify that any neurons surviving in the absence of NGF were indeed sympathetic. Counts of TH-labeled neurons were also performed to confirm the counts of phase-bright neurons.

To determine whether neurons surviving in the absence of NGF but in the presence of VSM differentiated as well as survived, one index of neuronal differentiation was assessed. The number of neurons extending processes greater than three cell body lengths was assessed in a subset of +NGF and −NGF + VSM cultures.

The role of neurotrophin-3 (NT-3) was assessed in three experiments. Neuronal-VSM cultures were grown in the absence of NGF and the absence (−NGF + VSM) and presence (−NGF + VSM + NT-3) of an antibody that neutralized the activity of NT-3. The survival of neurons in these cultures was assessed by counting the numbers of phase-bright neurons in the cultures 5 days after the removal of NGF and NT-3 and the addition of VSM.

The role of endothelin (ET) in NGF-independent survival was also assessed. Initially, studies were performed to determine whether ET could promote the survival of postganglionic sympathetic neurons in the absence of NGF. For these experiments, neuronal cultures were grown in the absence of NGF and VSM and in the absence and presence of ET-1. Survival was assessed by electronically counting numbers of cells, as described above. As described above, counts were performed at 6 days, because preliminary studies indicated that at this time no neurons survived in the absence of NGF, VSM, and ET.

To determine whether ET contributed to the effects of VSM on survival of sympathetic neurons in neuronal-VSM cocultures, −NGF + VSM cultures were grown in the absence and presence of the ET antagonist PD-142,893. In these experiments, survival was assessed by counting the number of phase-bright neurons in the cultures 5 days after the removal of NGF, the addition of VSM, and the addition of PD-142,893. The effects of PD-142,893 were similarly assessed in +NGF cultures.

**Materials.** DMEM, penicillin-streptomycin, and glutamine were obtained from GIBCO Life Technologies (Gaithersburg, MD). FBS was from Summit Biotechnology (Fort Collins, CO). NGF was from Becton Dickinson (Bedford, MA). Mitomycin C, NGF antibody, NT-3 antibody, and α-smooth muscle actin antibody were purchased from Sigma (St. Louis, MO). Collagenase, hyaluronidase, and trypsin were purchased from Worthington Biochemicals (Lakewood, NJ). TH primary antibody and secondary antibodies were from Chemicon International (Temecula, CA). Rat tail collagen was provided by Dr. Carson Cornbrook, Department of Anatomy and Neurobiology, University of Vermont.

**RESULTS**

To test the hypothesis that vascular cells produce factors other than NGF that promote the survival of postganglionic sympathetic neurons, the survival of these neurons was assessed in the absence of NGF, but in the presence of vascular cells. NGF was eliminated from the cultures with an antibody that neutralized the activity of NGF. The vascular wall is composed primarily of the two cell types, VSM and ECs. The effects of both of these cells were assessed.
Figure 1A shows that the sympathetic neurons used in the present study were in fact dependent on NGF for survival. Sympathetic neurons were plated as described in MATERIALS AND METHODS. The day after plating, after growth arrest with mitomycin C, the cultures were transferred to media containing 50 ng/ml NGF or to media containing an antibody that neutralized the activity of NGF. The percentage of cells surviving after 5 days in culture is shown. In the presence of NGF 68 ± 8% of the cells survived; in the absence of NGF only 7 ± 2% of the cells survived.

The primary targets of sympathetic neurons in blood vessels are VSM. The effects of VSM on NGF-independent survival were thus assessed. Figure 1B shows neuronal survival in cultures grown in the absence of NGF and in the absence or presence of VSM. Survival was assayed 1, 2, and 5 days after the addition of NGF antibody or NGF antibody and VSM to the cultures. At all times studied, neuronal survival in the absence of NGF was enhanced by VSM. After 5 days in the absence of NGF, survival in the presence of VSM (28 ± 7%) was significantly greater than that in the absence of VSM (7 ± 2%). The survival of neurons grown for 5 days in the presence of VSM but in the absence of NGF (28 ± 7%, Fig. 1B) was less than that of neurons grown for 5 days in the presence of 50 ng/ml NGF (68 ± 8%, open bar, Fig. 1A).

As noted earlier, blood vessels are composed of VSM and ECs. ECs could also affect the survival of sympathetic neurons, and thus the effect of these cells on NGF-independent survival of sympathetic neurons was also assessed. Sympathetic neurons were grown in the absence of NGF and in the absence (Fig. 1, A and B) and presence of vascular ECs. The percentage of cells surviving after 5 days is shown in Fig. 1C. These data indicate that ECs did not promote NGF-independent survival of postganglionic sympathetic neurons.

In two of the experiments depicted in Fig. 1, TH immunohistochemistry was performed on neuronal cultures grown for 5 days in the presence of 50 ng/ml NGF (Fig. 2A) and on neuronal-VSM cultures grown for 5 days in the absence of NGF (Fig. 2B). This was done to determine whether the neurons surviving in the absence of NGF but in the presence of VSM expressed TH, and whether the numbers of TH-labeled neurons were the same as the numbers of neurons surviving as assessed in Fig. 1B. Figure 2 shows that the neurons in both cultures expressed TH. In these two experiments, counts of TH-labeled cells were equal to counts from the survival assay (6 and 13.7 cells/field for the neuronal cultures grown in the presence of NGF; 1.5 and 3.2 cells/field for the neuronal-VSM cultures grown in the absence of NGF).

The development of postganglionic sympathetic neurons requires not only that the neurons survive, but also that they differentiate and form appropriate connections with their targets. NGF promotes the differentiation as well as the survival of these neurons (1). Do VSM promote NGF-independent differentiation as well as NGF-independent survival of postganglionic sympathetic neurons? Process outgrowth is one component of neuronal differentiation that is enhanced by NGF (1). Process outgrowth (percentage of cells with processes) in neuronal-VSM cultures grown for 5 days in the absence of NGF was
assessed and compared with that in neuronal cultures grown in the presence of 50 ng/ml NGF. Almost 100% (92 ± 5) of neurons extended processes in cultures grown in the presence of NGF. Only 36 ± 10% of surviving neurons extended processes in cultures grown in the presence of VSM but in the absence of NGF (P < 0.05, unpaired t-test, n = 3).

It has been reported that NT-3 is produced by VSM (7) and that NT-3 promotes the survival of postganglionic sympathetic neurons (1, 26). If NT-3 was promoting the survival of neurons in the neuronal-VSM cocultures, then inhibiting its activity should decrease the survival of the neurons in these cultures. This was not the case. The effects of an antibody that neutralized the activity of NT-3 were assessed in three experiments. In these three experiments, the survival in the absence of NGF and in the presence of VSM was 22 ± 0.6%. The survival in the absence of NGF and in the presence of VSM and the NT-3 antibody was 21 ± 4% (P = 0.88, unpaired t-test, n = 3). These data suggest that it is highly unlikely that NT-3 mediates the NGF-independent survival in SCG/VSM cultures.

ET is a peptide produced by VSM (2) and postganglionic sympathetic neurons (4) and is found in neuronal-VSM cocultures (5). This peptide binds to postganglionic sympathetic neurons (6) and has been reported to modulate the function of these cells (14, 15, 23). ET promotes the survival of many cell types (10, 18, 19) including neurons (8). Thus ET could mediate or modulate the VSM-dependent survival of neurons observed in Fig. 1B. Figure 3A shows that ET promotes the survival of postganglionic neurons grown in the absence of NGF and VSM. Up to 17 ± 2% of the neurons survived in the presence of ET and in the absence of NGF; 0% of the neurons survived in the absence of ET and NGF. ET also enhanced the survival of sympathetic neurons grown in the presence of low concentrations of NGF (Fig. 3B). In the presence of 0.1 ng/ml NGF, 11 ± 4% of neurons survived in the absence of ET-1 (10 nM), whereas 28 ± 5% of the neurons survived in the presence of ET-1 (P < 0.05, unpaired t-test, n = 6). ET did not enhance the survival of sympathetic neurons grown in the presence of higher concentrations of NGF (0.3–3 ng/ml). To determine whether ET contributed to NGF-independent survival in neuronal-VSM cocultures, cultures were grown for 5 days in the absence and presence of an ET receptor antagonist PD-142,893 (16). Figure 3C shows that the survival of postganglionic sympathetic neurons is reduced in neuronal-VSM cocultures grown in the presence of PD-142,893. As noted earlier and in Fig. 1B, 28 ± 7% of the neurons survived in the neuronal-VSM cocultures grown in the absence of PD-142,893; only 14 ± 2% of the neurons survived in the presence of PD-142,893 (P < 0.05, unpaired t-test, n = 4). Figure 3C also shows that PD-142,893 did not affect the survival of neurons grown in the presence of 50 ng/ml NGF (P > 0.05, unpaired t-test, n = 4).

**DISCUSSION**

Target-derived trophic factors are required for the survival and thus appropriate development of many neurons (20). Most postganglionic sympathetic neurons are dependent on target production of NGF. Antibodies that neutralize the activity of NGF during the postnatal development of postganglionic sympathetic neurons prevent the survival of most of these neurons and thus the development of most peripheral sympathetic innervation (3, 9, 12, 24, 26). Some peripheral sympathetic innervation, including that to blood vessels, persists after neutralization of NGF activity (3, 9, 12, 24, 26). One explanation for this resistance to NGF deprivation is that these targets promote the survival of sympathetic neurons through NGF-independent mechanisms. The current study tested the hypothesis that vascular cells promote NGF-independent survival of postganglionic sympathetic neurons.

The present study indicates that cultured VSM promote the survival of cultured postganglionic sympathetic neurons in the absence of NGF. ECs did not...
promote NGF-independent survival. Thus VSM, the vascular cells that are the target of sympathetic innervation, produce factors other than NGF that promote neuronal survival. Sympathetic neuronal survival in the presence of VSM was less than that in the presence of 50 ng/ml NGF, indicating that the neurotrophic activity produced by the VSM in the present cultures is less efficacious than 50 ng/ml NGF.

The neurons present in neuronal-VSM cocultures grown for 5 days in the absence of NGF expressed TH. The expression of TH was not quantitated in the present study, but immunohistochemical analysis did not indicate that these neurons expressed less TH than those grown in the presence of NGF. Process outgrowth from the neurons grown with VSM in the absence of NGF was less than that from neurons grown in the presence of NGF. Cell size was not quantitated in the present study, but Fig. 2 suggests that the neurons grown in the cocultures were smaller than those grown in the presence of NGF. These data suggest that the factor or factors produced by VSM that promote(s) the survival of postganglionic neurons does not promote the differentiation of these neurons (1), at least not as well as NGF.

NT-3 is produced by VSM (7) and promotes the survival of sympathetic neurons (1, 26). Antibodies that neutralized the activity of NT-3 did not affect VSM-dependent survival. It is not clear why this is the case. NT-3 was not measured in the present study, and it is thus possible that this neurotrophic factor was not present in the neuronal-VSM cultures.

Several lines of evidence suggest that ET may be a vascular-derived survival factor for postganglionic sympathetic neurons. ET is produced by VSM (2) and found in neuronal-VSM cocultures (5). ET binds to and activates high-affinity receptors on postganglionic sympathetic neurons (6). Activation of these receptors on postganglionic sympathetic nerve terminals alters the function of these neurons (14, 15, 23). In addition, ET promotes the survival of many cells (10, 18, 19), including neurons (8). The present study indicates that when ET-1 was added to postganglionic sympathetic neurons grown in the presence of an antibody that neutralized the activity of NGF, up to 17% of the neurons survived. In the absence of ET, 0% of the neurons survived. In neuronal-VSM cocultures, an ET antagonist, PD-142,893, reduced neuronal survival. There are two receptors that mediate the actions of ET. The present study does not indicate which receptor promotes the survival of postganglionic sympathetic neurons. ET-1 binds to and activates both subtypes of ET receptors; PD-142,893 is an antagonist for both ET<sub>A</sub> and ET<sub>B</sub> receptors (16).

**Fig. 3.** Endothelin (ET) and the survival of postganglionic sympathetic neurons in neuronal-VSM cocultures. A: survival of neurons grown in the absence of NGF and in the absence and presence of ET-1. Survival was assessed 6 days after the removal of NGF and the addition of ET-1. At this time, 0% of the neurons survived in the absence of NGF and ET-1 (0 on the x-axis). One hundred percent survival represents the number of neurons counted immediately before the removal of NGF and the addition of ET-1. *Survival in the presence of ET-1 was significantly greater than that in the absence of ET-1 (means ± SE; n = 3; unpaired t-test; P < 0.05). B: survival of neurons grown in the presence of NGF and in the absence (filled circles) and presence (open circles) of 10 nM ET-1. *Survival in the presence of ET-1 was significantly greater than that in the absence of ET-1 (means ± SE; n = 6; unpaired t-test; P < 0.05). C: neuronal survival in neuronal cultures (−NGF) and neuronal-VSM cocultures (−NGF + VSM) grown in the absence (open bars) and presence (solid bars) of 10 μM PD-142,893, an endothelin receptor antagonist. *In the neuronal-VSM cocultures, survival in the presence of PD-142,893 was significantly less than that in the absence of PD-142,893 (means ± SE; n = 4; unpaired t-test; P < 0.05). PD-142,893 did not affect neuronal survival in cultures grown in the presence of NGF (means ± SE; n = 3; unpaired t-test; P > 0.05).
Survival in the cocultures grown in the presence of PD-142,893 (14 ± 2%) was greater than survival in the neuronal cultures grown in the absence of NGF and VSM (7 ± 2%). This suggests that an additional factor or factors, other than ET, promotes the survival of the neurons in the neuronal-VSM cocultures. Vascular endothelial growth factor (VEGF) is another growth factor produced by VSM that has been reported to support the survival of postganglionic sympathetic neurons. The role of VEGF in VSM-dependent survival of sympathetic neurons is currently being investigated. Another possibility is that 10 µM PD-142,893 did not completely antagonize the ET present in the neuronal-VSM cocultures. This possibility is unlikely. The concentration of the antagonist used has been reported to inhibit the activity of up to 10 nM ET (16). The concentration of ET in the neuronal-VSM cocultures was estimated to be less than 1 nM (2, 4).

ECs, like VSM, synthesize and secrete ET. Thus it is not clear why VSM, but not ECs, supported the survival of postganglionic sympathetic neurons. It is possible that ECs do not produce enough ET. It is known that rat ECs produce less ET than rat VSM (2). It is also possible that another trophic factor (such as VEGF) produced by VSM, but not ECs, is required for VSM to support neuronal survival.

Previous studies from this laboratory indicate that ET is also produced by postganglionic sympathetic neurons (4). The data in Figs. 1 and 3 indicate that when these neurons are grown in the absence of VSM, and in the absence of NGF, very few if any of the neurons survive. These data suggest that the amount of ET produced by the neurons themselves was less than that required for their survival. In the neuronal-VSM cocultures both neurons and VSM are producing ET (4), and the data in Figs. 1 and 3 suggest that the amount of ET in the coculture is enough to promote the survival of the neurons. Previous studies also from this laboratory report that VSM growth is stimulated in sympathetic neuronal-VSM cocultures and that this stimulation is mediated, at least in part, by ET (5). Thus ET produced by sympathetic neurons and VSM appears to play a role in coordinating the growth and development of both nerves and VSM in blood vessels.

The present findings indicate that in the absence of NGF, VSM and ET promote the survival of postganglionic sympathetic neurons. Under most physiological and/or pathological conditions, VSM synthesize NGF, and under most physiological and/or pathological conditions, VSM promotes the survival of postganglionic sympathetic neurons in the presence of NGF. This raises the question, would ET or other non-NGF vascular-derived factors affect the survival or development of sympathetic innervation in the presence of NGF. VSM did not enhance the survival of neurons grown in the presence of 50 ng/ml NGF (data not shown), a concentration of NGF that has been shown to be maximally effective at promoting sympathetic neuronal survival (Fig. 3B; see Ref. 13). This suggests that VSM-derived survival factors do not enhance the activity of maximally active concentrations of NGF. ET-1 did potentiate NGF-induced neuronal survival at low submaximal concentrations of NGF (Fig. 3B). It is known that not all postganglionic sympathetic neurons survive. In addition, in vivo studies in rats indicate that postnatal administration of NGF can increase the number of postganglionic sympathetic neurons and sympathetic innervation density in sympathetic targets including blood vessels (11, 25). These observations suggest that the concentration of NGF produced by VSM during the development of sympathetic innervation is not maximally inducing the survival of postganglionic sympathetic neurons and that ET or other VSM-derived factors could modulate neuronal survival.

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