Soluble Factors from Rat Basophilic Leukemia (RBL-2H3) Cells Stimulated Cooperatively the Neurite Outgrowth of PC12 Cells

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Culture media from rat basophilic leukemia cells (RBL-2H3) induced the neurite outgrowth of rat pheochromocytoma PC12 cells, a model system for neuronal differentiation. The extension of the neurite outgrowth was dependent on the culture time of RBL-2H3 cells in the DMEM medium. The DMEM medium conditioned by RBL-2H3 cells for 48 h induced neurite outgrowth of PC12 cells significantly. The neurite extension was much higher than that by medium containing 1 ng/ml nerve growth factor (NGF) but was rather lower than that by medium containing 10 or 50 ng/ml NGF. The neurite extension by 50 ng/ml NGF was completely suppressed by excess anti-NGF antibody (1—1.5 μ g/ml), while the extension by culture medium conditioned by RBL-2H3 cells for 48 h was not completely suppressed in the presence of the same amount of anti-NGF antibody. The neurite extension by the culture medium of RBL-2H3 cells was also suppressed by anti-interleukin (IL)-6 antibody (1 μ g/ml), although IL-6 itself (20 units) could scarcely induce the neurite outgrowth of PC12 cells. This suggests that IL-6 in the culture medium of RBL-2H3 cells could be effective in inducing the neurite extension in cooperation with NGF. In the presence of an excess of both anti-NGF and anti-IL-6 antibodies, the culture medium of RBL-2H3 cells induced the neurite extension of PC12 cells. This suggests that the action of the various factors from RBL-2H3 cells may be synergistic as far as the neurite outgrowth of PC12 cells is concerned.

Key words RBL-2H3 cell; PC12 cell; NGF; IL-6; neurite outgrowth

There is accumulating evidence of an interrelationship between neuroendocrinology and immunology: neuropeptides are known to directly affect a whole spectrum of immune functions, such as natural killer cell activity and immunoglobulin synthesis.^{1,2)} There has also been frequent observations of mast cells adjacent to nerves, coupled with the finding that mast cells are activated by a variety of neurotransmitters *in vitro*.¹⁻⁶⁾ However, the cellular interactions involved in the development and maintenance of nerve/mast cell association are still unknown. To study these interactions more closely, Bienenstock's group developed a tissue culture model of murine sympathetic neurons co-cultured with rat peritoneal mast cells or rat basophilic leukemia (RBL-2H3) cells, homologous with mucosal mast cells.¹⁻⁵⁾ In 3-d co-cultures, light microscopy and electron microscopy showed that neurites were in contact with peritoneal mast cells or RBL-2H3 cells but not with glial cells or fibroblasts.¹⁾

The rat pheochromocytoma PC12 cell line is a valuable model system for neuronal differentiation.⁷⁻¹¹⁾ In the presence of nerve growth factor (NGF), PC12 cells change their shape from round cells to neurite-extending cells and acquire the properties of sympathetic neurons. If PC12 cells are able to be co-cultured with peritoneal mast cells or RBL-2H3 cells, the cellular interactions involved in the development and maintenance of nerve/mast cell association need to be more closely studied than before. We have planned to study nerve/mast cell interaction using a model system of PC12 cells co-cultured with RBL-2H3 cells and here we have studied whether mediators from RBL-2H3 cells were able to stimulate neurite outgrowth of PC12 cells using cell culture inserts. The present results show that mediators from RBL-2H3 cells stimulated cooperatively the neurite outgrowth of PC12 cells as well as purified NGF and interleukin (IL)-6.

MATERIALS AND METHODS

DMEM was obtained from GIBCO (Grand Island, NY). FCS (fetal calf serum) was obtained from HyClone (Logan, UT) and HS (horse serum) was obtained from GIBCO. NGF (mouse submaxillary glands) was from UCB (Lake Placid, NY) and rat IL-6 (mouse and rat reactive recombinant IL-6) was from IBL (Gunma, Japan). Anti-NGF antibody from myeloma cells (clone 27/21) was obtained from Boehringer and anti-IL-6 antibody was from IBL. The NGF assay kit was obtained from Boehringer (Mannheim) and PC12 cells were obtained from RIKEN (Wako, Japan). RBL-2H3 cells were obtained as described in our previous papers.^{12,13)} PC12 cells and RBL-2H3 cells were grown at 37 °C, in an atmosphere of 5% CO₂ in DMEM with 5% fetal calf serum (FCS), 2.5% HS, 100 U/ml penicillin G and 100 μ g/ml streptomycin. The culture media were changed three times per week. PC12 cells proliferated with a round-shape phenotype without neurites in medium containing serum.

For the experiments of neurite outgrowth, PC12 cells were cultured for 12 h in tissue culture plates (Falcon, No. 3503: 12 wells) which contained DMEM medium without serum. Then, the medium was changed to a culture medium of RBL-2H3 cells with 5% FCS and 2.5% HS using the Falcon cell culture insert system from Becton Dickinson (Lincoln Park, NJ). The cell culture insert system involved the above tissue culture plate (Falcon, No. 3503: 12 wells) and cell culture inserts (Falcon, No. 3494: pore size=0.45 μ m). The cell culture inserts incorporated polyethylene tetraphthalate (PET) track-etched membranes which had symmetrical, cylindrical pores. Both sides of the membranes were treated for tissue culture and suitable for cell growth. The cell culture inserts were used with the companion tissue culture plate. Thus, only soluble factors from the culture medium of RBL-2H3 cells in the cell culture inserts were able to penetrate into the culture

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Fig. 1. Microscopic Images of the Neurite Extension of PC12 Cells Which Were Induced by Mediators from RBL-2H3 Cells or NGF

PC12 cells were cultured together with the culture medium of RBL-2H3 cells or NGF for 7 d at 37 °C. (a) As a control experiment, PC12 cells were cultured in DMEM medium containing 5% FCS and 2.5% HS alone. (b) PC12 cells were cultured in DMEM medium of RBL-2H3 cells after a 48-h incubation. (c) PC12 cells were cultured in DMEM medium containing 50 ng/ml NGF.

medium of PC12 cells in the companion tissue culture plate. After 3 d the culture medium in the cell culture inserts was changed to fresh DMEM medium of RBL-2H3 cells with 5% FCS and 2.5% HS and incubated for 4 more days.

The culture medium of RBL-2H3 cells was obtained by the following procedure. RBL-2H3 cells (10^6 cells/ml) were cultured in DMEM medium with 5% FCS and 2.5% HS for 6 or 48 h at 37 °C and the supernatants were collected by centrifugation twice at 1500 rpm. They were used for the culture medium of RBL-2H3 cells after a 6 or 48 h incubation.

The differential interference images of the neurite outgrowth were measured by a Zeiss confocal microscope (LSM 410).^{14,15} The percentages of cells with neurites were calculated by the following procedure. Random fields were scored and minimum 200 cells were counted per test well. Cells were considered positive for neurite outgrowth if they had a process extending >1 cell diameter.¹⁶ The length of the neurite extension was calculated from the total length of neurites in positive cells for outgrowth using a command, DISTANCE MEASURE, in the computer program associated with a confocal fluorescence microscope (LSM410, Zeiss).

RESULTS

PC12 cells proliferated with a round-shape phenotype without neurites in the DMEM medium containing serum. When the cells were starved of serum for 12 h, PC12 cells were synchronized in the 12 well plates, yielding a more homogeneous population of cells available to differentiate. To study the effects of soluble factors from RBL-2H3 cells on the neurite outgrowth of PC12 cells, we exchanged the serum-starved DMEM medium of PC12 cells in the wells using cell culture inserts for the culture medium (DMEM) of RBL-2H3 cells for 5% FCS and 2.5% HS. PC12 cells responded to soluble factors from RBL-2H3 cells and their neurite-outgrowth was observed by a confocal laser microscope (Zeiss LSM410) as shown in Fig. 1. PC12 cells responded to the DMEM medium conditioned by RBL-2H3 cells for 6 h, however, the response was much weaker than that in the case of medium for 48 h (see Fig. 1b). As a control experiment we exchanged the serum-starved DMEM medium of PC12 cells in the 12 well plates for the DMEM medium for 5% FCS and 2.5% HS alone. There was very lit-

Fig. 2. Measurements of Neurite Outgrowth of PC12 Cells

The percentage of PC12 cells with neurites was determined by the following methods. A random field was scored and a minimum of 200 cells was counted per test well. Cells were considered positive for neurite outgrowth if they had a process extending >1 cell diameter.¹⁶ The values were calculated after quadruplicate experiments. Wells were scored after a 7-d incubation. Bars represent standard deviations.

tle neurite extension of PC12 cells as shown in Fig. 1a. This suggested the possibility that PC12 cells could respond to soluble factors from RBL-2H3 cells in a dose-dependent manner.

For comparison, we performed similar experiments using DMEM medium containing NGF (1–50 ng/ml). In this experiment we exchanged the serum-starved DMEM medium of PC12 cells in the 12 well plates for the DMEM medium (with 5% FCS and 2.5% HS) containing 1, 10 or 50 ng/ml NGF. Neurite outgrowth was much more significant in the case of 10 and 50 ng/ml NGF as shown in Fig. 1c. However, 1 ng/ml NGF had little effect in inducing the neurite extension of PC12 cells. Following the previous procedure,¹⁶ we calculated the percentage of PC12 cells with extended neurites as shown in Fig. 2. In the absence of the culture medium of RBL-2H3 cells or NGF, virtually no neurite extension of PC12 cells was observed (see Fig. 2). The culture media of RBL-2H3 cells for a 6- and 48-h incubation scored 40–60% of PC12 cells with neurites. The percentage of cells with neurites was much higher in the case of 10 ng/ml or 50 ng/ml NGF (70–90%). However, the neurite extension was much less pronounced in the case of 1 ng/ml NGF. In this case the

Fig. 3. Length of the Neurite Outgrowth in PC12 Cells after a 7-d Incubation with the Media Described Below

In this experiment the total length of the extension in positive cells was calculated as described in Materials and Methods. (a) DMEM medium alone, (b) DMEM medium from RBL-2H3 cells after a 6-h incubation, (c) DMEM medium from RBL-2H3 cells after a 48-h incubation. (d), (e) and (f) DMEM medium containing 1 ng/ml, 10 ng/ml and 50 ng/ml NGF, respectively. Bars represent standard deviations.

neurite extension was 20%. Thus, the culture media of RBL-2H3 cells for the 6- and 48-h incubation induced the neurite extension of PC12 cells more effectively than 1 ng/ml NGF.

Next, we tried to measure the lengths of the neurites of PC12 cells after a 7-d culture using the culture medium of RBL-2H3 cells or NGF. In this experiment we calculated the total lengths of neurites in positive cells in Fig. 2. The average values of neurite outgrowth were 30 and 65 μm for the culture media from RBL-2H3 for the 6- and 48-h incubation, respectively and 25, 75 and 165 μm for 1, 10 and 50 ng/ml NGF, respectively. In the control experiments, the length of the neurites was about 5 μm as shown in Fig. 3.

Then, we added anti-NGF antibody to the culture medium of PC12 cells. Neurite outgrowth of PC12 cells was suppressed drastically by anti-NGF antibody in a dose-dependent manner. In a control experiment we added mouse non-specific antibody (1 μg/ml) to the culture medium of PC12 cells. Neurite outgrowth of PC12 cells was not suppressed in such experiments. Closed circles represent the culture medium of RBL-2H3 cells (48 h culture) and open circles NGF (50 ng/ml) as shown in Fig. 4. The neurite extension induced by NGF was completely inhibited by anti-NGF antibody (1–1.5 μg/ml), however, the extension induced by soluble factors from RBL-2H3 cells was not completely inhibited by the same amount of anti-NGF antibody (see Fig. 4).

Then, we measured the amount of NGF in the culture medium of RBL-2H3 cells for a 48 h incubation using an NGF-assay kit. The amount of NGF in the culture medium was calculated to be 0.26 (± 0.08) ng/ml. The amount of NGF in the culture medium of RBL-2H3 cells (48 h) was much less than the amount of NGF used in the experiments shown in Figs. 2 and 3. This suggested the possibility that other mediators besides NGF should be effective in inducing the neurite outgrowth of PC12 cells in cooperation with NGF.

Next, we measured the effect of anti-IL-6 antibody on the neurite outgrowth of PC12 cells. The neurite outgrowth induced by the culture medium of RBL-2H3 cells was apparently suppressed by anti-IL-6 antibody (1 μg/ml) as shown in Fig. 5c. The neurite outgrowth induced by the soluble factors from RBL-2H3 cells was reduced to two thirds of the out-

Fig. 4. Effects of Anti-NGF Antibody Concentration on the Neurite Extension of PC12 Cells

The neurite extension was suppressed by anti-NGF antibody in a dose-dependent manner. The horizontal axis shows the concentration of anti-NGF antibody. (●), The neurite outgrowth was induced by the mediators from RBL-2H3 cells after 48 h incubation. (○), The neurite outgrowth was induced by 50 ng/ml NGF. Bars represent standard deviations.

Fig. 5. Cooperative Effects of Mediators from RBL-2H3 Cells on the Neurite Outgrowth of PC12 Cells

(a) Neurite outgrowth of PC12 cells induced by the culture medium of RBL-2H3 cells after a 48-h incubation. (b) Neurite outgrowth of PC12 cells induced in a similar fashion as (a) in the presence of 1 μg/ml anti-NGF antibody. (c) Neurite outgrowth of PC12 cells induced in a similar fashion as (a) in the presence of 1 μg/ml anti-IL-6 antibody. (d) Neurite outgrowth of PC12 cells induced in a similar fashion as (a) in the presence of both anti-NGF (1 μg/ml) and anti-IL-6 (1 μg/ml) antibody. Bars represent standard deviations.

growth without anti-IL6 antibody. However, hardly any neurite extension was induced by IL-6 (20 units) alone (data not shown). In the presence of both anti-NGF and anti IL-6 antibodies, the neurite extension was further reduced to one third of the value in the absence of any antibodies, as shown in Fig. 5d. The neurite outgrowth was shortest in the presence of both anti-NGF and anti-IL-6 antibodies (see Fig. 5d) but the neurite extension was not completely suppressed to the background level shown in Fig. 3a. This suggested that several soluble factors from RBL-2H3 cells could induce the neurite outgrowth of PC12 cells in a cooperative fashion.

DISCUSSION

The rat pheochromocytoma PC12 cell line is a valuable model system for neuronal differentiation. In the presence of

NGF, PC12 cells change their shape from round cells to neurite-extending cells and acquire properties of sympathetic neurons.⁷⁻¹¹⁾ In addition, it was shown that IL-6 was able to induce neuronal differentiation of PC12 cells through specific receptors.^{8,9,11,17)}

In the present experiments we showed that the culture medium of RBL-2H3 cells was able to induce the neurite outgrowth of PC12 cells as well as NGF. The neurite extension induced by the soluble factors of RBL-2H3 cells was significantly suppressed by anti-NGF antibody (1 μ g/ml) and/or anti-IL-6 antibody (1 μ g/ml). Although IL-6 (20 units) itself induced hardly any neurite extension of PC12 cells, IL-6 induced the neurite extension of PC12 cells in cooperation with NGF.^{11,17)} In addition, the neurite extension induced by the soluble factors of RBL-2H3 cells was not completely suppressed by the presence of both anti-NGF and anti-IL-6 antibodies. This suggests that other soluble factors from RBL-2H3 cells besides NGF and IL-6 could be effective in inducing the neurite outgrowth of PC12 cells.

At present we have not yet determined what kind of soluble factors in RBL-2H3 cells promote the neurite outgrowth of PC12 cells. However, several cytokines and soluble factors are known to be released from mast cells and basophils, e.g., IL-3, IL-4, IL-5, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF)- α , leukemia inhibitory factor (LIF), macrophage inflammatory protein (MIP).¹⁸⁻²⁰⁾ Thus, these cytokines and/or soluble factors from RBL-2H3 cells might induce the neurite outgrowth of PC12 cells as shown in Fig. 5d. In the present experiments we suggest that several soluble factors from RBL-2H3 cells could induce the neurite outgrowth of PC12 cells in a cooperative fashion. It seems that this kind of cooperation is important for nerve/mast cell interaction.

In a previous paper Bienenstock's group showed that a functional interaction between mast cells and peripheral nerves occurred at sites of association *in vivo*.¹⁾ To study these interactions, they developed a tissue culture model of murine sympathetic neurons (superior cervical ganglia) co-cultured with RBL-2H3 cells (homologous to mucosal mast cells). In their experiments apparent contacts between neu-

rites and RBL-2H3 cells were detected. From their results they speculated that several factors from RBL-2H3 cells could induce neurite/RBL contacts. Thus, the soluble factors described in the present experiment may be also involved in the induction of the neurite outgrowth of superior cervical ganglia as well as that of PC12 cells.

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