BINDING AND THERMAL DISSOCIATION OF NERVE GROWTH FACTOR AND ITS RECEPTOR ON HUMAN MELANOMA CELLS

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SUMMARY: The interaction between mouse nerve growth factor (NGF) and its receptor was studied on live and formaldehyde-fixed human melanoma cells in culture. These cells contain 5-8 x 10^5 NGF receptors per cell. The pH optima of this ligand-receptor association was 6.4. The kinetics of dissociation at 4°C was similar for the fixed and live cells; at 22°C, NGF readily dissociated from the fixed cells whereas the live cells showed little dissociation. Radioactive NGF which had been dissociated at 4°C from NGF-receptor containing cells was able to rebind with greater efficiency. With the dissociation of NGF from the cell surface, there was a concomitant increase in the number of available receptor sites. The initial events in the interaction of NGF and its receptor on human melanoma cells are reversible.

INTRODUCTION: Nerve growth factor (NGF) (1-3) interacts on an embryologically discrete class of cell types in culture, those derived from the neural crest (4). This group of cells includes sympathetic and sensory ganglia (5,6) and neuroblastomas (7). In a previous publication (8), we have shown that human melanomas in culture have high levels of specific receptors for NGF, probably reflecting their ancestral tissue of origin. In the present work, we further characterize the interaction between NGF and its receptor on human melanoma cells in culture.

MATERIALS AND METHODS

Cells and Media. The human melanoma line A875 used in these experiments has been described (8,9). These cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% fetal calf serum (Colorado Serum Co.).

Iodination of NGF. All experiments were performed with the 2.5S subunit of mouse NGF (10). Preparation of [^{125}I]NGF used in these experiments has been described (8). The specific activities of preparations used were 5-20 μCi/μg.

Formaldehyde Fixation. A875 cells were seeded in FB-16-24TC Linbro plates 16 hr before use and grown to a density of 40,000 cells per well. The wells were washed twice with Dulbecco's modification of Eagle's minimal media (DMEM) and 1 ml of 0.5% formaldehyde in DMEM was added to the cells for 20 min at 22°C. The fixative was removed and the cells were washed four times with DMEM. Plates prepared in this manner showed 90% of original binding activity after fixation. They could be stored at -60°C for two months without significant loss of binding capacity.

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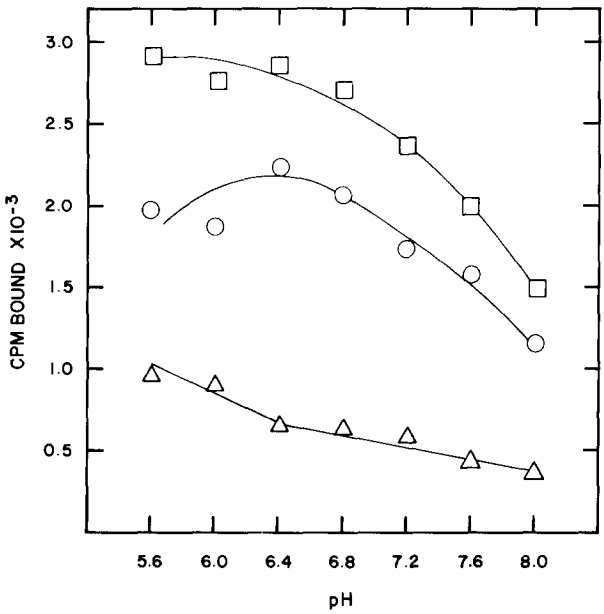

Figure 1: Binding of NGF to melanoma cells at different pHs.

Twenty-four hr before use, A875 cells were seeded in Linbro FB-16-24TC plates at a density of 2 x 10^5 cells per well. The cells were washed twice with buffered solutions containing 100 mM NaCl, 1 mM $CaCl_2$, 0.1% bovine plasma albumin, and 50 mM N,N'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) at various pHs, adjusted by titrating with hydrochloric acid or sodium hydroxide. Three ng of ^{125}-labeled NGF was added to the cells at the different pHs. Control wells at each pH received excess unlabeled NGF (2 µg) to determine the nonspecific binding. After a 60-min incubation at 22°C the cells were washed, lysed, and the bound radioactivity determined as described in the Materials and Methods. The total counts bound (□), the nonspecific counts bound (Δ), and the difference between them, the specific counts bound (o), are shown.

Binding Assays. The procedure for binding [^{125}I]NGF to the receptor-bearing A875 cells has been described (8). This protocol was modified for binding [^{125}I]NGF to formaldehyde-fixed cells in FB-16-24TC Linbro plates by substituting 0.5 ml of 5% sodium dodecyl sulfate for the usual lysing buffer. This fluid was transferred to scintillation vials containing 15 ml of Aquasol (New England Nuclear) and the wells were washed twice with the lysing buffer (0.5% sodium dodecyl sulfate, 10 mM Tris pH 7.4, and 1 mM EDTA).

RESULTS: Figure 1 shows the pH optimum for binding [^{125}I]NGF to the human melanoma cell line, A875. It is apparent that acid conditions increased both specific and nonspecific binding. The nonspecific binding rose rapidly when the pH was decreased below 6.4. The pH optimum for specific binding, obtained by subtracting the nonspecific binding from the total binding, was pH 6.4. In previous work (10) and in the other experiments presented in this publication,

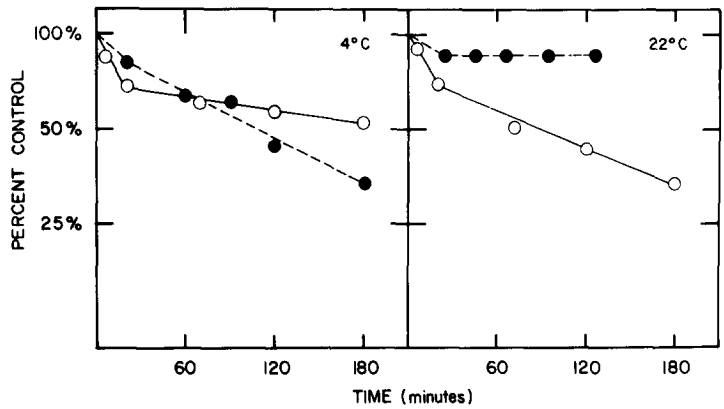

Figure 2: Dissociation of [^{125}I]NGF from formaldehyde-fixed and viable cells.

A875 cells were grown to a density of 4 x 10^4 per well in FB-16-24TC Linbro plates. The cells were either fixed with formaldehyde as described in the Materials and Methods and stored at -60°C or used as live monolayers. Both plates of these formaldehyde-fixed cells (o) and of viable cultures (•) were washed twice with the binding buffer and incubated with [^{125}I]NGF (3 ng/well in 250λ of binding buffer) for 60 min at 22°C. [^{125}I]NGF was removed and the wells were washed four times with binding buffer. Samples were incubated with 250λ of binding buffer for various times at either 4°C or 22°C and duplicate wells were washed twice and solubilized as described; the bound [^{125}I]NGF was determined as described.

binding was done at pH 6.8 in a solution containing Dulbecco's modification of Eagle's medium.

In order to compare the kinetics of dissociation of [^{125}I]NGF on formaldehyde-fixed and viable cells, bound [^{125}I]NGF was determined as a function of time of dissociation at 4°C and 22°C. The $T_{1/2}$ for dissociation on live cells at 4°C has been shown to be approximately 2 hr (8,11). As seen in Fig. 2, bound [^{125}I]NGF dissociated from fixed cells at both temperatures tested, though essentially no dissociation occurred from live cells at 22°C. The failure of viable cells to dissociate [^{125}I]NGF, and the capacity of fixed cells to do so, may imply an active sequestration of the bound ligand by growing cultures.

To determine whether the [^{125}I]NGF released from the surface receptors of the fixed and live cells was still active, previously bound [^{125}I]NGF was collected in fresh binding buffer as it dissociated from A875 at 4°C. As seen

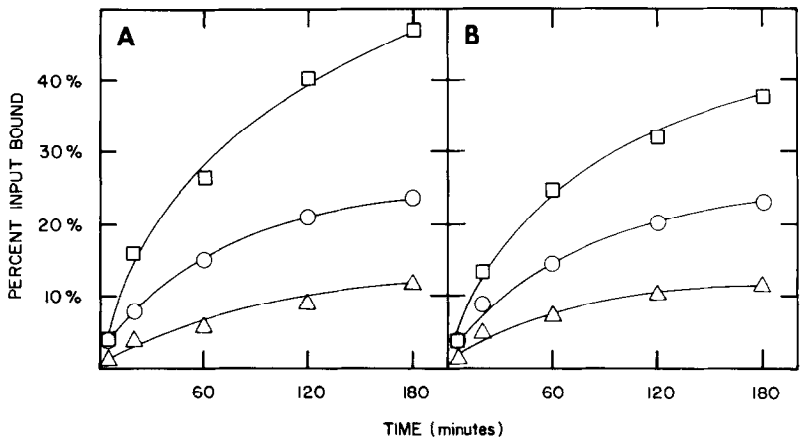

Figure 3: Recycling of dissociated [^{125}I]NGF.

Parallel cultures of A875 were grown to a density of 4 x 10^6 cells per T-75 (Falcon); (A) was fixed with formaldehyde as described in the Materials and Methods and (B) was used with the cells remaining viable. These flasks were incubated with 200 ng of [^{125}I]NGF in 8 ml of the binding buffer for 120 min at 37°C. The unbound material was removed, the cells were washed, and 8 ml of fresh binding buffer was added to the culture for 120 min at 4°C to collect dissociated [^{125}I]NGF. Fresh (o), unbound material (Δ) and dissociated (□) [^{125}I]NGF were diluted to 8000 cpm per ml and tested for their ability to bind to A875 (6 x 10^4 cells/well grown in FB-16-24TC Linbro plates) using 250 µl of each solution per well. The ordinate represents the percent of input cpm which specifically bound to the cells.

in Fig. 3, the percent of binding activity in a radiolabeled preparation of NGF can be enhanced by such a recycling experiment. The [^{125}I]NGF which had not bound to cells after a 120-min incubation, in contrast, was less able to bind when recycled on fresh cells.

In Fig. 4, the ability of receptors to bind new NGF molecules, after having dissociated previously bound NGF, was tested. In this experiment, some of the cells were prebound with saturating amounts of unlabeled NGF. As this cold NGF dissociated at 4°C, as evidenced by a concurrent dissociation curve with [^{125}I]NGF, the available surface receptors were determined at various times by removing the dissociated unlabeled NGF and exposing the cells to [^{125}I]NGF for 60 min. As shown in Fig. 4, the cells bound increasing amounts of [^{125}I]NGF with time, showing that the cellular receptors which had bound and released unlabeled NGF were competent in their ability to bind [^{125}I]NGF upon reexposure.

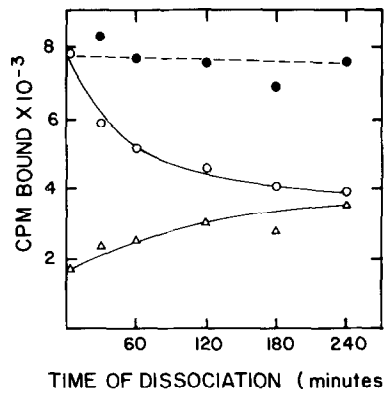

Figure 4: Recycling of NGF receptors.

Cultures of A875 were grown to a density of 2.7 x 10^5 cells per 20 cm^2 petri dish. After washing two times with the binding buffer, 20 ng of [^{125}I]NGF (33,000 cpm) was added in 1.5 ml to half the dishes (o) for a 60-min incubation at room temperature. [^{125}I]NGF was removed and the cells were washed four times with cold binding buffer. 1.5 ml of cold binding buffer was added. The samples were incubated at 4°C and, at various times, duplicates were lysed and the [^{125}I]NGF remaining was determined.

After washing the other dishes twice, 1.5 ml of the binding buffer with unlabeled NGF at 200 ng/ml was added for a 60-min incubation at 22°C (Δ). The cells were washed four times and 1.5 ml of fresh binding buffer was added at 4°C. At various times, the buffer containing dissociated unlabeled NGF was removed from the dishes and 20 ng of radiolabeled NGF in 1.5 ml of binding buffer was incubated with the culture for 60 min at 4°C. The dishes were washed and the bound radioactivity was determined. The counts remaining after dissociation (o), the counts binding to newly available receptors (Δ), and the sum of these (•) are plotted as a function of time of dissociation.

The sum of the counts remaining after dissociation and the counts binding to newly available receptors remained constant as a function of time of dissociation. This implies a one-to-one correspondence in the dissociated receptors' ability to accept new radiolabeled ligands in place of the unlabeled molecules which had dissociated. At 4°C, there is no synthesis of new receptors by the melanoma cells (data not shown).

DISCUSSION: Under the conditions used it is possible to bind NGF, and perhaps other molecules that recognize the NGF receptors, in such a way that both the ligand and the receptor can be dissociated and can function again.

Cells can be fixed with formaldehyde and stored frozen. [^{125}I]NGF which binds to such cells can be dissociated from the fixed receptor. This binding

opens the possibility that melanoma cells can be used to purify NGF and related substances from cell cultures and tissue fluids. Preliminary evidence suggests that supernatants of the NGF producing mouse sarcoma 180 cells can be concentrated and small amounts of the concentrates iodinated to yield radiolabeled material which specifically binds to human melanomas. This bound material may be dissociated from the melanoma lines and shown to be greatly enriched for a protein which specifically binds to the receptor-bearing melanomas. This procedure may provide a gentle means of purifying NGF-related growth factors.

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REFERENCES

1. Levi-Montalcini, R., and Hamburger, V. (1953) J. Exp. Zool., 123, 233-288.

2. Cohen, S., and Levi-Montalcini, R. (1956) Proc. Nat. Acad. Sci. USA, 42, 571-574.

3. Levi-Montalcini, R., and Angeletti, R. U. (1968) Physiol. Rev., 48, 534-569.

4. Colombre, A., Johnson, M., and Eston, J. (1974) Dev. Biol., 36, F1-F5.

5. Levi-Montalcini, R., and Angeletti, R. E. (1963) Dev. Biol., 7, 653-659.

6. Stoekel, K., Schwab, M., and Thoenen, H. (1975) Brain Res., 89, 1-14.

7. Revoltella, R., Bertolini, L., Pediconi, M., and Vignett, R. (1974) J. Exp. Med., 140, 437-451.

8. Fabricant, R. N., De Larco, J. E., and Todaro, G. J. (1977) Proc. Nat. Acad. Sci. USA, 74, 565-569.

9. Giard, D. S., Aaronson, S. A., Todaro, G. J., Arnstein, P., Kersey, J. H., Dosik, H., and Parks, W. P. (1973) J. Nat. Cancer Inst., 51, 1417-1423.

10. Bocchini, V., and Angeletti, P. U. (1969) Proc. Nat. Acad. Sci. USA, 64, 787-794.

11. Herrup, K., and Shooter, E. M. (1975) J. Cell Biol., 67, 118-128.