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HUMAN FIBROBLAST CELLS SYNTHESIZE AND SECRETE NERVE GROWTH FACTOR IN CULTURE

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SUMMARY: Using our enzyme immunoassay system developed for recombinant hNGF, we examined the synthesis and secretion of human NGF (hNGF) by human fibroblast (WS-1) cells. The amount of the factor secreted by WS-1 cells increased linearly and a significant amount of NGF was detected in the conditioned medium of WS-1 cultures. WS-1 NGF showed properties identical to those of recombinant human NGF in immunoreactivity and molecular weight. An increase in cell density or the withdrawal of serum from the culture medium caused a drastic decrease in the rate of NGF secretion. These results suggest that WS-1 cells are able to synthesize and secrete hNGF in culture and that the synthesis/secretion is regulated in a growth phase-dependent manner.

Nerve growth factor (NGF) is a target-derived trophic protein that is known to play an important role in the development and survival of peripheral sensory and sympathetic neurons as well as cholinergic neurons of the basal forebrain (1-4). Studies using Northern blotting and enzyme immunoassay (EIA) methods indicate that certain types of cells such as peripheral fibroblast cells (3, 5-8), smooth muscle cells (3,9), epithelial cells (10), Schwann cells, and astroglial cells from mouse and rat (11,12) synthesize and secrete NGF. We have shown that mouse fibroblast cells from heart and astroglial cells synthesize and secrete an extremely large amount of mouse NGF (mNGF) (6,12). However, since human NGF (hNGF) is not detected by the EIA for mNGF, owing to the low level of crossreactivity between the two factors (13), the extent to which hNGF levels change in neuronal disorders and the primary source of biosynthesis of hNGF in vivo are still unknown.

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Recently, we developed a two-site sandwich EIA for hNGF using polyclonal antibody against recombinant hNGF (13). This is a very sensitive and practical assay system for hNGF that can detect as low as 1 pg/ml of hNGF, whereas our EIA system for mNGF could detect hNGF only at a concentration above 100 pg/ml. Thus, our new EIA for hNGF is much more suitable to detect small physiological amounts of hNGF.

Using this highly sensitive enzyme immunoassay (EIA) system, we examined whether or not human foreskin fibroblast cell (WS-1) can synthesize and secrete NGF. This report describes the synthesis and secretion of a large amount of an hNGF-like immunoreactive factor by WS-1 cells in culture.

MATERIALS AND METHODS

Materials

Recombinant human nerve growth factor (rhNGF) and anti-rhNGF antiserum were prepared as previously described (14). DMEM was obtain from Nissei; FCS, from Bocknek; BSA, from Armour; actinomycin D, from Makor; cycloheximide, from Nakarai Chemicals Co., Ltd.; cytosine-β-D-arabinofuranoside, from Sigma; cell culture vessels and EIA plates were purchased from Falcon; and standard proteins for molecular weight determination, from Pharmacia.

Cell culture

WS-1 cells were purchased from Flow. Cell were grown in tissue culture flasks (bottom surface, 25 cm^2) or in 24-well plates (2.1 cm^2) in the presence of DMEM supplemented with 10% FCS in an humidified atmosphere of 5% CO_2 at 37 °C. The cells were fed every 3 days. Protein concentrations were determined by the Bradford Coomassie blue technique with BSA as the standard. One mg of cell protein is equivalent to 2.1×10^6 cells.

Two-site EIA

The EIA is based on the sandwiching of antigen between anti-NGF antibody IgG coated on polystyrene plates and biotinylated anti-NGF antibody IgG (13). The bound antibody complex was quantified with streptavidin linked-β-D-galactosidase.

Preparation of cell extracts

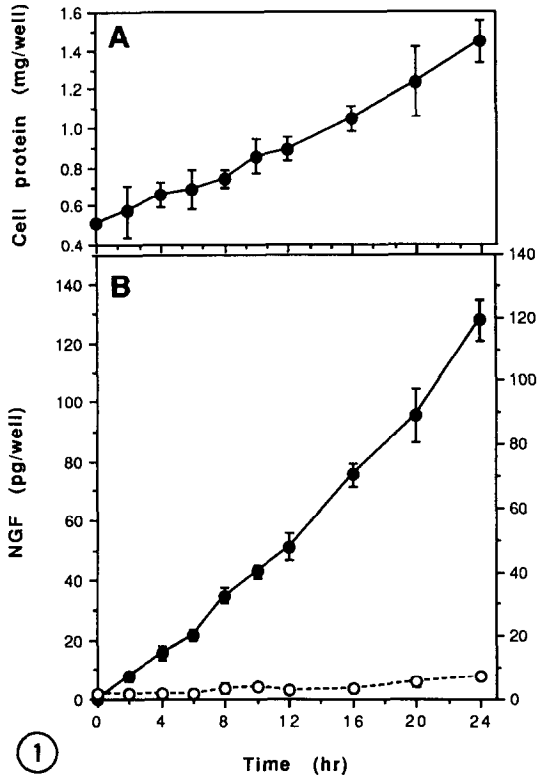
Cells were washed twice with PBS and scraped from the wells in 0.15 ml of 0.1M NaOH. NaOH extracts were neutralized with an equal volume of 0.1M HCl, and total cell protein was determined in an 25-μl aliquot of the extract by the Bradford Coomassie Blue technique with BSA as standard. For NGF analysis, the extract were homogenized with 0.75 ml of 0.1M Tris-HCl buffer, pH 7.6, containing 0.4M NaCl, 1mM $MgCl_2$, 0.1% BSA, and 0.1% NaN_3; and a 0.02-ml aliquot was applied to the EIA system.

RESULTS AND DISCUSSION

WS-1 cells in culture secreted a molecule that immunologically cross-reacted with rhNGF. This molecule is hereafter called WS-1 NGF. Fig.1 shows the time course of secretion of WS-1 NGF. When WS-1 cells reached subconfluence in 24-well plates, the medium was changed. The conditioned medium (CM) was collected at the culture times of 0, 2, 4, 6, 8, and 10 hr; and the

amount of WS-1 NGF in each CM sampled was measured by the two-site EIA specific for rhNGF. The NGF content in the CM increased in proportion to the culture time, when the cells were in the exponential growth phase. The content of intracellular NGF was low compared with the amount of extracellular NGF and remained essentially constant. These results indicate that NGF is synthesized by WS-1 cells during their incubation in culture and secreted rapidly.

The molecular properties of the WS-1 NGF were investigated. The immunoreactivity of WS-1 NGF in the CM was compared with that of authentic rhNGF by the EIA. WS-1 NGF and rhNGF were serially diluted with DMEM containing 10% FCS and used in the EIA. As shown in Fig.2, the dose-response curve obtained with WS-1 NGF was identical to that of rhNGF. This result indicated that there was no antigenic difference between WS-1 NGF and rhNGF.



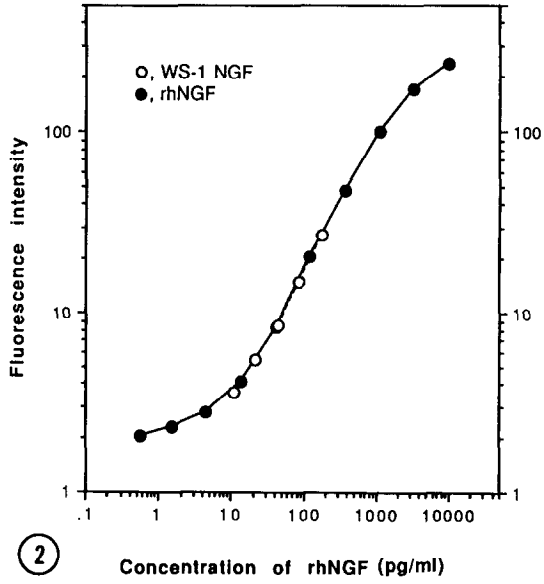


Fig.1. Change in the amounts of extracellular WS-1 NGF, intracellular WS-1 NGF, and cell protein of WS-1 cells during culture.
When the cells reached the subconfluent stage in 24-well culture plates, the culture medium was changed to fresh DMEM containing 10% FCS (0.4 ml). At the indicated times, the amounts of cell protein (A), and extracellular (●) and intracellular (o) WS-1 NGF (B) were determined by EIA. Each point is the mean ± S.E. of four determinations.

Fig.2. Antigenic relationship between WS-1 NGF and rhNGF.
CM of WS-1 cells and rhNGF were serially diluted 3-fold and 2-fold, respectively, with DMEM containing 10% FCS, and a 0.02-ml aliquot of each dilution was used for the EIA. Each point is the mean; S.E. is not shown, as it was less than the width of the symbol.

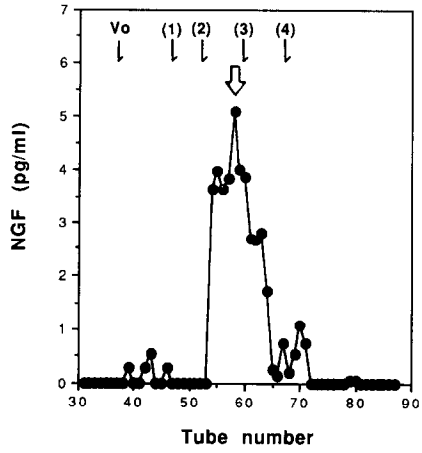


Fig.3. Gel filtration analysis of WS-1 NGF in WS-1 cell CM on a column of Bio-gel P-100. A 0.35-ml volume of WS-1 cell CM (190 pg WS-1 NGF/ml; 24 hr culture) was applied to a Bio-gel P-100 column (1.1x57.0 cm) equilibrated with 0.1M Tris-HCl buffer, pH 7.6, containing 0.4M NaCl, 1mM $MgCl_2$, 0.02% NaN_3, and 2% BSA. Fractions of 0.5 ml were collected at a flow rate of 7.5 ml/hr, and a 0.02-ml aliquot of each fraction was examined by EIA. Values are expressed as the mean of four determinations. Calibration of the column for estimation of the molecular weight of WS-1 NGF was performed by use of standard proteins: Vo) blue dextran; 1)BSA (Mr=67K); 2) ovalbumin (Mr=43K); 3) chymotrypsinogen A (Mr=25K); 4) cytochrome c (Mr=12.5K). The open arrow and the solid ones indicate the respective positions where rhNGF and marker proteins were eluted.

Then the molecular weight of WS-1 NGF was determined by gel filtration on a column of Bio-gel P-100. As shown in Fig.3, WS-1 NGF was eluted from the column at a position identical to that of rhNGF. The position of rhNGF, which was determined by a separate run under the same conditions, is indicated as an open arrow in Fig.3. From calibration of the column with marker proteins, the molecular weight of the WS-1 NGF was estimated as 26 K daltons. In the dorsal root ganglion bioassay, the WS-1 NGF (0.1 ng WS-1 NGF/ml; measured by the EIA) elicited a typical neurite outgrowth (+1 response), and the effect was decreased by the addition of anti hNGF antibody IgG (data not shown). This level of activity was similar to that observed with rhNGF. These findings indicate that the material(s) with NGF-like properties in the CM of WS-1 cells is a molecule identical to the rhNGF.

Next we examined the relationship between NGF secretion and cell growth. Fig.4 shows the change in the cell number (A), the amount of NGF secreted into the medium per day (B), and the amount of NGF secreted per 10^6 cells per day (C) during growth of the WS-1 cells. The cells were introduced into the dishes as a small inoculum at day 0, proliferated exponentially, and reached confluence at day 7. The doubling time was estimated as 11.4 hr. The NGF content in the CM is considered to be the amount of NGF synthesized since secretion was rapid and the

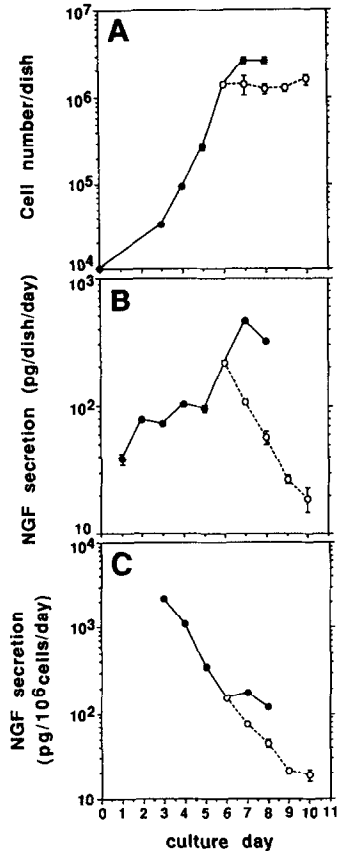


Fig.4. Relationship between cell growth and NGF secretion by WS-1 cells.
A: Growth curve of WS-1 cells. B: Change in amount of NGF secreted/dish/day. C: Change in the amount of NGF secreted/10^6cells/day.

WS-1 cells were cultured at about $1x10^4$ cells/35 mm dish in 1 ml of DMEM containing 10% FCS. On day 6, the medium of some of the dishes was replaced with 1 ml of DMEM containing 0.5% BSA, and the cells were then cultured for an additional 4 days. All CM were collected and replaced with fresh media every 24 h. An aliquot of each CM (0.02 ml) collected was used in the EIA for the measurment of its NGF content. The closed circles with solid line indicate the values in the CM with DMEM containing 10% FCS, while the open circles with dashed lines indicate the values in the CM with 0.5% BSA. Each point is the mean $\pm$ S.E. of four determinations.

intracellular content of NGF was low (Fig.1). The amount of NGF secreted/dish increased with the increase in cell number when the cells were in the exponential growth phase, and gradually decreased after confluence was reached (Fig.4B). The amounts of NGF secreted/10^6 cells are shown in Fig.4C. An over twenty-fold difference was observed between the maximal (2ng/10^6 cells) and the minimal (120pg/10^6 cells) values. Serum deprivation also caused decreased secretion of NGF by WS-1 cells. These observations suggest that NGF synthesis/secretion by WS-1 cells may be regulated in a growth phase-dependent manner.

The effects of actinomycin D, an inhibitor of DNA-dependent RNA synthesis, cycloheximide, an inhibitor of proteinsynthesis, and cytosine arabinoside, an inhibitor of DNA polymerase, on

WS-1 NGF content in the CM were examined next. When the cells were incubated for 24 hr in the presence of actinomycin D (11.1μg/ml) or cycloheximide (100μg/ml), the respective amount of WS-1 NGF in the CM decreased to 5.5±1.5 % (n=4) or 17.3±2.1 % (n=4) of the value in the absence of the drug. In contrast, cytosine arabinoside did not show any appreciable effect on synthesis and secretion of WS-1 NGF, even at the concentration of 1mM. These results demonstrate that the increase in WS-1 NGF in WS-1 cell cultures is due to DNA transcription-dependent protein synthesis and not to DNA replication.

It has been reported that mouse and rat fibroblast cells and Schwann cells synthesize and secrete NGF. However, NGF secretion from human tissue had not been documented until now, and this domonstration is attributable to our two-site enzyme immunoassay system that allows sensitive, quantitative, and reliable determination of hNGF (13). In this report, we have shown direct evidence that human foreskin fibroblast (WS-1) cells in culture actually synthesize and secrete into the medium a large amount of molecules identical to hNGF in term of immunoreactivity (Fig.2), molecular weight (Fig.3), and neurite outgrowth-stimulating activity. The manner of NGF synthesis and secretion resembles that of mouse fibroblast cells and astroglial cells (6,14), i.e., these three cell types synthesize NGF in a DNA transcription-dependent and growth-dependent manner and secrete it quickly. It is probable that the expression of some genes relevant to cell growth is associated with the upregulation of NGF synthesis. In the human peripheral nervous system fibroblast cells in the sympathetically innervated end organ may synthesize and secrete NGF for use by neurons for their survival and maintenance of function as is the case in the mouse and rat.

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