Nerve Growth Factor Is Mitogenic for Cancerous but Not Normal Human Breast Epithelial Cells*

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We show here that nerve growth factor (NGF), the archetypal neurotrophic factor, is able to stimulate the proliferation of breast cancer cells (MCF-7 and MDA-MB-231 cell lines), although it is unable to stimulate growth of normal breast epithelial cells (NBEC). This stimulation induced cells in the G0 phase to reenter the cell cycle, as well as shortening cell cycle duration. Immunoblotting experiments revealed that both the two cancer cell lines and the NBEC express high affinity (p140trkA) and low affinity (p75) NGF receptors. Inhibition of the NGF growth-promoting effect by the drugs K-252a and PD98059 indicated that activation of Trk-tyrosine kinase activity and the mitogen-activated protein kinase cascade are necessary to obtain the mitogenic effect. Activation of mitogen-activated protein kinase can be detected in breast cancer cells after 10 min of NGF stimulation, whereas no change was detected in NBEC. These results demonstrate that NGF is a mitogenic factor for human breast cancer cells and that it might constitute a new regulator of breast tumor growth.

The growth of breast cancer cells can be regulated by various growth factors that stimulate or inhibit their proliferation and/or differentiation (1). For example, epidermal growth factor, insulin-like growth factors, and transforming growth factor α can all stimulate the proliferation of breast cancer cells, whereas mammary-derived growth factor inhibitor and transforming growth factor β inhibit their growth. In fact, with the exception of mammary-derived growth factor inhibitor, these molecules were not first identified as growth regulators in breast cancer cell assays but only secondarily appeared to be implicated in mammary tumor development.

Nerve growth factor (NGF)1 is the prototypical member of the neurotrophin polypeptide family (2), which includes brain-derived neurotrophic factor (BDNF) and neurotrophins 3–6 (NT-3, NT-4/5, and NT-6). NGF is essential for the survival and differentiation of central and peripheral neuronal cells, and its role in the development and regeneration of the sympathetic and sensory nervous system has been extensively described (3). It has been shown to interact with two types of membrane binding sites: the high affinity receptor tyrosine kinase (p140trkA), which corresponds to the TrkA proto-oncogene, and an accessory receptor known as p75 for which the precise function is still controversial but does not involve tyrosine kinase activity (4, 5). In addition to its neurotrophic function, several other activities for NGF have been described. For example, exposure to an NGF gradient is chemotactic for melanocytes (6), and antibodies directed against either NGF or p75 strongly inhibit the migration of Schwann cells (7). NGF has also been reported to stimulate the proliferation of a number of cell types such as chromaffin cells (8) and lymphocytes (9). Blocking antibodies directed against NGF can inhibit the cellular growth of keratinocytes, which led to the idea that NGF might be an endogenous mitogenic factor produced by these cells (10). The potential mitogenic activity of NGF raises the question of its possible implication in carcinogenesis. Until now, evidence implicating NGF in cancer was derived from human prostatic cells where production of NGF induces tumor cell growth and invasion (11, 12). This effect is mediated by p140trkA and p75 and suggests that NGF is involved in prostatic carcinogenesis.

On the basis of these previously reported non-neurotrophic activities of NGF, we have investigated here the potential activity of this factor in breast cancer, which is one of the most frequent forms of human cancer. We demonstrate for the first time that NGF is mitogenic for breast cancer cells, whereas it has no effect on the proliferation of normal breast epithelial cells. The NGF interaction is mediated through p140trkA and p75 receptors, and such stimulation of breast cancer cell growth involves both tyrosine kinase activation and the MAP kinase pathway.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal anti-nerve growth factor receptor antibodies (clone 8211) were purchased from Boehringer Mannheim, and polyclonal anti-p140trkA antibody (trk 763) was from Santa Cruz. Cell culture reagents were from Eurobio (France). Recombinant human nerve growth factor was from R & D Systems (France). K-252a (inhibitor of Trk-tyrosine kinase activity) and PD98059 (inhibitor of MAP kinase cascade) were from Calbiochem (France). PY20 anti-phosphotyrosine, anti-MAP kinase (anti-ERK2) monoclonal antibodies, and protein A-agarose were purchased from Transduction Laboratories.

Cell Culture—The MDA-MB-231 and MCF-7 cancer cell lines were obtained from the American Type Culture Collection and routinely grown as monolayer cultures. Cells were maintained in minimal essential medium (Earle’s salts) (Eurobio) supplemented with 20 mM Heps, 2 g/liter sodium bicarbonate, 2 mM l-glutamine, 10% fetal calf serum, 100 units/ml of penicillin-streptomycin, 50 μg/ml gentamycin, 1% of nonessential amino acids, and 5 μg/ml of insulin. Normal human breast epithelial cell (NBEC) cultures were established as described previously from reduction mammoplasty (women aged 20–40 years) obtained in the Department of Plastic Surgery (Dr. Pellerin) of the Medical University of Lille (France). Rat pheochromocytoma cells (PC12) were grown in Dulbecco’s modified minimum essential medium (Eurobio) supplemented with 3 g/liter sodium bicarbonate, 4 mM l-glutamine, 10% fetal calf serum, 5% horse serum, and 100 units/ml of penicillin-streptomycin. All cells were maintained in a humidified atmosphere of 95% air and 5% carbon dioxide at 37 °C.

Growth Assay—Experiments were performed as follows. 35-mm di-
ameter dishes were inoculated with 2 × 10⁴ cells/dish in 2 ml of medium containing 10% fetal calf serum. After 24 h, cells were washed twice with serum-free medium. Next day, the medium was replaced with 2 ml of medium containing different concentrations of NGF. When studying NGF effects as a function of time, a concentration of 100 ng/ml was used, and cells were counted after 1, 2, and 3 days of stimulation. To study the effect of K-252a and PD98059, various concentrations of the inhibitors were added simultaneously with NGF (100 ng/ml). After 2 days of NGF exposure, cells were harvested by trypsinization and counted using an hemocytometer.

Cell Cycle Parameter Study—Measurements of cell cycle parameters were performed following methods previously described (14). The percentage of cells in S + G2 + M phases was determined after Feulgen staining and microscopic image analysis on a Samba 200 (TITN) processor. The nuclei were analyzed automatically by calculating parameters related to the densitometry and texture of chromatin. The proportion of cells in S + G2 + M phases was determined with computerized integrated optical density histograms. Cell cycle duration was determined after bromodeoxyuridine incorporation (15).

NGF Receptor Immunoblotting—Subconfluent cell cultures were harvested by scraping in serum-free medium. After centrifugation (1000 g, 5 min), the pellet was treated with lysis buffer (0.3% SDS, 200 mM dithiothreitol) and boiled 5 min. The lysates were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane (Immobilon-P, Millipore), and electroblotting (100 V, 75 min), and probed with either rabbit anti-Trk polyclonal antibody (2 μg/ml, Santa Cruz) or mouse anti-p75 monoclonal antibody (1 μg/ml, Boehringer Mannheim) at 4 °C overnight. The membrane was then incubated at room temperature for 3 h with goat anti-rabbit immunoglobulin G-conjugated peroxidase for TrkA detection and goat anti-mouse immunoglobulin G for p75 detection. The reaction was revealed using the chemiluminescence kit ECL (Amersham Pharmacia Biotech) with Kodak X-Omat AR film.

Detection of MAP Kinase Activation—Proteins were extracted in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% SDS, 1% Nonidet P-40, 10 μM sodium orthovanadate) prior to immunoprecipitation. Preclearing was done with protein A-agarose (10 μl/250 μl) (60 min, 4 °C). After centrifugation (10,000 g, 2 min), the supernatant was incubated with monoclonal anti-MAPK (anti-Erk2) antibody (10 μl/250 μl, 60 min, 4 °C). Protein A-agarose (10 μl) was added for 60 min (4 °C) and then pelleted by centrifugation (10,000 g, 2 min). The pellet was then rinsed three times with lysis buffer and boiled for 5 min in Laemmli buffer. After SDS-PAGE and electroblotting, nitrocellulose membranes were blocked with 3% bovine serum albumin. Membranes were then treated with PY20 antibody overnight at 4 °C, rinsed, and incubated with a horseradish peroxidase-conjugated anti-mouse IgG for 3 h at room temperature. Membranes were rinsed overnight at 4 °C before visualization with ECL.

RESULTS

The Effect of NGF on Breast Epithelial Cell Proliferation—The effects of various concentrations of NGF were assessed after 48 h of exposure. The growth stimulatory effect of NGF on the breast cancer cell lines was dose-dependent (Fig. 1A). The highest level of stimulation was observed at a concentration of 100 ng/ml, for which the cell number was approximately 3-fold greater than for the unstimulated situation, and corresponded to approximately 70% of the level obtained in presence of 10% fetal calf serum. The effect of the duration of NGF exposure was estimated after 1, 2, and 3 days of stimulation at an NGF concentration of 100 ng/ml (Fig. 1B). For the cancer cell lines, an increase in cell number was noticeable after 24 h. The mitogenic effect of NGF was also observed for two other breast cancer cell lines: T47-D and BT20 (data not shown). This was in contrast to the NBEC, for which we did not find any NGF growth stimulation. On the contrary, NBEC can be stimulated to grow in the presence of epidermal growth factor (50 ng/ml) or in the presence of fetal calf serum (5%).

The percentage of cells in S + G2 + M phases and cell cycle duration in the presence or absence of 100 ng/ml of NGF are shown in Table I. MCF-7 cells treated with NGF displayed an increase of 2–3-fold in the percentage of cells in S + G2 + M phases and a decrease in cell cycle duration from 45 to 30 h. No change in any of the measured cell parameters was detected for NBEC in the presence of NGF.

![Fig. 1. NGF effect on normal and breast cancer epithelial cells.](http://www.jbc.org/)

**TABLE I**

| Cell cycle parameter | S + G2 + M Cell cycle duration |
|----------------------|-------------------------------|
|                       | %                             | h                             |
| **MCF-7**             |                               |                               |
| Control               | 13 (11–14)                    | 45 (43–48)                    |
| NGF                   | 29 (25–30)                    | 30 (27–32)                    |
| **MCF-7**             |                               |                               |
| Control               | 21 (20–22)                    | 32 (31–33)                    |
| **MCF-7**             |                               |                               |
| Control               | 34 (32–35)                    | 27 (24–30)                    |
| **NBEC**              |                               |                               |
| Control               | 11 (10–12)                    | 50 (46–53)                    |
| **NBEC**              |                               |                               |
| Control               | 13 (10–15)                    | 50 (46–53)                    |

Immunodetection of NGF Receptors—Normal and breast cancer epithelial cells exhibited a band of TrkA immunoreactivity at approximately 140 kDa after Western blotting (Fig. 2A). The anti-TrkA antibody used in this study does not cross-react with the other neurotrophin receptors TrkB and TrkC. The anti-p75 antibody gave rise to one immunoreactive band at 75 kDa for all cell types studied (Fig. 2B). These molecular masses of 140 and 75 kDa correspond to what is obtained with PC12 cells, which have been extensively used to study NGF receptors (4). The intensity of the p75 and TrkA immunoreactivity was about the same in the cancer cells as in NBEC.

Tyrosine Kinase and MAP Kinase Activation—K-252a and PD98059 induced a dose-dependent inhibition of the NGF growth stimulatory effect on MCF-7 and MDA-MB-231 breast cancer cells (Fig. 3A). This indicated that Trk-tyrosine kinase activity was about the same in the cancer cells as in NBEC.
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activity as well as MAP kinase cascade are implicated in NGF-induced intracellular signaling. Indeed, anti-MAPK immunoprecipitation followed by anti-phosphotyrosine immunoblotting showed a band corresponding to the extracellularly regulated kinases Erk (44 kDa). MAPK tyrosine phosphorylation level was increased by NGF in the MDA-MB-231 and MCF-7 cancer cells after 10 min of stimulation, whereas no modification was observed in NBEC (Fig. 3B).

DISCUSSION

We have shown here for the first time that NGF can activate the proliferation of human breast cancer cells. Although NGF has been extensively studied for its neurotrophic properties, relatively few cellular types have been shown to have their growth stimulated by this factor. Human B lymphocytes can be induced to grow and differentiate by NGF (9), and in human epidermis, NGF is produced by keratinocytes to stimulate their cell proliferation with an autocrine loop (10) and melanocyte proliferation with a paracrine pathway (6). NGF also stimulates the proliferation and invasion of prostate cancer cells and uses TrkA/p75 to do so (11, 12). The reduced expression of p75 has been observed in benign and malignant prostate tissue, and a complete loss of p75 has been reported in metastatic cells (4, 5); the signaling pathway used by this factor in breast cancer cells is thus likely to be similar. Absence of NGF growth responsiveness in NBEC indicates the specificity of breast cancer cells in their sensitivity to NGF. To our knowledge, NGF is the first factor to be identified as specifically stimulating the growth of tumorous, as opposed to normal breast epithelial cells. This suggests that NGF could have a crucial function in the initiation and progression of human breast tumors. NBEC present both types of receptors, and the MAP kinase cascade can be activated in these cells because they are proliferating in response to epidermal growth factor. The fact that in NBEC, NGF does not induce MAP kinase activation suggests that these cells might lack a functional signaling protein or that they might be inhibited in their response to this factor. Alternatively, NBEC might respond to NGF in different ways than just the stimulation of cell proliferation. It is now established for neuronal cells that NGF controls the process of apoptosis; however, the mechanism of this effect is still not fully understood at the receptor level, and different hypotheses involving p75 alone or in combination with Trk have been proposed (20). Because NBEC present both types of receptors, NGF stimulation of normal breast cells might participate in the control of cell survival. Under our experimental conditions, we were not able to observe any increase in NBEC, survival rates with NGF treatment. However, the culture medium used here is well formulated for NBEC survival, because we usually observe less than 10% of cell death; a precise study of cell survival in the presence of NGF would require further investigation.

Because the effect of NGF on breast epithelial cells has not hitherto been suspected, the expression of NGF in mammary gland has not been extensively studied. Evidence for the presence of NGF in breast tissue include NGF immunoreactivity (21) and biological activity (22) in murine milk. In the human, immunoreactivity for NGF has been found in breast capsule...
implants (23). However, in these studies, the source of NGF has not been clarified, although in the mouse it does not seem to be affected by removal of the submaxillary glands, which are known to be a major source of NGF. Using our cell culture system, we have not detected any NGF immunoreactivity in either normal or breast cancer epithelial cells (data not shown), suggesting that this factor is not produced by these cells in significant quantities. It is known that NGF is produced by targets of sympathetic innervation and is found in circulating blood (24), but the precise origin of NGF in breast cannot yet be precisely defined. Our finding that NGF is a mitogenic factor for human breast cancer cells and not for normal breast epithelial cells strongly suggests that this factor is involved in the growth of human breast tumors. Little is known at present about NGF expression and function in mammary gland, and our present data indicate the need to further explore the role played by NGF in breast carcinogenesis. A better understanding of NGF function in breast cancer should open new perspectives for the detection, prognosis and treatment of this pathology.

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