Nerve Growth Factor Exerts Differential Effects on the Growth of Human Pancreatic Cancer Cells

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ABSTRACT
Nerve growth factor (NGF) exerts both stimulatory and inhibitory effects on neuronal and certain nonneuronal tumors with the effect based on the type of tumor. We investigated NGF and its receptors (TrkA and p75) in pancreatic cancer cells (PANC-1, MIA-PaCa-2, CAPAN-1, ASPC-1, and T3M4) by reverse transcription-PCR, Western blot analysis, NGF ELISA, and growth assays. NGF mRNA was present at comparable levels in all five pancreatic cancer cell lines. TrkA expression was relatively high in PANC-1 and MIA-PaCa-2 cells and low in CAPAN-1, ASPC-1, and T3M4 cells. p75 expression was high in PANC-1, MIA-PaCa-2, and T3M4 cells, moderate in CAPAN-1, and low in ASPC-1 cells. By ELISA assay, the intracellular NGF content in all cell lines was ~40 pg/10^6 cells. NGF content increased significantly in PANC-1 and MIA-PaCa-2 cells when these cells were cultured with serum-free media, whereas there was no change in the other cancer cell lines. PANC-1 and MIA-PaCa-2 cells but not the other cell lines released NGF in the culture media. Exogenous NGF stimulated the growth of PANC-1 and MIA-PaCa-2 cells, inhibited the growth of T3M4 and CAPAN-1 cells in a dose- and time-dependent manner, and did not affect the growth of ASPC-1 cells. NGF led to the phosphorylation of TrkA, mitogen-activated protein kinase (MAPK), and p38 MAPK but not stress-activated protein kinase/c-Jun NH2-terminal kinase in PANC-1 and MIA-PaCa-2 cells. In contrast, in the other pancreatic cancer cell lines none of these kinases were phosphorylated by NGF. In conclusion, the effects of NGF on pancreatic cancer cell growth are dependent on the expression levels and the balance of its TrkA and p75 receptors. NGF-induced pancreatic cancer cell growth seems to be mediated through the phosphorylation of TrkA and subsequently via MAPK. These results point to a previously unknown autocrine/paracrine pathway in pancreatic cancer, suggesting that NGF-TrkA interactions are important factors influencing cell growth and spread in this malignancy.

INTRODUCTION
NGF was the first critical member of the neurotrophin polypeptide family to be discovered. This family also includes brain-derived neurotrophic factor, and neurotrophins NT-3, NT-4/5, NT-6 (1), and NT-7 (2). NGF binds to two distinct receptors: the high affinity TrkA receptor with tyrosine kinase activity and the low affinity p75 receptor without tyrosine kinase activity (1). In past years the role of NGF in some neuronal tumors as well as in nonneuronal tumors such as prostate, lung, and breast cancers has been investigated, and this research has suggested that NGF has the potential to exert mitogenic and growth-inhibitory effects, depending on the underlying cell type. For example, NGF induces tumor cell growth and invasion in human prostate, breast, and lung cancer cells (3–5), whereas in human small cell lung cancer cell lines, NGF remarkably inhibits the proliferation rate of the cancer cells, prevents their anchorage-independent clonal growth in soft agar, impairs their invasive capacity in vitro, and abolishes their tumorigenic potential in nude mice (6). Although the exact mechanisms for the almost opposite functions of NGF in different tumor cells are not readily apparent, it seems that its receptors TrkA and p75 play important roles in these processes. Recent studies indicate that in the presence of the TrkA receptor, p75 can participate in the formation of high-affinity binding sites, resulting in enhanced NGF responsiveness and growth-promoting signals. In the absence of TrkA receptors, however, p75 is capable of mediating signals that lead to cell death (5, 7). NGF binding to the TrkA receptor induces tyrosine phosphorylation of TrkA at the Tyr-490 tyrosine residue, followed by the binding of the intracellular protein Shc to this site. Phosphorylated Shc activates MAPKs (Erk1 and Erk2) via several intracellular signaling molecules, resulting in cell proliferation (8). The role of NGF in pancreatic cancer pathogenesis has not been elucidated. Recently, NGF overexpression was reported in resected human pancreatic cancer samples, and the presence of NGF was associated with neuronal invasion and spreading.
of pancreatic cancer cells along the nerves (9). However, NGF has been reported to inhibit the invasiveness of some pancreatic cancer cell lines (10). Nonetheless, CEP-701, a Trk tyrosine kinase inhibitor, has recently been shown to have antitumor effects in pancreatic cancer cell xenografts in nude mice (11, 12). However, the effects of NGF on pancreatic cancer cell growth and the involved extra- and intracellular pathways are currently not known. Therefore, in the present study, we examined the effects of NGF on pancreatic cancer cell growth and further studied whether the presence of its receptors, TrkA and p75, influences NGF functions in pancreatic cancer.

**MATERIALS AND METHODS**

**Cell Culture.** Human pancreatic cancer cells were routinely cultured in DMEM (PANC-1 and MIA-PaCa-2) or RPMI 1640 (ASPC-1, CAPAN-1, and T3M4) supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin (complete medium) at 37°C in 95% air and 5% CO₂.

**RNA Extraction and Semiquantitative RT-PCR.** Total RNA was extracted by the single-step acid guanidinium isothiocyanate phenol chloroform method (13). Total RNA was first quantitated, and quantitative PCR analysis was performed as reported previously (14). In brief, the cDNA was first synthesized, and quantitative PCR analysis was performed in parallel experiments for NGF, TrkA, and p75 to get the specific products and to avoid a plateau effect. The following primers were used: NGF (sense), 5'-ATA CAG GCC GAA CCA CAC TC-3'; NGF (antisense), 5'-TGC TCC TGT GAG TCC TGT TG-3'; TrkA (sense), 5'-CCA TTT CAC TCC TCG GCT CAG T-3'; TrkA (antisense), 5'-GCC GAC CCC CAG ATT TCA TCA C-3'; p75 (sense), 5'-CCC TGG CCG TTG GAT TAC AC-3'; and p75 (antisense), 5'-GAG ATG CCA CTG TCG CTG TG-3' (Amplimmun, Madulain, Switzerland). The sizes of amplification products used by the above PCR primers are 313 bp for NGF, 401 bp for TrkA, and 351 bp for p75. All PCR products were cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI), and their authenticities were confirmed by sequencing using the dye terminator method (ABI 373A; Perkin-Elmer, Rot Kreuz, Switzerland). All experiments were performed in triplicate.

**Western Blotting.** Pancreatic cancer cells were washed twice with 1X PBS (pH 7.4), lysed with lysis buffer, and incubated on ice for 15 min. The lysis buffer contained 150 mM NaCl and 10 mM Tris-HCl (pH 7.5) supplemented with a protease inhibitor mixture (Boehringer Mannheim, Mannheim, Germany). The lysates were collected and centrifuged at 4°C for 30 min with 14,000 rpm to remove the insoluble material. The protein concentration of the supernatant was measured by spectrophotometry using the BCA protein assay method (Pierce, Rockford, IL). A total of 40 µg of protein/ lane was loaded on 12% (for NGF) or 7.5% (for TrkA and p75) SDS-polyacrylamide gels and separated by electrophoresis. After transfer to nitrocellulose membranes, blots were incubated with specific polyclonal rabbit or goat antibodies detecting human NGF (1:1,000; Serotec, Ltd., Oxford, United Kingdom), TrkA (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA), and p75 (1:1,000; Santa Cruz Biotechnology), respectively. After washing, blots were incubated with antirabbit or antigoat IgG (1:3,000; Bio-Rad, Hercules, CA) and conjugated with horseradish peroxidase, and visualization was performed by the enhanced chemiluminescence method (Amersham International, Buckinghamshire, United Kingdom).

For detection of phosphorylation of signaling kinases, cells were first cultured in complete medium and subsequently incubated in medium with 0.5% FBS for 24 h. Cells were then treated with 100 ng/ml NGF for 5, 15, and 30 min. Cells were lysed, protein was extracted, and the concentration was measured as described above. The intracellular phosphorylation of TrkA at tyrosine 490 (Tyr-490), MAPK, p38 MAPK, and SAPK/JNK was detected by polyclonal rabbit antihuman phosphospecific TrkA (Tyr-490: 1:500), phospho-MAPK (Erk1/2: 1:1000), phospho-p38 MAPK (1:1000), and phospho-SAPK/JNK (1:1000) antibodies, respectively (New England Biolabs, Beverly, MA).

**Measurement of NGF by ELISA.** A two-site ELISA (NGF EmaxTM ImmunoAssay System; Promega Corp., Madison, WI) was used to quantitate NGF content in pancreatic cancer cell extracts and cultured media. The cancer cells were grown in complete medium up to 50–70% confluence, washed twice with PBS (pH 7.4), and then incubated with serum-free conditioned medium at 37°C in 95% air and 5% CO₂. The culture medium was collected on days 1, 2, and 3, and whole-cell lysates were collected on days 0, 1, 2, and 3. Lysates of the cells cultured with complete medium including 10% FBS were also collected at the same time points as the controls. ELISA assay was performed according to the manufacturer's instructions. Briefly, the 96-well flat-bottomed immunoplates were coated with 100 µl well of 0.5 µg/ml polyclonal anti-NGF antibody (NGF pAb) at 4°C for 18 h and washed once with 1× PBS. Blocking of nonspecific binding was done with 200 µl of 1× blocking and sample buffer for 1 h at room temperature. One hundred µl of cell lysate or cultured media were added to the coated wells, incubated with immobilized anti-NGF pAb for 6 h at room temperature, and washed five times. Afterward the plates were incubated with monoclonal anti-NGF antibody at 4°C for 12 h. After five washings, the plates were incubated with antirat IgG conjugated with horseradish peroxidase for 2.5 h at room temperature. After five washings, TMB peroxidase substrate was added for 10 min at room temperature. The color reaction was stopped with 1× phosphoric acid, and the light absorbance was measured at 450 nm using a microplate reader.

**Cancer Cell Growth Assay.** Pancreatic cancer cell growth was evaluated by the SRB method (15). Briefly, pancreatic cancer cells were plated overnight at a density of 3000 cells/well in 96-well plates. The cells were washed twice with PBS and subsequently incubated in serum-free medium containing ITS1 liquid medium supplement (Sigma Chemical Co., St. Louis, MO) in the presence of different concentrations (0, 0.8, 1.6, 3.2, 6.3, 12.5, 25, 50, 100, and 200 ng/ml) of human recombinant NGF (Calbiochem-NOVAbiochem Corp., La Jolla, CA) for 48 h. For studying the time-dependent effects of NGF on cancer cell growth, a concentration of 100 ng/ml NGF was...
used, and the SRB assay was performed after an incubation of 1, 2, 3, and 4 days.

Cells were washed three times with PBS (pH 7.4), fixed with 10% trichloroacetic acid for 60 min at 4°C, and then washed for five times in deionized water and stained with 0.4% SRB solution (Sigma) for 15 min at room temperature. Unstained SRB was removed by washing the cells with 1% glacial acetic acid. Afterward, the cells were dried, and the stained cells were dissolved with 10 mM Tris-HCl. The absorbance value was measured at 540 nm. All experiments were performed in triplicates and repeated three times.

**Statistical Analysis.** The data are expressed as mean ± SE. The statistical analysis was performed using Student’s t test. Significance was defined as P < 0.05.

**RESULTS**

**Semiquantitative Determination of NGF, TrkA, and p75 mRNA by RT-PCR.** Quantitative RT-PCR was used to detect the expressions of NGF, TrkA, and p75 mRNA, because the expression levels were below the level of detection by Northern blot analysis using 20 μg of total RNA. In all five pancreatic cancer cell lines (PANC-1, MIA-PaCa-2, CAPAN-1, ASPC-1, and T3M4), NGF mRNA was present at comparable levels (Fig. 1). In the case of TrkA mRNA, relatively high expression levels were present in PANC-1 and MIA-PaCa-2 cells and lower levels in CAPAN-1, ASPC-1, and T3M4 cells (Fig. 1). The mean densitometric TrkA mRNA expression values in PANC-1 and MIA-PaCa-2 cells were 9.3-fold (P < 0.01) and 10.6-fold higher than those of ASPC-1, CAPAN-1, and T3M4 cells. p75 mRNA expression was high in PANC-1, MIA-PaCa-2, and T3M4 cells, moderate in CAPAN-1 cells, and low in ASPC-1 cells (Fig. 1).

**Western Blot Analysis.** NGF, TrkA, and p75 protein levels were evaluated by Western blot analysis. There was a good relationship between the Western blot analysis and the results obtained by quantitative RT-PCR. In all five pancreatic cancer cell lines, NGF was detected with a single 14.4-kDa band at similar intensity (Fig. 2). TrkA was detected in PANC-1 and MIA-PaCa-2 cells at relatively high levels, whereas it was weak in the other three cancer cell lines (Fig. 2). The intensity of the p75 band was high in PANC-1, MIA-PaCa-2, and T3M4 cells, moderate in CAPAN-1 cells, and low in ASPC-1 cells. In PANC-1, MIA-PaCa-2, and T3M4 cells, the intensity of the p75 band was comparable (Fig. 2).

**Measurement of NGF Content in Pancreatic Cancer Cells and Cultured Media.** NGF was also quantitatively measured by ELISA in the five pancreatic cancer cell lines and in the corresponding serum-free medium. NGF was present in the cell extracts of all five cancer cell lines. On day 0, NGF concentration was 44.6 ± 1.2 pg/10⁶ cells in PANC-1 cells and 46.4 ± 3.7 pg/10⁶ cells in MIA-PaCa-2 cells. Intracellular NGF concentration increased during the incubation with serum-free culture medium, and maximum levels were observed at day 1 in
Effects of NGF on Pancreatic Cancer Cell Proliferation.
Growth-stimulatory effects of NGF were observed in two pancreatic cancer cell lines (PANC-1 and MIA-PaCa-2), and this effect was dose and time dependent (Fig. 4). A significant stimulation was evident at a concentration of 100 ng/ml NGF after 48 h, with the cell number increasing by ~1.3-fold ($P < 0.05$) in PANC-1 cells and 1.8-fold ($P < 0.05$) in MIA-PaCa-2 cells in comparison with the control group. In contrast, in T3M4 cells, NGF inhibited cell growth, and the cell number decreased 2.5-fold ($P < 0.05$) compared with the control group. As shown in Fig. 4B, both the growth-stimulatory and -inhibitory effects of NGF were time dependent. NGF did not show any influence on the growth of CAPAN-1 and ASPC-1 cells (Fig. 4A).

Receptor Phosphorylation and Downstream Signaling.
Because it is established that NGF transmits its signals via TrkA phosphorylation and subsequently via MAPK in certain cell lines, we next investigated whether NGF was capable of triggering signaling through these pathways in pancreatic cancer cell lines as well. NGF stimulated TrkA phosphorylation in PANC-1 and MIA-PaCa-2 cells in a time-dependent manner. The effect occurred after 5 min and reached a maximum after 15 min of NGF treatment (100 ng/ml). In addition, NGF stimulated MAPK and p38 MAPK phosphorylation in a similar time-independent manner. However, NGF-induced SAPK/JNK phosphorylation was not detectable in these two pancreatic cancer cell lines (Fig. 5). Furthermore, NGF did not stimulate TrkA, MAPK, p38 MAPK, or SAPK/JNK phosphorylation in ASPC-1 (Fig. 5), T3M4, and CAPAN-1 cells.

DISCUSSION
Pancreatic cancer is one of the most aggressive human malignancies, characterized by rapid tumor progression, difficulty of early diagnosis, and poor prognosis, even after radical resection (16, 17). Many growth factors and their corresponding tyrosine kinase receptors are overexpressed in this malignancy and stimulate pancreatic cancer cell growth (16, 18). For example, the concomitant presence and overexpression of the EGF receptor and its ligands EGF, TGF-$\beta$, and/or amphiregulin is associated with enhanced tumor aggressiveness and shorter survival periods after tumor resection (19, 20). Furthermore, the growth-inhibitory effects of the TGF-$\beta$ superfamily of serine-threonine kinase receptors and their ligands are often altered in pancreatic cancer cells either by an imbalance in the expression of the signaling receptors (21, 22), mutations in intracellular signaling genes (smad4; Ref. 23), and/or by up-regulation of TGF-$\beta$ pathway signaling inhibitors (smad6 and smad7; Refs. 24 and 25).

NGF binds independently to two different receptors, the TrkA tyrosine kinase receptor and the p75 neurotrophin receptor. In addition to promoting cell differentiation and survival, NGF can paradoxically also induce cell death. Although the mechanisms for these divergent effects are still not completely known, available data suggest that in cells where p75 is coexpressed with Trk receptors, p75 functions with the Trk receptors to enhance the responsiveness to their ligands or facilitates apoptosis resulting from neurotrophin withdrawal. In cells lack-
ing TrkA expression, NGF and other neurotrophins binding to p75 activate signaling cascades that may in some circumstances result in apoptosis (7, 26). TrkA-mediated rescue involves not only activation of survival signals but simultaneously also suppression of death signals mediated by p75 (27). Some studies have demonstrated that the duration and magnitude of NGF signaling depends on the ratio of p75 to TrkA (28), yet nothing is known about the correlation between p75/TrkA expression and the effects of NGF in pancreatic cancer cells. We now show for the first time, that in pancreatic cancer cells with high levels of NGF, the growth of PANC-1 and MIA-PaCa-2 cells was stimulated, the growth of T3M4 and CAPAN-1 cells was inhibited, and no effect was observed in ASPC-1 cells. Data are expressed as a percentage of increase or decrease of untreated controls and are means; bars, SE. *, P < 0.05; **, P < 0.01 in comparison to non-NGF-stimulated cells.

**Fig. 4** Effects of NGF on pancreatic cancer cell growth in vitro as determined by the SRB assay. A, dose-dependent effects of NGF on pancreatic cancer cell growth. Cells were incubated with 0, 0.8, 1.6, 3.2, 6.3, 12.5, 25, 50, 100, and 200 ng/ml NGF for 48 h. The growth of PANC-1 and MIA-PaCa-2 cells was stimulated, the growth of T3M4 and CAPAN-1 cells was inhibited, and no effect was observed in ASPC-1 cells. Data are expressed as a percentage of increase or decrease of untreated controls and are means; bars, SE. *, P < 0.05; **, P < 0.01 in comparison to non-NGF-stimulated cells. B, time-dependent effects of NGF on pancreatic cancer cell growth. Cells were incubated with 100 ng/ml NGF for 1, 2, 3, and 4 days. Data are expressed as the percentage of increase or decrease of untreated controls and are means; bars, SE. *, P < 0.05; **, P < 0.01 in comparison to non-NGF-stimulated cells.
of TrkA and p75 (PANC-1 and MIA-PaCa-2), NGF exhibited growth-stimulatory effects. In contrast, in pancreatic cancer cells with low levels (or lack) of TrkA and high levels of p75 (T3M4 and CAPAN-1), NGF exerted inhibitory or no effects on pancreatic cancer cell growth. When these two receptors were simultaneously expressed at low levels, NGF did not exert any growth-influencing effects (ASPC-1). Our present results that the different effects of NGF on pancreatic cancer cells are dependent on the expression ratio of TrkA and p75 are also strongly supported by the above-mentioned findings in other cell systems.

RT-PCR, Western blot analysis, and NGF ELISA revealed that NGF is produced in all five pancreatic cancer cell lines at similar levels. However, only PANC-1 and MIA-PaCa-2 cells released NGF into the culture medium. The reason, however, that some pancreatic cancers secrete NGF whereas others do not remains to be elucidated in future studies. Regardless, our results indicate that NGF may exert growth-stimulatory effects through previously unrecognized autocrine and/or paracrine mechanisms in some pancreatic cancer cells, as has been shown in other cancers (29, 30).

In human pancreatic cancers, high levels of NGF are present in the cancer cells, whereas TrkA is present in the perineurium (9). Released NGF from pancreatic cancer cells might activate TrkA in a paracrine manner and thereby influence nerve growth, perineural invasion, and pain, as has been hypothesized before (9). The signaling pathways that lead to growth stimulation in pancreatic cancer cells have thus far not been elucidated. In PC12 cells and human breast cancer cells, NGF signaling occurs through TrkA and subsequently by MAPK phosphorylation (31). In the present study, we examined TrkA phosphorylation as well as MAPK, p38

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**Table 1** Expression of NGF, TrkA, and p75 and effects of exogenous NGF on pancreatic cancer cell growth

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<th>NGF (protein)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NGF in culture medium&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TrkA (protein)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P75 (protein)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Growth effects&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Phosphorylation&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>MIA-PaCa-2</td>
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<td>CAPAN-1</td>
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<td>T3M4</td>
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<sup>a</sup> As determined by Western blot analysis: –, absent; ±, faint; +, weak; ++, moderate; ++++, strong.

<sup>b</sup> As determined by ELISA: +, present; –, absent.

<sup>c</sup> As determined by SRB assay: ↑, stimulatory effect; ↓, inhibitory effect; →, no effect.

<sup>d</sup> As determined by Western blot analysis: +, phosphorylation present; –, phosphorylation absent.
MAPK, and SAPK/JNK phosphorylation. NGF stimulated TrkA phosphorylation and MAPK, p38 MAPK, but not SAPK/JNK, phosphorylation in PANC-1 and MIA-PaCa-2 cells, which both express high levels of TrkA and p75, indicating that the mitogenic signaling of NGF is mediated, as in other cells, via MAPK phosphorylation. In the other cell lines, TrkA, MAPK, p38 MAPK, and SAPK/JNK phosphorylation were not induced by NGF, which might be caused by the low levels of TrkA in these cells. These findings also imply that the growth-inhibiting effects of NGF in T3M4 and CAPAN-1 cells are not mediated via the MAPK/SAPK/JNK pathways. Although novel aspects of the NGF/TrkA signaling cascade have been elucidated in the present study, further studies are needed to show how growth inhibition by NGF is mediated in pancreatic cancer cells.

In conclusion, it appears that in pancreatic cancer cells the growth effects of NGF depend on the expression levels and ratio of TrkA and p75. NGF has the potential to promote pancreatic cancer cell growth through autocrine and/or paracrine mechanisms via MAPK. Taken together with previous observations (11, 12), these findings suggest that modalities aimed at abrogating NGF/TrkA signaling might be potentially effective as a future therapy in pancreatic cancer.

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