Expression and Activation of the Nerve Growth Factor Receptor TrkA in Serous Ovarian Carcinoma

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ABSTRACT

Purpose: The purpose is to analyze the possible correlation between expression and activation of the high-affinity nerve growth factor (NGF) receptor TrkA, cell cycle protein expression, and disease outcome in serous ovarian carcinoma. In addition, we wished to study the possible link between expression of NGF, a novel angiogenic factor and its receptor TrkA, and the expression of factors involved in angiogenesis in effusions and solid tumors.

Experimental Design: Sections from 80 malignant effusions and 65 corresponding solid tumors were evaluated for protein expression of NGF, TrkA, and phospho-TrkA (p-TrkA). Effusions were additionally studied for expression of p53, p21WAF1/CIP1, Ki-67, and the phosphoinositol-3-kinase/AKT pathway (3, 7). AKT receptor activation correlates with poor outcome in advanced stage serous ovarian carcinoma.

Results: NGF, TrkA, and p-TrkA were expressed in carcinoma cells in effusions in 60 of 80 (75%), 64 of 80 (80%), and 15 of 80 (19%) specimens, respectively. In solid tumors, p-TrkA expression was more frequent (52 of 65 tumors; 80%) and was accompanied by p-TrkA expression in endothelial cells. NGF colocalized with bFGF protein (P = 0.016) and mRNA (P = 0.032) in effusions, and with VEGF (P < 0.001) and bFGF (P = 0.008) in solid tumors. In survival analysis, expression of p85-PARP (P = 0.017) and cytoplasmic TrkA (P < 0.001) in effusions predicted better outcome, whereas membrane expression of p-TrkA in solid tumors correlated with poor survival (P = 0.004). Diffuse expression of p53 and Ki-67 was often seen using IHC, whereas p21WAF1/CIP1 and p85-PARP expression was infrequent and focal. None of these correlated with NGF or TrkA expression or activity.

Conclusions: Coexpression of NGF with molecules involved in angiogenesis and p-TrkA expression in endothelial cells suggest that the proangiogenic role attributed to NGF in vitro and in vivo may be relevant in clinical cancer. Expression of p85-PARP as a marker of apoptosis and cytoplasmic expression of TrkA (probably representing non-glycosylated receptor) predict better outcome, whereas p-TrkA activation correlates with poor outcome in advanced stage serous ovarian carcinoma.

INTRODUCTION

Neurotrophins are a family of growth factors, consisting, at present, of the prototype compound NGF2 and additional members such as brain-derived neurotrophic factor, and the neurotrophins NT-3, NT-4, and NT-6 (1–4). Neurotrophins bind to the TrkA, TrkB, and TrkC receptors. NGF binds to the specific high-affinity tyrosine kinase receptor TrkA. TrkA receptors possess a tyrosine kinase catalytic domain. The structure of the TrkA-NGF complex has recently been published and contains one conserved motif, common to all neurotrophins, and one specific motif for TrkA-NGF binding (5). NGF ligand binding to TrkA receptors activates TrkA autophosphorylation at several sites such as tyrosine 490, promoting Src homology and collagen binding and phosphorylation, which in turn leads to coupling of growth factor receptor binding protein to son of sevenless complexes and activation of Ras (6, 7). The net result in the central nervous system is differentiation and survival of neuronal cells, mainly of sympathetic and sensory systems (2, 7). Inhibition of Ras activity leads to neuronal death, whereas deletion in neurofibromatosis-1, a Ras inhibitor, allows for neuronal survival in the absence of neurotrophins in vitro (7). Ras is able to activate two major intracellular signal transduction pathways: the mitogen activated protein kinase pathway and the phosphoinositol-3-kinase/AKT pathway (3, 7). AKT appears to be the main (although not the only) target for phos-
phosphoinositol-3-kinase signaling and is responsible for 80% of NGF-induced survival in sympathetic neurons, mainly through phosphorylation of Bad and Forkhead 1 (7). NGF signaling is able to induce survival and proliferation but can also induce differentiation without proliferation (8). This is mediated by TrkA signaling, which has been shown to inhibit cell proliferation through a variety of mechanisms, including increased nuclear expression of p53 and direct activation of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 promoter in PC-12 cells (9–11). TrkA itself has been shown to associate with p53 and was phosphorylated in presence of the latter even in the absence of NGF (12, 13).

In addition to these well-defined effects of TrkA-mediated signaling in neural networks, recent data has suggested that NGF may have additional biological roles by demonstrating that NGF is an angiogenic factor in human endothelial umbilical vein cells (14). This finding supports earlier findings that have documented the proangiogenic effects of NGF in the developing central nervous system (15), NGF-induced expression of inflammatory markers in skin vessels (16), and NGF-mediated increase in vascularization of brain malignancies such as gliomas (17).

Although originally isolated from neural tissues, a growing body of evidence supports a role for Trk receptors in tumorigenesis in nonneural tumors, primarily carcinomas (18–20). Rearrangement or mutation of the TrkA gene, resulting in constitutive activation of the receptor, has been observed in colon and thyroid papillary carcinomas, as well as in acute myeloid leukemia (reviewed in Ref. 18). Reduced TrkB and elevated TrkA and TrkC expression showed an association with tumor progression in medullary thyroid carcinoma (21). Conversely, the expression of TrkA as a proto-oncogene has been associated with better prognosis in neuroblastoma (22, 23). In addition to the alteration in structural and expression of Trk receptors, intracellular signaling appears to be dysregulated in cancer cells (18). These studies suggest a novel emerging concept in cancer that NGF and other neurotrophins may be involved in the growth of certain nonneural tumors by paracrine and/or autocrine regulation via humoral-tumoral-stromal Trk interaction.

Ovarian carcinoma is the most lethal gynecologic tumor. Although recent advances in chemotherapy regimens have led to somewhat prolonged disease-free survival, essentially no effect on long-term survival has been achieved. Although this mainly reflects the late detection of this tumor, it also owes to our poor understanding of changes undergone by ovarian cancer cells along tumor progression because most studies focus exclusively on primary tumors. As part of our efforts to define events involved in ovarian cancer evolution and metastasis, we have recently reported on the expression of the NGF receptors TrkA and the low-affinity neurotrophin receptor p75 in effusions, primary and metastatic tumors of serous ovarian carcinoma patients (19). Expression of TrkA predominated in both solid tumors and effusions, but cancer cells in the latter compartment showed reduced expression of TrkA and elevated expression of p75 compared with solid tumors. Activation (autophosphorylation) of TrkA, using an antibody against the tyrosine 490-phosphorylated form of TrkA, was found in a few effusions.

The objective of this study was to further investigate the biological role of the NGF-TrkA axis in ovarian carcinoma by studying NGF and TrkA protein expression in effusions and solid tumors from a new cohort of 72 ovarian carcinoma patients, with longer follow-up periods compared with the previous study. In addition, p-TrkA expression was analyzed in solid tumors for the first time. Finally, the possible correlation between NGF, TrkA, and p-TrkA expression and the expression of angiogenic molecules, p53, p21WAF1/CIP1, the M, 85,000-cleaved product of PARP as an indicator of apoptosis (24, 25) and of the proliferation marker Ki-67 was studied.

**Materials and Methods**

**Effusion Specimens**

The material consisted of 80 fresh nonfixed peritoneal and pleural effusions submitted to the Division of Cytology, Department of Pathology, Norwegian Radium Hospital, during the period of January 1998 to November 2000. Specimens were obtained preoperatively, intraoperatively, or at disease recurrence from 69 patients diagnosed with serous ovarian carcinoma and 3 patients diagnosed with primary peritoneal carcinoma. Effusion specimens consisted of 57 peritoneal and 23 pleural effusions. All effusion specimens, as well as relevant clinical data, were obtained from the Department of Gynecologic Oncology, Norwegian Radium Hospital. Specimens submitted to our laboratory arrived within minutes after tapping and were processed immediately. Cells were suspended and frozen in RPMI + DMSO at −70°C. Smears and cellblock sections from all specimens underwent morphological evaluation by three experienced cytopathologists and were additionally characterized using immunocytochemistry with broad antibody panels against cancer and mesothelial epitopes, as detailed previously (26–28). Clinicopathological data are presented in Table 1.

**Tumor Specimens**

Sixty-five surgical specimens, consisting of primary tumors (n = 28) and metastatic lesions (n = 37) of the above
patients were additionally studied. Metastases consisted of 19 omental, 9 peritoneal, and 6 intestinal biopsies, as well as 3 lesions from other sites. Formalin-fixed, paraffin-embedded tissue blocks were obtained from archival material at the Department of Pathology, Norwegian Radium Hospital. All tissue specimens underwent microscopic confirmation of diagnosis, tumor type, and histological grade, following established criteria (29).

Immunohistochemical Analysis

**TrkA, p-TrkA, and NGF.** Sections from all 80 malignant effusions and 65 solid tumors were stained for these proteins. Staining for TrkA protein was performed using the very selective 203 anti-TrkA antibody developed at National Cancer Institute, NIH (Frederick, MD) and kindly donated by David R. Kaplan (30), which has been extensively used in many studies. Staining for NGF was performed using an antibody described previously (31). This rabbit polyclonal antibody selectively cross-reacts with proNGF and mature NGF but lacks any cross-reactivity with other neurotrophins such as brain-derived neurotrophic factor, ciliary neuropathic factor, NT-3, or NT-4 as previously (31). This antibody was evaluated for NGF staining.

For p-TrkA, a mouse monoclonal IgG1 antibody was raised against a decapeptide corresponding to an amino acid sequence containing a phosphorylated tyrosine residue (tyrosine 490) of human TrkA. The antibody was isolated from a serum-free hybridoma culture medium (32) by sequential affinity chromatography on unphosphorylated and phosphorylated peptide-Sepharose gels. The antibody reacts specifically with phosphorylated TrkB or TrkC. The antibody was concentrated to 150 μg/ml in PBS containing 0.1% sodium azide and 0.1% gelatin and stored at 4°C. Staining procedure was identical to the one described above for TrkA. Positive immunostaining with this antibody indicates that the TrkA receptor was autophosphorylated at tyrosine 490, as shown in our previous report (19). Positive control for these antibodies consisted of a specimen of immature ovarian teratoma containing primitive neural elements.

**Cell Cycle Proteins.** All 80 effusions were stained for the proliferation marker Ki-67 (clone Ki-S5; Dako), the p85 fragment of PARP (Promega, Madison, WI), p53 (DO-1 antibody; Santa Cruz Biotechnology, Santa Cruz, CA) and p21WAF1/CIP1 (Oncogene Research Products, San Diego, CA). Staining for Ki-67 and p21WAF1/CIP1 was performed using the biotin streptavidin peroxidase method (supersensitive immunodetection LP000-UL; BioGenex, San Ramon, CA). Staining for p85-PARP and p53 was performed using the Envision method (Dako). Microwave pretreatment in citrate buffer (pH 6) was used for all antibodies, 4 × 5 min for PARP and Ki-67, 2 × 5 min for p53 and p21WAF1/CIP1.

**Angiogenic Proteins.** Angiogenic protein expression was studied in 30 effusions. Polyclonal antibodies against VEGF (Zymed, San Francisco, CA), IL-8 (Biosource International, Camarillo, CA), and bFGF (Sigma, Saint Louis, MO) were used. Using the antibodies directed against VEGF and bFGF, pretreatment consisted of microwave oven antigen retrieval for 4 × 5 min in citrate buffer. No pretreatment was used using the anti-IL-8 antibody (33).

Negative controls in all reactions consisted of sections that underwent a similar staining procedure, with the exclusion of primary antibody application or that were stained with mouse myeloma protein of the same isotype as the primary antibody used. Two ovarian carcinoma biopsies in which immunoreactivity for the studied antigens was previously demonstrated were used as positive controls.

**Evaluation of IHC Results.** Membrane and cytoplasm immunoreactivity was scored for TrkA, whereas only the latter was evaluated for NGF staining. p-TrkA expression was scored at the membrane, cytoplasm, and nucleus. Nuclear immunoreactivity was interpreted as positive for cell cycle proteins, whereas cytoplasmic expression of angiogenic molecules was the parameter scored. The extent of staining was scored using the following scale: 0 = no staining; 1 = staining of 0–5% of tumor cells; 2 = staining of 6–25% of cells; 3 = staining of 26–75% of tumor cells; and 4 = staining of 76–100% of tumor cells. A minimum of 500 cells, when present, was evaluated. Evaluation was done without knowledge of patient clinical data.

**IB**

Thirty-two malignant effusions (21 peritoneal, 11 pleural) were studied. Frozen samples were thawed, washed twice in

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Table 2  NGF, TrkA, p-TrkA and cell cycle protein expression in 80 effusions

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Cellular location</th>
<th>0%</th>
<th>1–5%</th>
<th>6–25%</th>
<th>26–75%</th>
<th>76–100%</th>
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<td>TrkA</td>
<td>Membrane</td>
<td>47 (59%)</td>
<td>20 (25%)</td>
<td>8 (10%)</td>
<td>5 (6%)</td>
<td>0 (0%)</td>
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<tr>
<td>TrkA</td>
<td>Cytoplasm</td>
<td>16 (20%)</td>
<td>0 (0%)</td>
<td>6 (7%)</td>
<td>11 (14%)</td>
<td>47 (59%)</td>
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<tr>
<td>p-TrkA</td>
<td>Membrane</td>
<td>69 (86%)</td>
<td>7 (9%)</td>
<td>5 (4%)</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
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<tr>
<td>NGF</td>
<td>Nucleus</td>
<td>72 (90%)</td>
<td>5 (6%)</td>
<td>1 (1%)</td>
<td>2 (3%)</td>
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<tr>
<td>Ki-67</td>
<td>Nucleus</td>
<td>2 (3%)</td>
<td>18 (22%)</td>
<td>22 (28%)</td>
<td>33 (41%)</td>
<td>5 (6%)</td>
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<tr>
<td>p85-PARP</td>
<td>Nucleus</td>
<td>38 (48%)</td>
<td>36 (45%)</td>
<td>5 (6%)</td>
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<td>16 (20%)</td>
<td>10 (12%)</td>
<td>23 (29%)</td>
<td>10 (12%)</td>
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<tr>
<td>p21WAF1/CIP1</td>
<td>Nucleus</td>
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<td>23 (29%)</td>
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<td>0 (0%)</td>
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* Total = 15 positive specimens, of which 7 showed membrane expression alone, 4 nuclear expression alone, and 4 immunoreactive at both sites.
Fig. 1  IHC results in effusions. A, TrkA staining in a peritoneal effusion. The majority of carcinoma cells show cytoplasmic immunoreactivity, whereas some cells show additional localization to the cell membrane. B, p-TrkA membrane immunoreactivity in a peritoneal effusion. C, TrkA translocation to the nucleus in 5–25% of cancer cells in a peritoneal effusion. D, NGF expression in a peritoneal effusion. All tumor cells show cytoplasmic immunoreactivity. E, Ki-67 expression in a peritoneal effusion. Five to 25% of tumor cells show nuclear staining. F, p53 accumulation in the specimen shown in A. Seventy-five to 100% of tumor cells show nuclear staining. G, focal expression of p85-PARP in a peritoneal effusion. Less than 5% of tumor cells show nuclear staining but many reactive cells surrounding tumor cells express p85-PARP. H, focal expression of p21\^WAF1/CIP1 in a peritoneal effusion. Less than 5% of tumor cells show nuclear staining. I, another peritoneal effusion, showing no immunoreactivity for p21\^WAF1/CIP1 in tumor cells, whereas reactive cells, including both leukocytes and reactive mesothelium, are stained.
TrkA, Angiogenic Genes, and Cell Cycle Proteins

Search Genetics (Huntsville, AL). The probe sequences (5' transcripts of VEGF, bFGF and IL-8 were obtained from Research Genetics (Amersham Pharmacia, Buckinghamshire, United Kingdom). Specific antisense oligonucleotide DNA probes for mRNA expression were chosen with the objective of showing some negative specimens and all three pTrkA-positive effusions because only 3 of 32 cases were positive for this receptor.

<table>
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<th>Molecule</th>
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<th>Weak</th>
<th>Moderate</th>
<th>Strong</th>
<th>Total</th>
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<td>0</td>
<td>32</td>
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<tr>
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<td>0</td>
<td>0</td>
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<tr>
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<td>13</td>
<td>14</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>p21^{WAF/CIP1}</td>
<td>3</td>
<td>13</td>
<td>9</td>
<td>6</td>
<td>31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data refer only to expression at predicted molecular sizes (see “Discussion” section).
<sup>b</sup> One failed reaction.

cold PBS, and lysed in NP40 ice-cold lysis phosphorylation buffer [1% NP40, 10% glycerol, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 100 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin, pepstatin, and aprotinin] to preserve the integrity and phosphorylation activity of samples proteins. The lysates were then sonicated and clarified by centrifugation, and protein quantification was done by Bradford analysis. Twenty-five μg of total cellular protein were loaded into each lane, separated by electrophoresis through SDS-12% PAGE, blotted onto immobilon-P-membranes (Millipore Corporation, Bedford, MA) and blocked in 5% dry milk in tris buffered saline-tween. The filters were subsequently hybridized with the antibodies against TrkA, p-TrkA, p53, and p21^{WAF/CIP1} used for IHC. A mouse monoclonal antibody against α-tubulin (clone 57; Oncogene) was used as loading control. After washing in TBS-T, bound antibody was visualized using peroxidase-conjugated antimouse IgG and the enhanced chemiluminescence detection system (Amersham Pharmacia, Buckinghamshire, United Kingdom). Negative controls consisted of antibody in the absence of lysate. Positive controls for the IB procedure consisted of the WM35 melanoma cell line (34).

**Blocking/Competition Experiment.** To verify the specificity of the TrkA reaction, a specific blocking antibody was used. Five-fold excess of blocking peptide in 500 μl of PBS was used. Incubation was for 2 h in room temperature.

**Oligonucleotide Probes**

Specific antisense oligonucleotide DNA probes for mRNA transcripts of VEGF, bFGF and IL-8 were obtained from Research Genetics (Huntsville, AL). The probe sequences (5'-3') were as follows (35): bFGF, 5'-CGGGAGGCCCGCGTCGCGGCA-3'; IL-8, 5'-CTCCACAACCTCTCGACCC-3'; and VEGF, 5'-TGTGATGTTGGACCTCAGTGCGGU-3'.

A poly d(T)20 oligonucleotide (Research Genetics) was used to verify the integrity and lack of degradation of mRNA in each sample. DNA probes for VEGF, bFGF, and IL-8 were hyperbiotinylated. Stock dilution was prepared with a resulting equal concentration for all probes. The stock dilution was diluted with probe diluent (Research Genetics) immediately before use. Specific sense oligonucleotides were used for the evaluation of nonspecific activity for each probe.

**mRNA ISH**

mRNA expression of angiogenic molecules was studied in 63 effusions and 65 corresponding solid tumors. Sections (4-μm thick) of formalin-fixed, paraffin-embedded specimens were mounted on ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA). Sectioning was performed in RNase-free water. ISH was carried out using the microprobe manual staining system (Fisher Scientific; Ref. 36). Hybridization of the probes was carried out as described previously (37). A positive enzymatic reaction in this assay stained dark blue. Known positive controls were used in each hybridization reaction. These consisted of two ovarian carcinomas for which positive hybridization was reproducible in a previous study. Controls for endogenous alkaline phosphatase for all probes included treatment of the sample in the absence of the probe and use of chromogen alone.

**Evaluation of ISH Results.** The presence mRNA of angiogenic molecules in carcinoma and stromal cells was scored. Staining extent was scored as detailed for IHC. In addition, staining intensity was scored as absent (n = 0), weak to moderate (n = 1), or intense (n = 2). Evaluation was done without knowledge of patient clinical data or other expression data.

**Statistical Analysis**

Statistical analysis was performed applying the SPSS-PC package (version 10.1; SPSS, Chicago, IL). P < 0.05 was considered statistically significant. Full clinical and pathological data were available for all patients. Studies of the association between molecule expression in effusions and clinicopatholog-
clinical parameters were undertaken using the two-sided $\chi^2$ test. These consisted of analyses of the association between IHC results and effusion site, FIGO stage, tumor grade, and chemotherapy status (pre-versus posttreatment specimen). Analysis of the association between TrkA, $p$-TrkA, and NGF expression and that of cell cycle and angiogenic molecules were similarly performed using the two-sided $\chi^2$ test. Univariate survival analyses were executed using the Kaplan-Meier method and log-rank test. Expression categories in these tests were clustered so as to allow for a sufficient number of cases to be included in each category.

RESULTS

Trk-A, $p$-TrkA, and NGF Expression Is Independent of Cell Cycle Protein Expression in Effusions. In this analysis, we investigated the possible correlation between TrkA and NGF expression and cell cycle protein expression to define whether the findings in cells of neural origin are of relevance in ovarian carcinoma. Although NGF and TrkA protein expression was relatively frequent in carcinoma cells in effusions, $p$-TrkA was found in only a minority of specimens (Table 2; Fig. IA–D). As predicted for metastatic cancer cells, $K_i$-67 expression and p53 accumulation were frequently found (Table 2; Fig. 1, E and F). Conversely, $p21^{WAF1/CIP1}$ and $p85$-PARP expression, when present, were limited to few (<5%) tumor cells (Table 2; Fig. 1, G and H), whereas staining of reactive cells, mostly lymphocytes, was distinctly more common (not scored; Fig. 1I). When the presence of proteins at their predicted molecular weight was evaluated, IB demonstrated expression of TrkA in 9 of 32, $p$-TrkA in 3 of 32, $p21^{WAF1/CIP1}$ in 31 of 32, and p53 in 28 of 31 cases (one failed reaction for p53; Table 3; Fig. 2). Additional truncated forms were seen for p53 (data not shown). Use of blocking peptide for TrkA resulted in elimination of its expression in all positive cases (Fig. 2). Ovarian carcinoma cells in pleural ($n = 23$) and peritoneal ($n = 57$) effusions showed comparable expression of all markers. Similarly, no association was seen between protein expression of the studied molecules and patient age, FIGO stage, tumor grade, the extent of residual disease, or chemotherapy status (data not shown). Trk-A, $p$-TrkA, and NGF expression showed no association with that of p53, $K_i$-67, $p21^{WAF1/CIP1}$, or $p85$-PARP.

Trk-A Activation Is a Frequent Event in Solid Ovarian Carcinoma. Because activation of TrkA plays a significant role in the clinical behavior of various solid tumors and because NGF is angiogenic, we wished to investigate the expression of these molecules in solid primary and metastatic tumors. TrkA protein membrane and cytoplasmic expression was detected in carcinoma cells in 57 of 65 (88%) and 63 of 65 (97%) solid tumors, respectively (Table 4; Fig. 3A). Although these figures exceeded those seen in effusions in both this and our earlier report, the difference was still more pronounced using the $p$-TrkA antibody because $p$-TrkA membrane and cytoplasmic expression was seen in 50 of 65 (77%) and 52 of 65 (80%) tumors, respectively (Table 4; Fig. 3B). As in effusions, a small subset of carcinomas ($n = 6$) showed unequivocal nuclear immunoreactivity (Table 4; Fig. 3C). Interestingly, intratumoral and peritumoral vessels showed intense membrane expression of $p$-TrkA in 60 specimens, including cases that were $p$TrkA-negative in tumor cells (Fig. 3D). NGF expression was seen in cancer cells in 52 of 65 (80%) tumors (Table 4; Fig. 3E).

NGF and TrkA Are Coexpressed with Angiogenic Genes. Because NGF is an angiogenic molecule, we wished to study the possible relationship between NGF and TrkA expression and the synthesis of three angiogenic molecules that have been previously studied in this cohort. mRNA expression of bFGF predominated in both biopsy and effusion specimens, followed by IL-8, whereas VEGF mRNA expression was infrequent, as previously reported (Refs. 33, 38; Table 5; Fig. 3, F–I). In effusions, NGF expression correlated with that of bFGF protein ($P = 0.016$), as well as with intense bFGF mRNA expression ($P = 0.032$; Table 6). In solid tumors, the presence of NGF in tumor cells correlated with high VEGF mRNA expression in both tumor ($P = 0.002$ for both intensity and extent) and stromal ($P < 0.001$ for intensity, $P = 0.002$ for extent) cells (Table 7). In addition, NGF and bFGF mRNA showed significant coexpression in tumor cells ($P = 0.008$; data not shown). Finally, TrkA membrane expression in tumor cells correlated with intense stromal expression of VEGF mRNA ($P = 0.017$; Table 8).

Trk-A, $p$-TrkA, and $p85$-PARP Are Novel Predictors of Disease Outcome in Ovarian Cancer. In the final part of the analysis, we wished to evaluate the potential prognostic role of the molecules studied. In this cohort, no patients were lost to follow-up. Follow-up period ranged from 1 to 83 months (mean = 27 months). Forty-nine patients died of disease, 18 patients were alive with disease, and 5 patients were free of disease at the time of last follow-up. In survival analysis, cytoplasmic expression of TrkA ($P < 0.001$) and nuclear expression of $p85$-PARP ($P = 0.017$) in tumor cells in effusions predicted better outcome (Fig. 4, A and B). In solid tumors, expression of $p$-TrkA at both the membrane ($P = 0.004$) and cytoplasm ($P = 0.042$) predicted poor survival (Fig. 4, C and D). As previously reported, angiogenic gene expression showed no correlation with disease outcome ($P > 0.05$, data not shown). Neither did
Fig. 3  Immunohistochemistry and mRNA ISH in solid tumors. A–F, TrkA, p-TrkA, and NGF. A, TrkA protein membrane expression in a primary serous ovarian carcinoma. All cells express TrkA. B, p-TrkA protein membrane expression in a primary serous ovarian carcinoma. C, p-TrkA protein nuclear expression in a cutaneous metastasis. The majority of tumor cells are positive. This pattern was observed in six carcinomas. D, p-TrkA protein membrane expression in peritumoral vessels in the vicinity (left) of a primary carcinoma. Unequivocal membrane expression is seen. E, vascular p-TrkA expression at a peritumoral site in the tumor shown in C. F, NGF expression in cancer cells in a uterine metastasis of a serous ovarian carcinoma. All cells show cytoplasmic immunoreactivity. G–I, expression of mRNA of angiogenic molecules. G, d(T) control for mRNA integrity in a primary ovarian serous carcinoma. All cells are labeled (nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate staining). Inset shows an intestinal metastasis negative for VEGF. Cells are counterstained with nuclear fast red. H, weak diffuse VEGF mRNA expression in both the tumor and the stromal cell compartments. I, intense expression (dark blue) of the bFGF in the two compartments.
malignancies, including ovarian carcinoma (18–21) and recent studies in murine models have demonstrated the role of neurotrophin signaling in tumor growth and metastasis (39, 40). However, the biological roles of the NGF-TrkA autocrine loop in ovarian carcinoma are poorly defined at present. In this study, we attempted to study the correlation between these molecules and disease outcome, as well as their association with expression of cell cycle molecules and angiogenic factors.

We have previously reported on the reduced expression of TrkA in ovarian carcinoma cells in effusions compared with corresponding solid tumors (19). In this study, a similar and more pronounced phenomenon was seen for p-TrkA. However, unlike experimental models (39, 40), this down-regulation does not appear to be related to reduced cell proliferation, as evidenced both by the lack of correlation with Ki-67 counts and by the generally high expression of this marker in effusions. The possibility that the reduced expression of TrkA is associated with the alternative expression of other tyrosine kinases receptors such as epidermal growth factor, c-erbB-2, and fibroblast growth factor receptors in effusions deserves additional research.

Interestingly, nuclear carcinoma cell-specific expression of p-TrkA was detected in a few specimens, both primary and metastatic. The biological significance of this finding, which is well documented for other kinases involved in signal transduction, is unknown to us at present and requires additional investigation.

The comparison between pleural and peritoneal carcinoma cells did not reveal differences in the expression of TrkA, p-TrkA, NGF, or the cell cycle markers detailed below, in agreement with our previous studies of ovarian carcinoma cells in effusions (19, 27, 28, 33, 41–47). The present findings for

**DISCUSSION**

Overexpression of growth factor receptors such as tyrosine kinase receptors is one of the phenotypic changes providing cancer cells with the ability to override normal cell growth regulatory mechanisms. Expression of TrkA, the high-affinity receptor for NGF, has been documented in several epithelial malignancies, including ovarian carcinoma (18–21) and recent

*Table 5 Angiogenic gene expression in 63 effusions and 65 solid tumors*

<table>
<thead>
<tr>
<th>Molecule</th>
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<th>Percentage of stained cells</th>
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<tr>
<td>bFGF</td>
<td>Effusions</td>
<td>Carcinoma</td>
<td>35</td>
<td>31</td>
</tr>
</tbody>
</table>

*P = 0.002.*

*Table 6 The association between protein expression of NGF and mRNA expression of bFGF in carcinoma cells in effusions (n = 63 specimens)*

<table>
<thead>
<tr>
<th>NGF</th>
<th>bFGF</th>
<th>Absent</th>
<th>Weak</th>
<th>Intense</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>1</td>
<td>10</td>
<td>2</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Focal (1–25%)</td>
<td>2</td>
<td>6</td>
<td>13</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Diffuse (26–100%)</td>
<td>0</td>
<td>10</td>
<td>19</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>26</td>
<td>34</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>

*P = 0.032.*

*Table 7 The association between expression of NGF in carcinoma cells and mRNA expression of VEGF in solid tumors (n = 65 specimens)*

A. VEGF tumor cells

<table>
<thead>
<tr>
<th>NGF</th>
<th>Absent</th>
<th>Focal (1–25%)</th>
<th>Diffuse (26–100%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Focal (1–25%)</td>
<td>6</td>
<td>9</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Diffuse (26–100%)</td>
<td>17</td>
<td>6</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>15</td>
<td>15</td>
<td>65</td>
</tr>
</tbody>
</table>

B. VEGF stromal cells

<table>
<thead>
<tr>
<th>NGF</th>
<th>Absent</th>
<th>Focal (1–25%)</th>
<th>Diffuse (26–100%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Focal (1–25%)</td>
<td>5</td>
<td>10</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Diffuse (26–100%)</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>17</td>
<td>15</td>
<td>65</td>
</tr>
</tbody>
</table>

*P = 0.002.*

The expression of p53, Ki-67, or p21WAF1/CIP1 show such correlation (P > 0.05, data not shown).

**DISCUSSION**

Overexpression of growth factor receptors such as tyrosine kinase receptors is one of the phenotypic changes providing cancer cells with the ability to override normal cell growth regulatory mechanisms. Expression of TrkA, the high-affinity receptor for NGF, has been documented in several epithelial malignancies, including ovarian carcinoma (18–21) and recent
NGF and TrkA provide additional evidence in support of our hypothesis that ovarian carcinoma cells in pleural effusions closely resemble those in peritoneal effusions and possess no phenotypic or genotypic advantages that can support the classification of isolated pleural effusion as FIGO stage IV disease. Furthermore, carcinoma cells at both effusion sites undergo significant biological alterations compared with cells at the primary tumor site, suggesting that the cells in ascites are truly metastatic.

p53 and p21WAF1/CIP1 are two powerful regulators of the cell cycle, the latter through inhibition of the cyclin-dependent kinase 2, involved in the G1-S transition (48). The role of the NGF-TrkA autocrine loop in activation and expression of p53 and p21WAF1/CIP1 has been documented in PC12 cells (9–11). Our findings do not support such role in ovarian carcinoma cells because expression of p53 and p21WAF1/CIP1 did not correlate with NGF and TrkA expression or with activation of TrkA. We observed an almost universally low expression of p21WAF1/CIP1 in cancer cells in our material, with expression in blots reflecting inflammatory and mesothelial cell contribution. This was accompanied by high expression of cyclin-dependent kinase 2 in all effusions using IB (data not shown). Blots for p53 have shown additional truncated forms, possibly representing mutated protein, in many specimens. It therefore appears that the dysregulation of cell cycle events in ovarian carcinoma in effusions also involves the dissociation from NGF-TrkA signaling.

Fig. 4 Kaplan-Meier survival curves showing the correlation between TrkA, p85-PARP, and p-TrkA and disease outcome. A, the correlation between cytoplasmic expression of TrkA in carcinoma cells in effusions and OS. Patients with tumors showing expression in >25% of cancer cells (n = 22, discontinuous line) had a median OS of 20 months compared with 31 months for patients with tumors showing >25% expression (n = 58, continuous line; P < 0.001). The death of the last patient among the poor survivors, 65 months after diagnosis, has been censored but is shown in B. B, the correlation between p85-PARP expression in effusions and OS. Patients with specimens showing any number of p85-PARP-positive cells (n = 42, continuous line) had a median OS of 37 months, compared with 23 months for patients with p85-PARP-negative tumors (n = 38, discontinuous line; P = 0.017). C, the correlation between membrane expression of p-TrkA in carcinoma cells in solid tumors and OS. Patients with p-TrkA-negative tumors (n = 15, continuous line) had a median OS of 83 months compared with 24 months for patients with tumors showing staining of 1–5% of cells (n = 30, discontinuous line) and 25 months for patients with expression exceeding 5% of cells (n = 20, dotted line; P = 0.004). D, the correlation between cytoplasmic expression of p-TrkA in carcinoma cells in solid tumors and OS, showing results that are comparable with those in C. Patients with p-TrkA-negative tumors (n = 13, continuous line) had a median OS of 46 months compared with 24 months for patients with tumors showing staining of 1–5% of cells (n = 32, discontinuous line) and 23 months for patients with expression exceeding 5% of cells (n = 20, dotted line; P = 0.042).
PARP-1, together with PARP-2, is a member of a family of enzymes involved in DNA repair after damage by various agents such as alkylating agents, ionizing radiation, and oxidants (24, 25). PARP-1 appears to protect cells from apoptosis after minor DNA damage, but its excessive expression leads to NAD⁺ consumption and cell death through apoptosis or necrosis (25). Ample experimental data, mainly through studies of knockout mice, support the role of PARP-1 in tumorigenesis, and mice lacking both p53 and PARP-1 develop a large variety of tumors. Data regarding human tumors is lacking to date, and mutations or loss of heterozygosity of the PARP-1 gene have not been reported, despite alterations of the gene site on chromosome 1q41-42 in breast, liver, and brain tumors (24). To the best of our knowledge, our data are the first to document a predictive role for p85-PARP in human cancer. Surprisingly, any degree of apoptosis, as reflected by p85-PARP expression, including that limited to <5% of tumor cells, was associated with improved outcome. The peritoneal space is a hypoxic environment in which cancer cells are removed from the more efficient supply of nutrients and oxygen through blood vessels. The apparent total immunity from apoptosis shown in some of the studied tumors in effusions may therefore signify aggressive behavior and enhanced refractoriness to both hypoxia and chemotherapy.

As opposed to the consistent role of TrkA in predicting better prognosis in tumors of neural origin such as neuroblastoma (22, 23), the prognostic role of neurotrophin receptor expression in epithelial malignancies remains largely unknown. Absence of TrkA expression correlated with advanced disease stage, high histological grade, and poor survival in esophageal carcinoma (49). However, this finding was not reproduced in multivariate survival analysis in which disease stage was the only significant predictor of survival (49). In agreement with this finding, TrkA mRNA expression, evaluated by real-time reverse transcription-PCR, predicted improved survival in breast carcinoma (50). However, these studies did not evaluate the expression of activated receptor, which is the biologically active molecule. We now report that cytoplasmic expression of TrkA in effusions predicts better outcome. This may reflect the presence of nonglycosylated receptor, a finding that is in agreement with the finding of bands at Mᵦ 110,000 in IB. The difference between survival analysis results of TrkA expression in effusions in this study compared with our earlier report (19) may result from both the larger cohort of patients with clinical follow-up (72 compared with 55 patients) and the longer follow-up period (mean = 27 months compared with 19 months) in this study. More significantly, activation of TrkA, as evaluated with use of the anti-p-TrkA antibody, predicted worse survival. The main reason for the difference between the positive predictive role in effusions and the negative one in solid tumors is probably because nonactivated TrkA does not have the biological activity of the activated receptor. However, we believe that this can also be attributed to the phenotypic and genotypic differences between ovarian carcinoma cells in solid tumors and effusions, as we have been shown in previous studies (19, 27, 28, 33, 42). To the best of our knowledge, this is the first evidence linking activation of the TrkA receptor with disease outcome in human carcinoma.

Calza et al. (15) have recently demonstrated the role of NGF in angiogenesis, a finding that was confirmed by a subsequent study of human endothelial umbilical vein cells (14), but the issue of the angiogenic effect of NGF-TrkA signaling in human cancer has not been investigated previously. Two findings in the current study suggest such a role in ovarian carcinoma: first, coexpression of NGF and TrkA with angiogenic molecules known to be expressed in this tumor. Second—and the finding we regard as the most exciting in this study—unequivocal expression of activated TrkA on endothelial cell membranes, both inside the tumor and in its vicinity. These findings provide new information regarding the possible induction of angiogenesis in solid ovarian carcinoma and, if substantiated, may aid in designing future antiangiogenic therapy for this malignancy.

In conclusion, the expression and activation of the neurotrophin receptor TrkA is reduced in effusions compared with solid ovarian carcinoma and appears to be independent of cell cycle progression in this tumor. Taken together with p85-PARP, TrkA and p-TrkA provide novel prognostic markers in ovarian carcinoma. The evidence for an angiogenesis-promoting role for the NGF-TrkA autocrine loop may aid in understanding the function of neurotrophins in human carcinoma.

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Expression and Activation of the Nerve Growth Factor Receptor TrkA in Serous Ovarian Carcinoma

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