Tumor Necrosis Factor and Its Receptors in Human Ovarian Cancer

Potential Role in Disease Progression

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Abstract

The gene for tumor necrosis factor, TNF, was expressed in 45 out of 63 biopsies of human epithelial ovarian cancer. In serous tumors, there was a positive correlation between level of TNF expression and tumor grade. TNF mRNA was found in epithelial tumor cells and infiltrating macrophages, whereas TNF protein localized primarily to a subpopulation of macrophages within and in close proximity to tumor areas. mRNA and protein for the p55 TNF receptor gene localized to the tumor epithelium and tumor, but not to stromal macrophages. The p75 TNF receptor was confined to infiltrating cells. Cells expressing TNF mRNA were also found in ovarian cancer ascites and TNF protein was detected in some ascitic fluids. In 2 out of 12 biopsies of normal ovary, TNF mRNA was detected in a minority of cells in the thecal layer of the corpus luteum. Serum levels of TNF and its soluble receptor did not correlate with extent of TNF expression in matched biopsies. Northern and Southern analysis revealed no gross abnormality of the TNF gene. The coexpression of TNF and its receptor in ovarian cancer biopsies suggests the capacity for autocrine/paracrine action. TNF antagonists may have therapeutic potential in this malignancy. (*J. Clin. Invest.* 1993. 91:2194–2206.) Key words: IL-1 • IL-6 • macrophages • in situ hybridization • epithelial

Introduction

When the gene encoding human TNF was cloned in 1984 (1), there was great interest in this factor as a new therapy for cancer. Both the historical background (2) and preclinical studies with partially purified material (3) appeared to support these early hopes. Recombinant TNF was selectively cytotoxic for some tumor cell lines (4) and caused necrosis of certain experimental syngeneic murine tumors (5) and human tumor xenografts (6). However, in contrast to the antitumor action observed in these earlier studies, a number of biological activities were identified that could promote the growth and invasive capacity of tumors (7). TNF may act directly as a growth factor for fibroblasts (8) and thus may contribute to the generation of tumor stroma (9). TNF also has wide ranging effects on endothelial cells including promoting chemotaxis, the induction of

proliferation, and stimulation of angiogenesis (10, 11). TNF upregulates certain metalloproteinase genes (12, 13) that are associated with high invasive activity and metastatic potential (14). Further actions include alterations in the expression of surface adhesion molecules (15, 16) increasing tumor cell binding to endothelium (17). TNF also has potent effects on osteoclast activity, thereby stimulating bone resorption (18). The role of TNF in cancer cachexia is still contentious, though experiments in rat models have provided some evidence that suggest a role in this complex wasting syndrome (19).

We have previously demonstrated that TNF can promote tumor progression in vivo. In xenograft models, intraperitoneal injection of recombinant human TNF causes ascitic human ovarian cancer cells to clump and form multiple solid tumors on the peritoneal surface (20). Moreover, cells transfected with TNF exhibited enhanced invasive capacity in nude mice, an effect that could be neutralized with antibodies to TNF (21). Other groups have shown that certain human tumor cell lines express TNF in vitro and that prolonged exposure to TNF led to the development of resistance to the cytotoxic effects of TNF and induced constitutive secretion of TNF by these cells (22).

We and others (23, 24) have demonstrated TNF expression in colorectal carcinoma in vivo by Northern analysis of extracted mRNA. In situ hybridization localized this expression to < 0.1% of infiltrating macrophages. No TNF gene expression was found in the neoplastic epithelial cells in these cancers. Similar results have been seen in studies on breast carcinomas (Miles, D., L. Happerfield, S. Naylor, L. Bobrow, and F. R. Balkwill, manuscript submitted for publication). In a preliminary study of 14 cases of human ovarian cancer (25), we found a different pattern of TNF gene expression, with localization to tumor epithelial areas. Of the common human cancers, ovarian carcinoma offers an insight into differing modes of progression; spreading by local expansion, direct invasion, transcoeloemic spread, and lymphatic or vascular invasion. The animal data and our initial findings indicate that human ovarian cancer offers a unique system for the analysis of TNF and its role in tumor biology.

This paper describes the detailed analysis of TNF cytokine and receptor distribution in 81 ovarian neoplasms, 12 samples of normal ovary, and 10 samples of ascites obtained from ovarian cancer patients. Using riboprobes to TNF and the p55 and p75 TNF receptors, we have studied mRNA expression by in situ hybridisation techniques, and related this to histologic grading of the tumor. Using immunohistochemistry, we have assayed for the presence of TNF and TNF receptor protein. The cause of dysregulated TNF expression has been investigated by Southern and Northern analyses of tumor. We have also assessed the interaction of macrophages and tumor cells in the tumor microenvironment and investigated the expression

of the TNF related, and possibly induced, cytokines, IL-1α, IL-1β, and IL-6. Results obtained have been correlated with levels of TNF and its soluble receptors in serum samples from some of these patients.

Methods

Patients/histological assessment

Tissues were obtained from unselected patients from several hospitals in the United Kingdom. Conventionally stained sections were used to identify the tumors according to International Federation of Gynaecology and Obstetrics classification (26). Thus of the 81 tumors, 63 were carcinomas (40 serous, 14 mucinous, five endometrioid, and one clear cell [mesonephroid], and three undifferentiated), and 18 tumors were either benign ovarian or nonovarian tumors (three thecoma, four mucinous cystadenomas, one squamous carcinoma of cervix, one leiomyoma, one leiomyosarcoma, three metastatic colonic carcinoma, one granulosa cell tumor, one immature teratoma, one endometrioma, one malignant mixed mullerian tumor, and one appendiceal mucinous carcinoma). After identification of the 11 borderline tumors (carcinoma of low malignant potential), a scoring system was used to grade the frankly malignant ovarian carcinomas taking into account architectural and cytological features (27). This grading was performed blind and independent of the assessments of immunostaining and in situ hybridization.

Tumor samples

Solid tumor specimens were snap frozen in liquid nitrogen immediately after removal from the patient. Normal ovarian tissue was also obtained from patients who had no evidence of ovarian neoplasia. For RNA isolation, solid tumor was cut into 10-μm slices on an ultramicrotome and transferred into 5 M guanidinium thiocyanate lysis buffer. For in situ hybridization, 5–7-μm sections were taken onto baked glass slides coated with triethoxysilylethanolamine (Sigma Immunochemicals, Poole Dorset, UK), air dried, fixed in freshly prepared 4% paraformaldehyde for 20 min, and stored dessicated at −70°C until required.

Cell lines

The HL60 human promyelocytic leukemic line was maintained in RPMI 1640 supplemented with 5% FCS. RNA was extracted for Northern analysis, and cytospins prepared for in situ hybridization after a 3-h incubation with 50 ng/ml PMA (Sigma Immunochemicals). This provided a source of TNF, IL-1α, and β mRNA for use as a positive control in all RNA analyses (28). Human foreskin fibroblasts were used as a source of IL-6 mRNA.

Probes

IN SITU HYBRIDIZATION

TNF. An antisense TNF riboprobe was generated from the Apa 1–cleaved pGEM1-hTNF containing a 1-kb sequence of the TNF cDNA (obtained from Prof. W. Fiers, University of Ghent, Ghent, Belgium) using T7 RNA polymerase (Promega Biotech, Madison, WI). The negative control was sense TNF generated from Bam H1 cleaved pGEM1-hTNF using SP6 RNA polymerase (Promega Biotech).

IL-1α and β. Antisense IL-1α and β probes were prepared from EcoRI cleaved pSPhIL-1α and β plasmids, respectively (containing 400 bp Hind III/EcoR1 fragment of the respective cDNAs, obtained from Dr. A. Shaw, Biogen, Geneva).

IL-6. The IL-6 probe was prepared from pGEM3 containing a 300-bp Taq 1-Xba 1 fragment of the IL-6 cDNA (originally obtained from Prof. W. Fiers).

p55 and p75 TNF receptor. The p55 and p75 TNF receptor riboprobes were generated from plasmids pGEM 3 Hup 55 (containing ~ 800 bp Bam H1/Bgl II fragment of the cDNA) and pGEM 3 Hup 75 (containing ~ 1,300 bp Bam H1/Bgl II fragment of the cDNA) (obtained from Prof. M. Feldmann, Sunley Research Institute, London, see reference 29).

In vitro transcriptions were performed using transcription kits (Promega Biotech, Southampton, UK) incorporating ^{35}S-UTP (Amersham International, Amersham, UK). Restriction enzymes were all obtained from Pharmacia fine Chemicals (Piscataway, NJ).

For Southern and Northern analyses, the Pst 1 fragment of p-hTNF-1 (obtained from Prof. W. Fiers) was labeled as outlined below.

In situ hybridization

In situ hybridization was carried out on cryostat sections under ribonuclease limited conditions using ^{35}S-labeled riboprobes as described previously (23).

The sections were examined by direct illumination and dark field microscopy using a Leitz Diaplan microscope. Positive cells were identified by deposition of silver grains per cell in a concentration over the perinuclear cytoplasm that exceeded the "background" and levels on sections exposed to the "sense" probes by a factor of ≥ 10, and also reproduced a similar labeling density and pattern on duplicate sections. For relative abundance of labeled cells, an arbitrary scoring system of average labeling was used. Measurements were based on the mean number of labeled cells per high power field (hpf).[1] A minimum of 10 randomly selected hpf and up to 50 hpf were counted using an eyepiece with a magnification of 10 and an objective lens with a magnification of 40 and field area = 3.2 mm².

In Table I, +++ indicates > 20 cells/hpf; ++ indicates 2–20 cells/hpf; + indicates 0.2–2 cells/hpf; and ± indicates < 0.2 cells/hpf. Tumors showing low densities of labeled cells were more easily assessed using dark field microscopy.

The focal nature of the expression meant that the range of expressing cells varied from 0 up to a maximum of, in one case, ~ 50 cells in a single hpf. The maximal expression was also noted in each case.

Immunohistochemistry

Serial cryostat sections were immunostained either by a standard peroxidase-antiperoxidase method (30) or the alkaline phosphatase antialkaline phosphatase technique (31) (reagents from Dako, High Wycombe, Bucks, UK) with the following primary antibodies:

Macrophage/histiocyte antigens. EBM11 (anti-CD68; Dako), Y1/82A (anti-CD68, gift of Dr. David Mason, Histopathology Dept., John Radcliffe Hospital, Oxford). Specificities of these antibodies are outlined in (32).

Epithelial markers. HMFG-2 (anti–human epithelial mucin monoclonal antibody, gift of Dr. J. Taylor, Imperial Cancer Research Fund [ICRF], London) neat supernatant; Cam 5.2 (anti–low molecular weight cytokeratin monoclonal antibody, gift of Dr. W. Bodmer, ICRF, London), diluted 1 in 10.

TNF protein. CB6 (anti–human TNF monoclonal antibody, gift of Dr. Sue Stephens, Celltech, Slough, UK), diluted 1 in 40. The specificity of staining was confirmed by positive staining of Chinese hamster ovary cells transfected with the TNF gene and competition with excess recombinant human TNF, and negative staining of control, neomycin-transfected Chinese hamster ovary cells (21).

TNF receptor. htr-9 (recognizes the p55 TNF receptor) and utr antibodies (recognizing the p75 TNF receptor) See reference 33.

Endogenous alkaline phosphatase activity was quenched with levamisole (31), endogenous peroxidase activity was blocked by preincubation with hydrogen peroxidase (30).

Isolation of DNA

DNA was isolated according to standard procedures (34).

Southern blot analysis

20 μg of DNA was digested with 100 U of Pst 1 at 37°C in appropriate digestion buffer for 3 h. After digestion, fragments were separated on

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1. *Abbreviation used in this paper: hpf, high power field.*

an 0.7% agarose gel buffered at 40 V overnight. Ethidium bromide was added to the gel at 0.5 mg/ml. 5 μg of λ DNA-Hind III φX -174 DNA-Hae III digest was run in one lane as size marker. After electrophoresis, the gel was depurinated in 0.25 M HCl for 15 min, neutralized in 0.4 M NaOH for 5 min, and then alkaline blotted (in 0.4M NaOH) onto genescreen. The membrane was rinsed in 2× SSC and left to air dry for 2 h. Blots were reprobed with a single copy probe to control for loading inaccuracies.

Isolation of cellular RNA

Total cellular RNA was isolated after centrifugation through cesium chloride followed by precipitation with 3 M sodium acetate and ethanol as described in (35).

Northern blotting analysis

15-μg aliquots of total cellular RNA were electrophoresed through a 1.4% agarose-formaldehyde denaturing gel and capillary blotted onto membranes (Biodyne A; Pall Ultrafine Filtration Corp, Glen Cove, NY). Membranes were hybridized to the ^{32}P-labeled inserts of human cDNA probes under standard conditions (36) and labeled with [^{32}P]-dCTP by the random priming method (37). Membranes were subsequently washed to high stringency and exposed to Kodak XAR5 film at −70°C with two intensifying screens (Dupont, Stevenage, Herts, UK).

Radiommunoassay for TNF

The concentration of TNF was determined by radioimmunoassay using TNF-α IRMA kit (Medgenix, Medgenix Diagnostics, Fleurus, Belgium). The sensitivity of this assay is < 10 pg/ml.

Soluble TNF receptor assay

These were performed by Dr. Dan Aderka in the laboratory of Prof. David Wallach at the Weizmann Institute (Rehovot, Israel) (38).

Results

Ovarian cancers

TNF expression. TNF gene expression was found in 45 out of 63 cases of ovarian cancer and was localized to epithelial tumor areas. These data are summarized in Table I. The majority of serous carcinomas contained labeled cells (29 out of 40) in clusters of two to five cells within the epithelial compartment, and not within the intervening desmoplastic stroma (Fig. 1, a and b). Up to 65% of cells in a maximal hpf were found to express TNF (see Fig. 1 a), though the focal nature of this expression meant that the mean percentage of expressing cells was lower (13% in this case). Fig. 2 shows the relationship between TNF expression and grade in the serous group. The level and frequency of TNF expression increases with progression from borderline (low malignant potential) to poorly differentiated grade 3 (Fisher's exact test, significance $P < 0.005$).

Undifferentiated and endometrioid carcinomas showed some TNF expressing cells, again within the epithelial compartment. While TNF mRNA expression was not extensive in these types of carcinoma, the numbers studied were relatively small, though representative of their frequency in the general population. No TNF expression was found in the single case of the relatively uncommon clear cell carcinoma of the ovary.

There was a significant difference in TNF gene expression between mucinous and serous tumors. Only 3 out of 14 mucinous tumors contained labeled cells, whereas the serous group showed 29 out of 40 TNF mRNA positive cases (Fisher's exact test P [two-tail] = 0.001).

Table I. Frequency and Extent of TNF Expression in Ovarian Neoplasms

Tumor type	Number of cases	Total number positive	+	++	+++
Serous	40	29	17	11	1
Undiff. Adenoca.	3	2	1	1	−
Mucinous	14	3	2	1	−
Endometrioid	5	2	1	1	−
Clear cell	1	0	−	−	−
Miscellaneous	18	1	1	−	−

+++, >20 cells/hpf; ++, 2–20 cells/hpf; +, 0.2–2 cells/hpf; ±, <0.2 cells/hpf. The different neoplasias studied were representative of their relative frequency in the general population. Undiff. adenoca., undifferentiated adenocarcinoma.

In the miscellaneous group of tumors, no labeling was observed in 17 out of 18 of the benign/mesenchymal tumor group. The one sparsely labeled tumor happened to be a recurrent colorectal carcinoma that showed labeling of < 0.1% of cells predominantly in the stroma, a pattern that we have previously described in a series of colorectal carcinomas (23).

Immunohistochemistry with TNF antibody and macrophage marker. Though it was clear that TNF expression was confined to the epithelial areas of the tumor, these are complex areas comprising epithelial tumor cells with stromal invaginations. These stromal invaginations carry tumor vasculature and a population of tumor infiltrating inflammatory cells, predominantly macrophages, with a minority of T and B lymphocytes as determined previously (25). Thus, it was possible that infiltrating macrophages were responsible for a proportion of the mRNA expression observed. We used specific antibodies to relate the location of macrophages and the epithelial tumor cells with the TNF mRNA expressing cells. At the same time, we also investigated the existence of immunoreactive protein with a characterised TNF monoclonal antibody (CB6; Celltech).

Macrophages were found within the epithelial areas of the tumor, but predominantly localized to the stromal areas of the tumor and the epithelial/stromal margin (Fig. 1, c and d). The stromal core of the tumor was found to contain the majority of the macrophages whereas TNF expression localized predominantly to the epithelial tumor cells adjacent to this infiltrate (clearly shown in Fig. 3). Those macrophages found within the epithelial areas of the tumor were evenly scattered, whereas TNF expression in the corresponding areas was found in aggregated foci in some areas and absent from others. In addition, numbers of TNF mRNA–expressing cells were focally greater than those positive for the macrophage marker CD 68 in some cases (see Fig. 1). TNF expression was not seen in macrophages within the central areas of tumor epithelial islands showing focal tumor cell necrosis.

The distribution of immunoreactive TNF protein appeared to be different from that of TNF mRNA (Fig. 1, e and f). In some cases, the epithelial areas contained a number of tumor cells and macrophages that stained with the anti-TNF monoclonal. However, the majority of immunoreactive TNF protein was detected in the stroma and at the epithelial/stromal borders also reflecting the distribution of macrophages. Despite the differing distributions of TNF mRNA and protein, and the
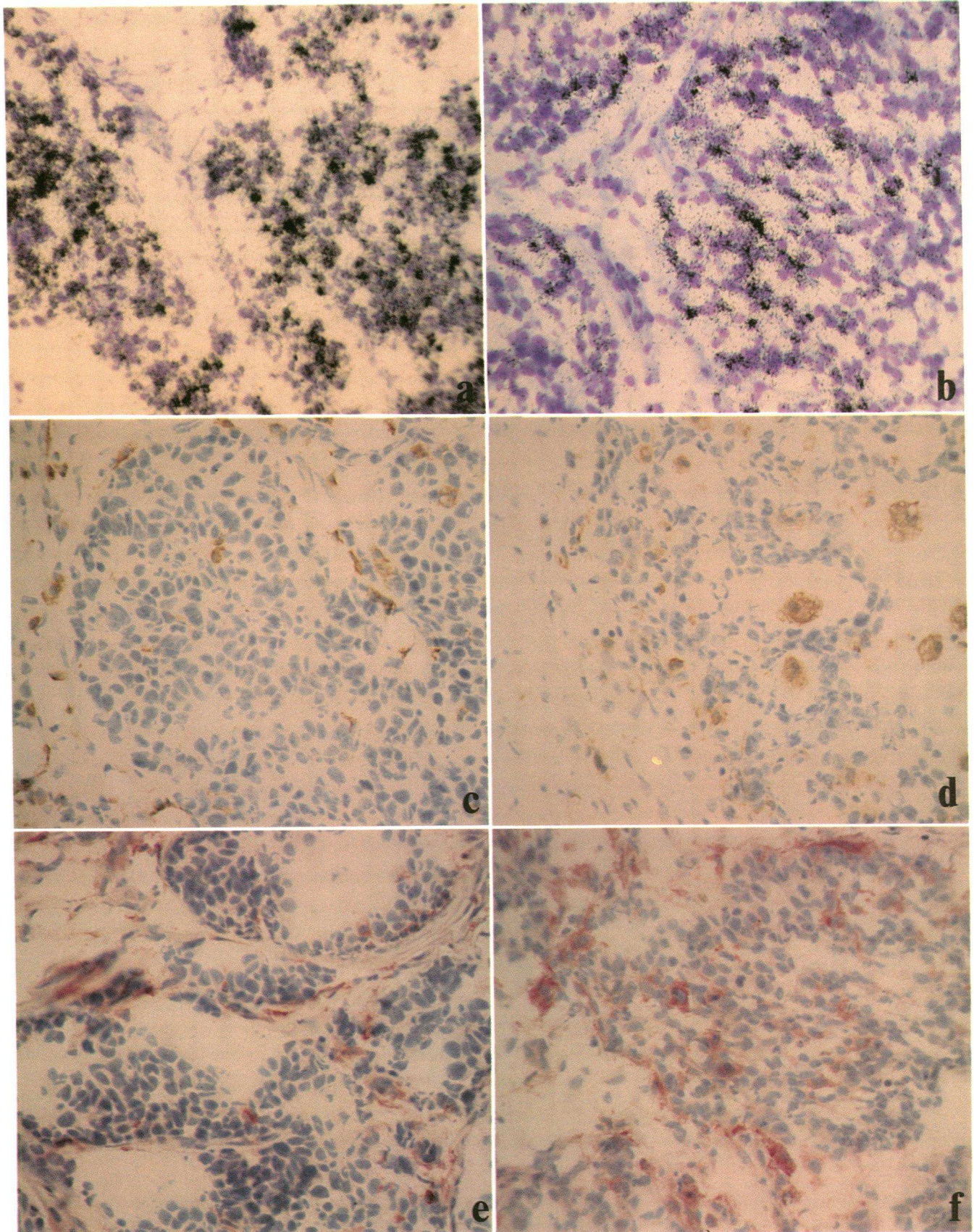
Figure 1. Relative localization of TNF mRNA, TNF protein, and macrophages in two cases of ovarian cancer as determined by in situ hybridization with a ^{35}S-labeled antisense TNF riboprobe (*a*) (×80), (*b*) ×140 and immunohistochemistry with the macrophage marker CD68 (Y1/82A), (*c*) ×140, (*d*) ×140 and immunohistochemistry with a TNF monoclonal antibody (CB6) (*e*, ×140; *f*, ×140). Cells expressing TNF mRNA (as determined by silver grain deposition after autoradiography for 10 d) outnumber those bearing the macrophage marker and also have a different distribution (malignant gland as opposed to luminal space). Distribution of TNF protein (red precipitate) is similar to that of the macrophage population (brown precipitate).
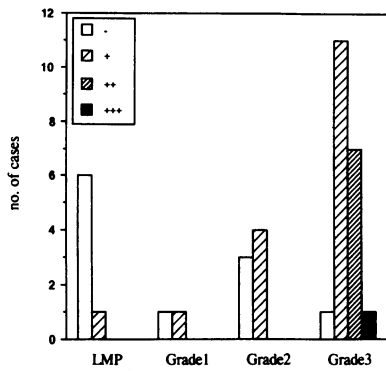
Figure 2. Histogram showing relationship of serous ovarian tumor grade with relative abundance of TNF gene expression as determined by in situ hybridization. +++, > 20 cells/hpf; ++, 2–20 cells/hpf; +, < 2 cells/hpf (see Table I and Methods). Poorly differentiated (grade III) carcinomas show significantly higher quantitative and qualitative levels of TNF expression ($P = 0.005$ Fisher's exact test). *LMP*, low malignant potential.

macrophage population, tumors with the highest overall densities of macrophages also had the highest indices of TNF mRNA labeling.

TNF receptor expression and immunolocalization. TNF p55 receptor expression was detected in all cases studied ($n = 12$). Of these cases, five were serous tumors, three were endometrioid, two were mucinousand, and two were undifferentiated. All but one of these had detectable TNF mRNA in the epithelial compartment. The p55 receptor expression was confined to the epithelial tumor cells yet in a more homogeneous distribution than that of TNF mRNA expression (see Fig. 4, *B* and *F*). The distribution and extent of labeling was more easily appreciated by dark field microscopy (see Fig. 4, *a, c,* and *e*). The macrophages within the glandular lumina did not appear to express the p55 receptor. In contrast, p75 TNF receptor expression was not seen in the malignant glandular epithelium, but was found at the tumor–stromal interface and over cells in the malignant gland lumina (Fig. 4 *d*). This related to the macrophage distribution on adjacent sections (not shown).

Immunohistochemistry with the htr antibody (recognizing the p55 receptor) showed a weak to moderate localization that was present in the majority of epithelial cells and appeared to reflect the in situ hybridization results. The utr antibody (recognizing the p75 receptor) localized to some cells in the stroma and within malignant glandular spaces corresponding to the p75 mRNA distribution and the distribution of macrophages. p75 immunostaining was not observed on epithelial cells.

Expression of TNF-related cytokines. Expression of other cytokines was investigated in relation to TNF expression. IL-1β expression was found in 23 out of 68 cases assessed. In 20 of these cases expressing IL-1β, TNF was also expressed. The distribution and extent of IL-1β mRNA was unlike that of TNF. IL-1β gene expression was scattered, found at lower levels (generally < 0.1% of the population, though in one case ≤ 23% of cells in the highest expressing area) and localized to the boundaries of stroma and epithelium. In many cases, IL-1β expression showed a similar distribution to that of the macrophage population. IL-1α mRNA was not detected in any of the cases, though it could be detected in the HL60 control. IL-6 expression was found in 2 out of 12 cases (both of which were TNF and IL-1β negative), and localized to stromal areas in clusters again usually in a single high power field consisting of 7 and 8% of the field.

Ovarian cancer ascites

Cytospin preparations from the ascitic fluids of nine patients were examined. Seven of the nine samples contained malignant cells. In the other two cases, one had inflammatory and mesothelial cells only, and the other showed marked degenerative changes with no recognizable malignant cells. In situ analysis for TNF mRNA showed clear labeling (relative to sense probed controls) in three of the seven cases containing malignant cells. One case showed light labeling on 45% of malignant cell clusters, another showed labeling on malignant cells and 1–2% of inflammatory cells (see Fig. 5), and the remaining case showed occasional labeling of mononuclear cells (< 1% total cell population) and no localization to the malignant cell population. Mesothelial cells did not show labeling in any of the cases studied.

Normal ovary

In 2 out of 12 biopsies of nonneoplastic ovary, a small proportion of cells expressed TNF mRNA. In both cases, these cells localized to focal areas of the externa theca of the corpus luteum (Fig. 6). Normal ovarian mesothelium, stroma, and follicles did not contain TNF mRNA as assessed by this methodology.

DNA and RNA analysis

To investigate gross genetic changes at the TNF locus, Southern blots were run on DNA extracted from 40 matched tumor and normal tissue samples in parallel with single copy controls. There appeared to be no significant amplification of the TNF gene in tumor relative to matched normal tissue and band shifts were not detected at this level of analysis, suggesting there are no gross abnormalities at the TNF locus in these tumors relative to the normal tissue controls. (Data not shown.)

Northern analysis was performed on five of the most positive cases to determine any aberrances in transcript size. No significant changes in the size of the TNF transcript were determined as assessed by electrophoretic mobility relative to an HL60 control RNA sample. (Data not shown.)

Serum and ascites assays for TNF and TNF receptor proteins

Levels of soluble TNF receptor and TNF protein in serum and ascites of ovarian cancer patients were also investigated (see Fig. 7).

In cases where matched material ($n = 42$) was available, there appeared to be no significant correlation with expression in the tumor and levels of TNF protein and receptors in the serum, although the two cases where the highest levels of TNF protein were detected also showed high levels of TNF expression within the tumor. Samples of matched serum and ascitic fluid were obtained from three patients. In these cases, levels of TNF protein were significantly higher in ascites than in the matched serum; i.e., in one case, levels of TNF were undetected in the serum, whereas in the ascites, levels of 206 pg/ml were recorded. Levels of soluble TNF receptor in the serum of these patients were not significantly higher than normal volunteers p55 receptor (normal range = 0.69–0.99 ng/ml [mean value of 11 volunteers = 0.8 ng/ml]; p55 patient range = 0.2–1.49 ng/ml [mean value of 21 patients = 0.7 ng/ml]; p75 receptor normal range = 1.1–2.2 ng/ml [mean value of 8 patients = 1.6 ng/ml, $n = 8$], p75 patient range = 0.52–4.32 ng/ml [mean
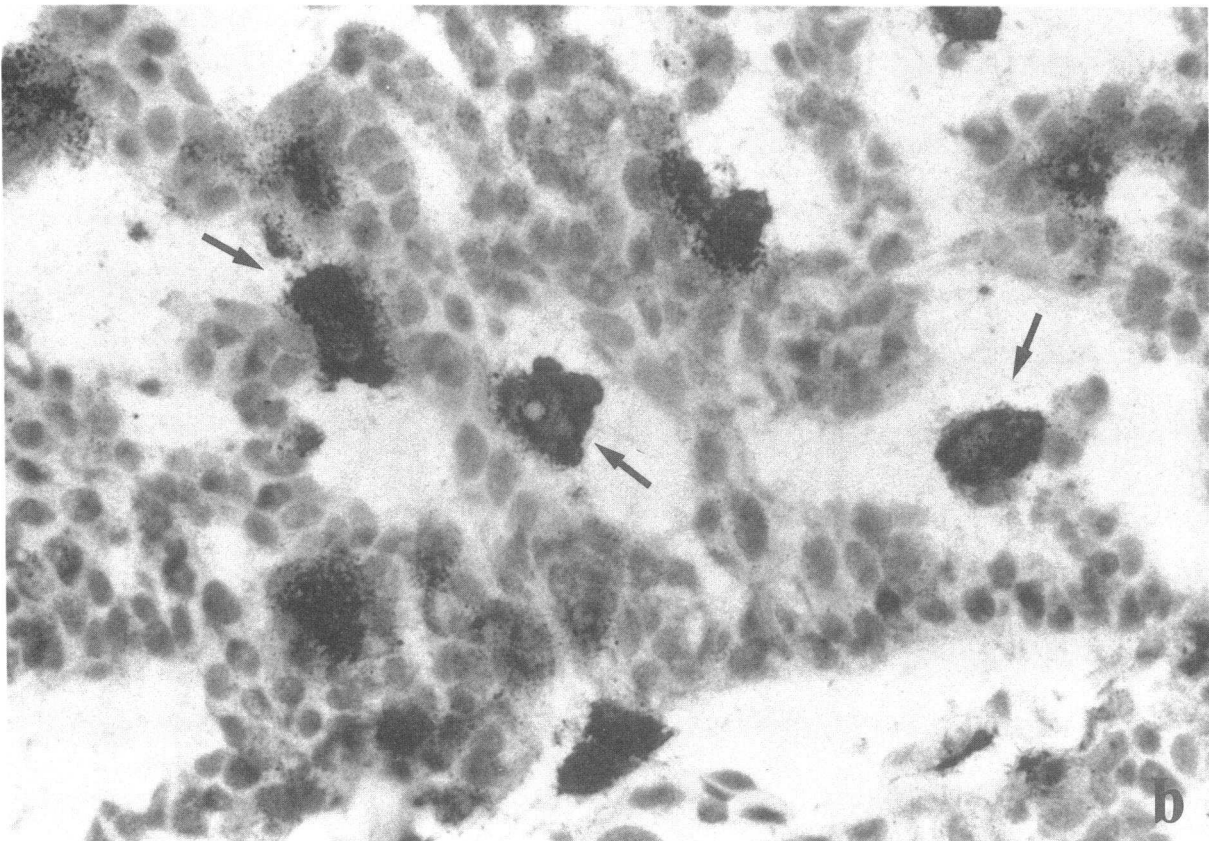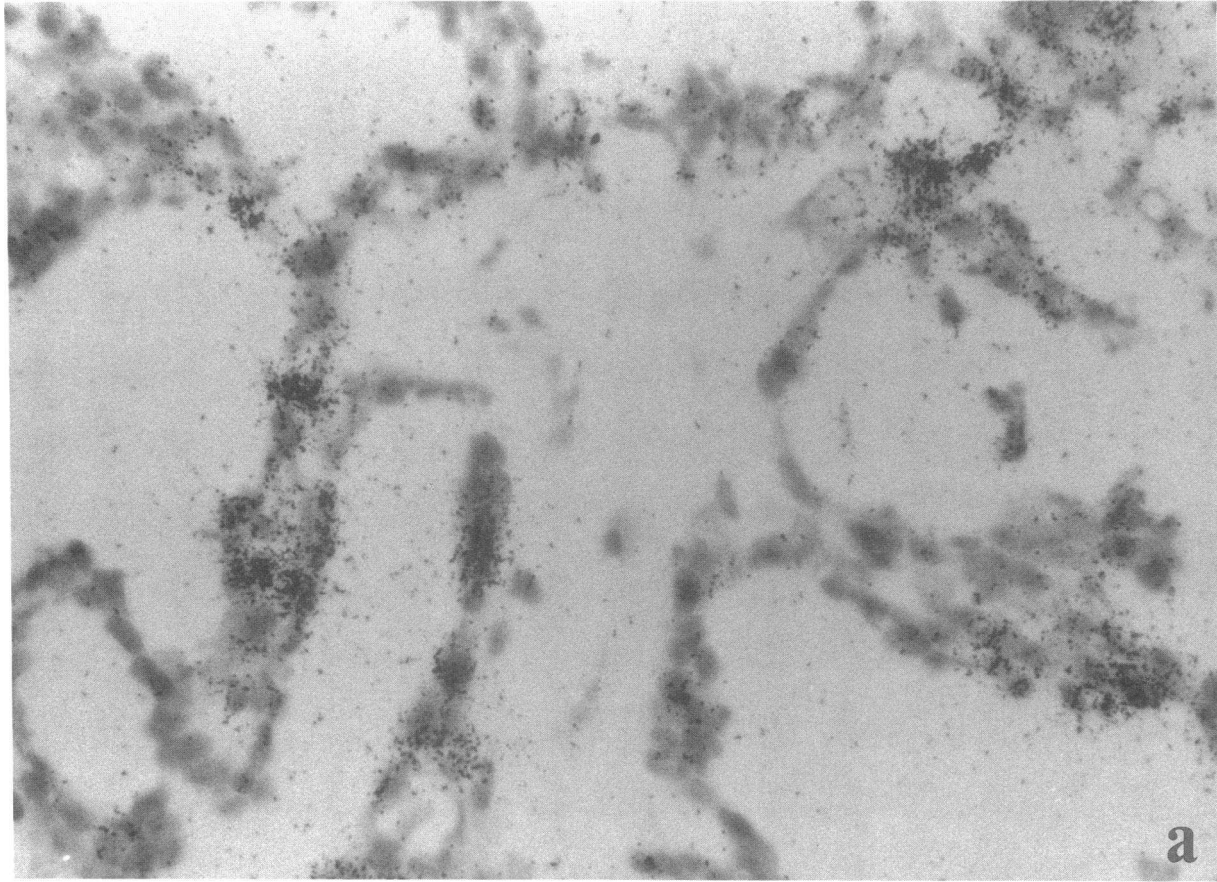
Figure 3. Relative distribution of TNF gene expression and macrophages. In situ hybridization with a ^{35}S-labeled antisense TNF riboprobe demonstrates that expression is confined to malignant epithelial cells as determined by silver grain deposition after autoradiography for 10 d (*a*) ×300. CD68 immunostaining of an adjacent section picks out isolated macrophages within the stromal and luminal space (*b*) ×300.
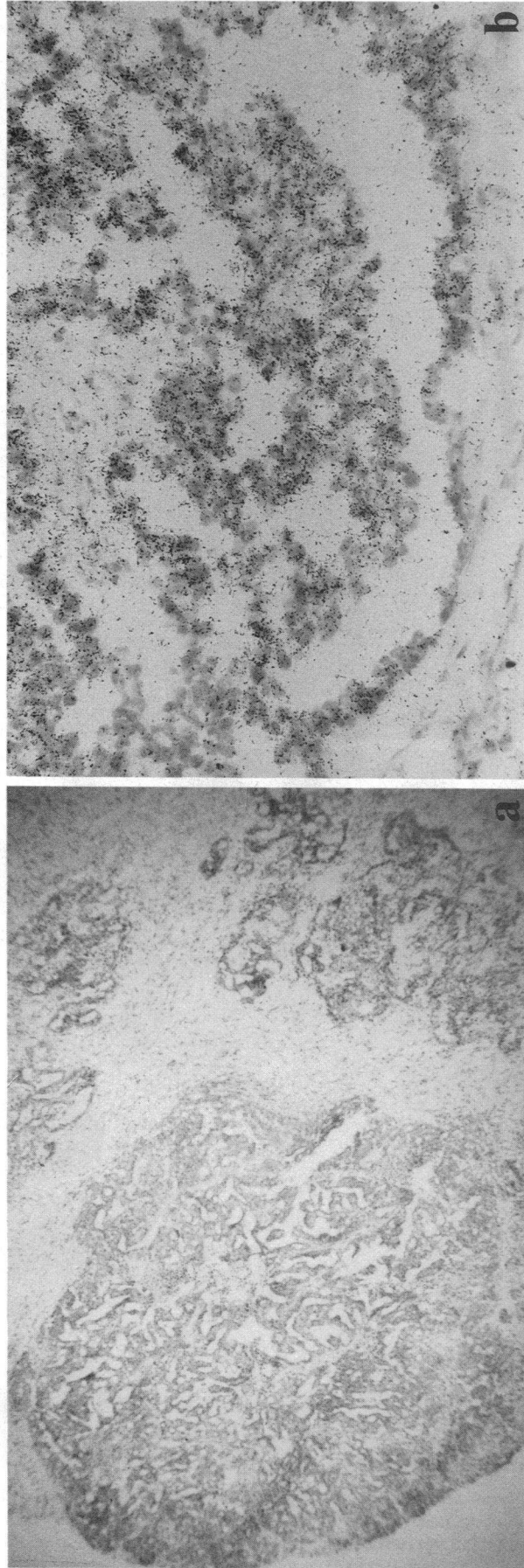
Figure 4. TNF p55 and p75 receptor mRNA expression in human ovarian cancer, relationship to TNF expression. Low power microscopy (×15) shows clear areas of tumor and stroma as revealed by haematoxylin and eosin staining (*a*). Dark field microscopy shows that the p55 TNF receptor is confined to the tumor areas (*c*), as is TNF expression (*e*). Silver grains appear white under dark field illumination. Note the more homogeneous nature of p55 receptor expression relative to that of TNF expression. Higher power bright field microscopy shows that diffuse p55 expression is confined to malignant epithelium within the tumor and not stromal areas (*b*)(×175), as is TNF gene expression (*f*) (×75), though this appears more focal. In contrast, p75 expression localizes to the luminal areas of the tumor (*d*) (×175), correlating with distribution of macrophages (see Fig. 3 *b*). Specific ³⁵S-labeled antisense riboprobes were used for in situ hybridization analysis. Localization determined by silver grain deposition after 10 d autoradiography.
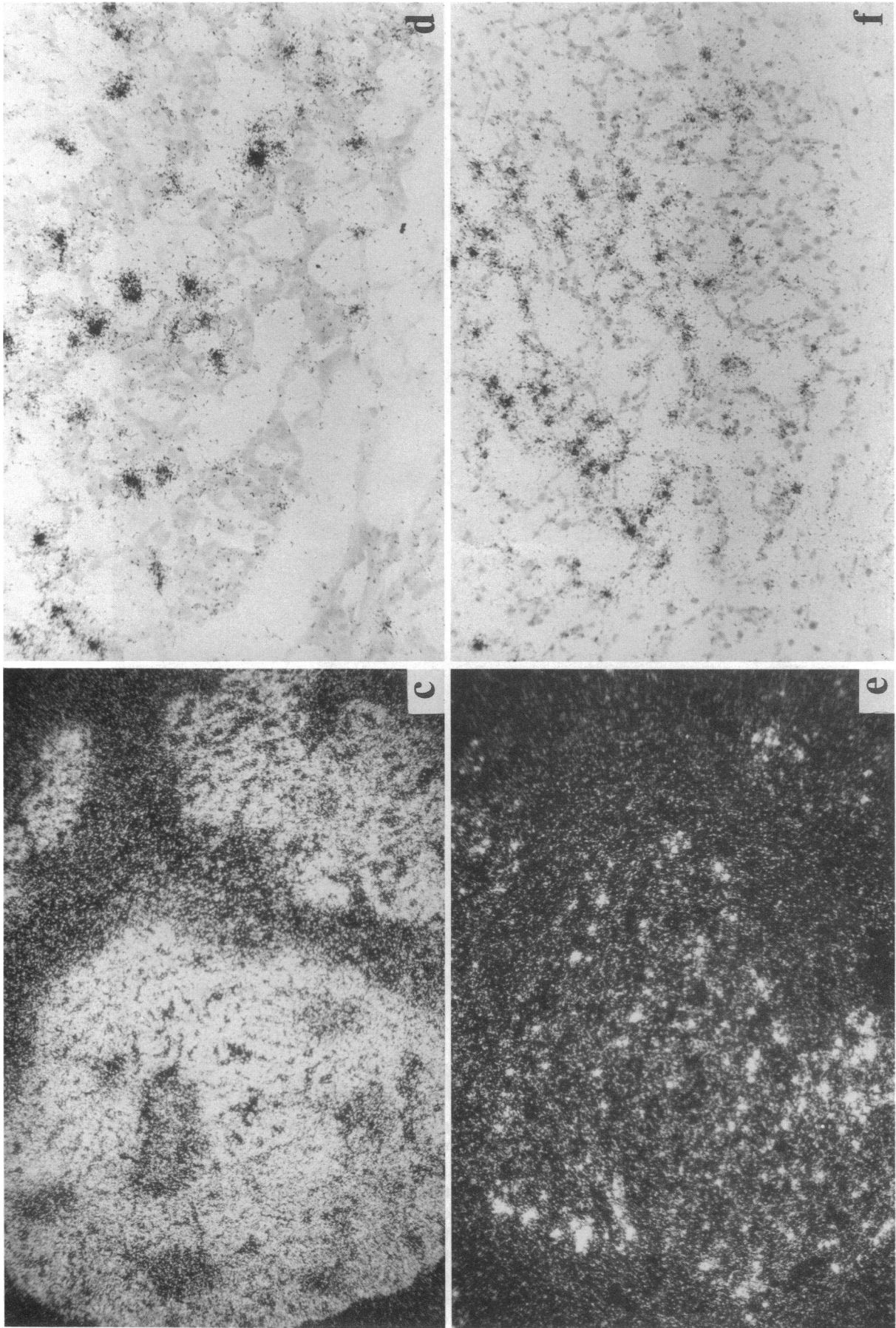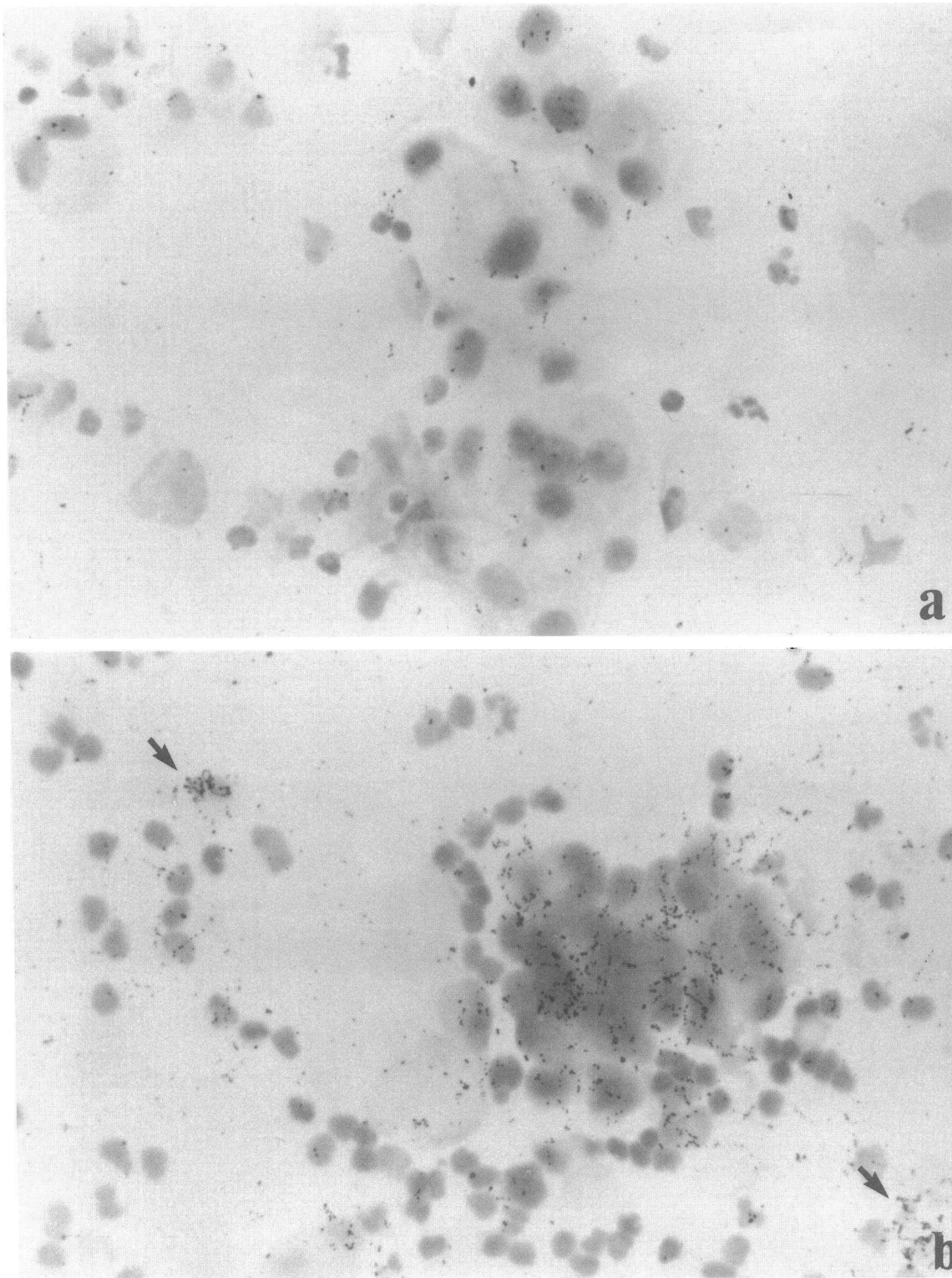
Figure 4 (Continued)

Figure 5. Expression of TNF mRNA in ovarian cancer ascites. No labeling is seen with the ^{35}S-labeled sense riboprobe control (*a*), whereas the antisense TNF riboprobe localizes to malignant cell clusters as well as some smaller inflammatory cells (*b*), *arrows.* ×300.

value of 21 patients = 1.5 ng/ml]). These levels did not relate to TNF mRNA expression in the tumor.

Discussion

In this study, we have demonstrated dysregulated expression and production of TNF and its receptors to an extent and dis-tribution that appears to be unique to ovarian cancer. We also demonstrate a positive correlation with TNF expression and tumor grade, suggesting that TNF production may enhance tumor development. The pattern of TNF expression in ovarian carcinomas is distinct from other tumors we and others have investigated; i.e., colorectal (23), cervical (unpublished obser-vations), and breast cancers (Miles, D., L. Happerfield, S. Nay-
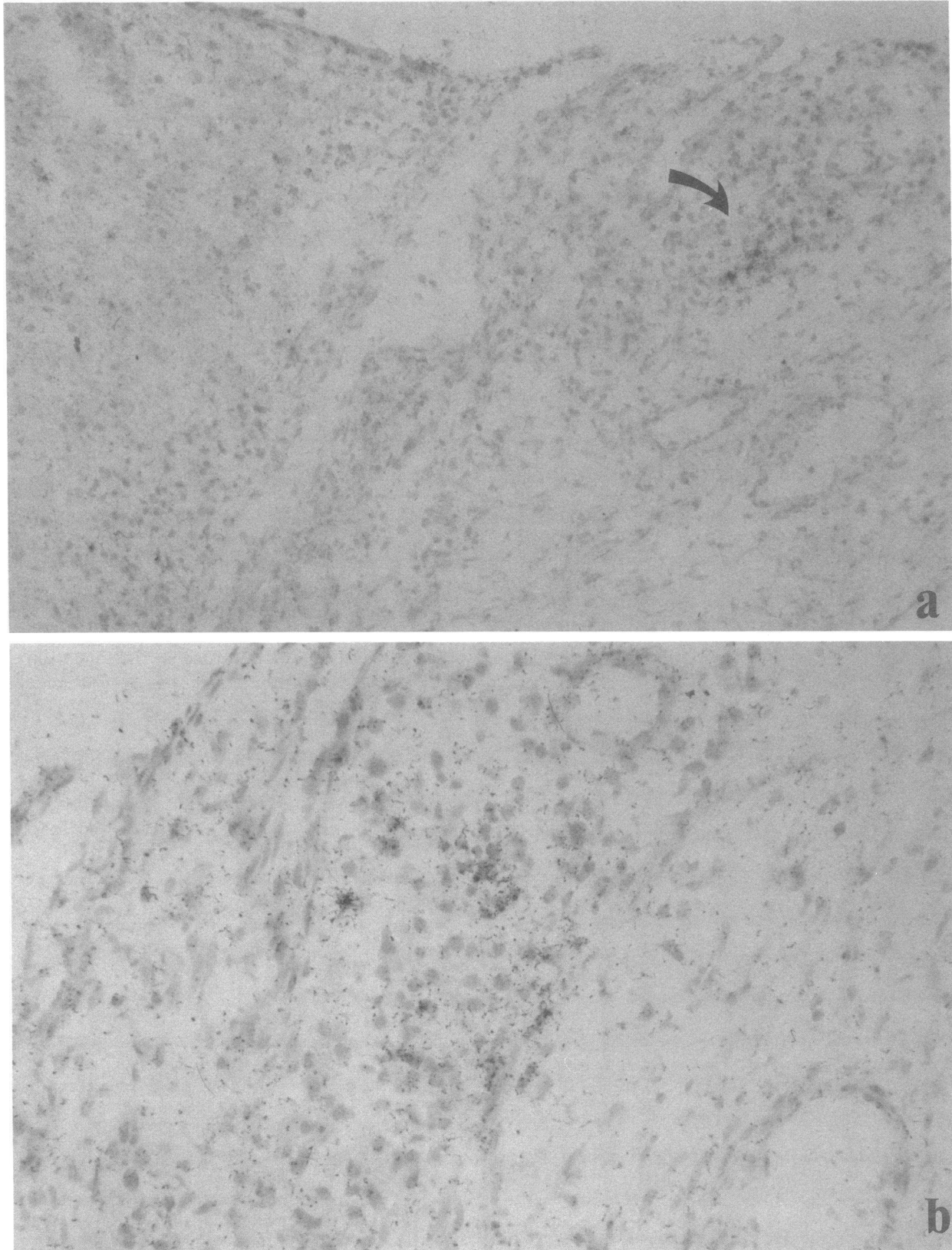
Figure 6. TNF mRNA expression in normal ovary. Area of expression arrowed (*a*), ×75, and at high power (*b*), ×175. Localization of ^{35}S-labeled antisense TNF riboprobe to cells of externa theca of corpus luteum. Epithelial, stromal, and mesothelial cells show no expression of the TNF.
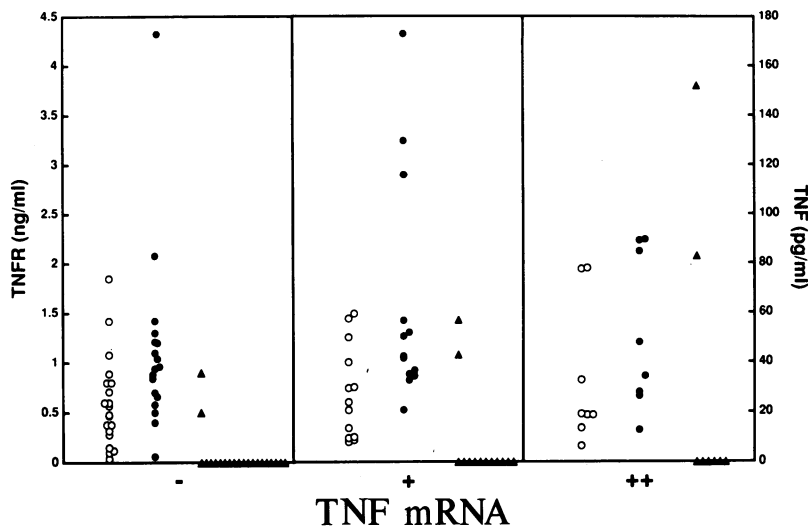
Figure 7. Serum levels of TNF protein (▲), p55 (○), and p75 (●) TNF receptor proteins grouped according to extent of TNF mRNA expression within the tumor (see Methods). There appears to be no significant correlation with expression in the tumor and levels of TNF protein and receptor in the serum, though the two cases where the highest levels of TNF protein were detected also showed higher levels of TNF expression within the tumor.

lor, L. Bobrow, and F. R. Balkwill, manuscript submitted for publication). TNF mRNA expression was particularly associated with serous tumors. Further assessment of the less common subtypes such as endometrioid and clear cell carcinomas is warranted.

TNF mRNA expression is confined to the tumor epithelial areas and the majority of cells responsible for this expression are the neoplastic epithelial cells with a minor and more variable contribution from infiltrating mononuclear cells. It is, perhaps, not surprising that the presence of TNF protein does not follow mRNA distribution considering the extent of posttranscriptional regulation in TNF production (39). Although a number of epithelial cells appear to contain immunoreactive TNF protein, the anti-TNF antibody predominantly stains cells with a macrophage distribution. Such disparities between mRNA and protein have been seen with TNF previously (40) and may be usual for rapidly secreted proteins, where the immunoreactivity reflects slower release and/or greater stability of protein, or even uptake by other cells. However, until antibodies are available that discriminate between free TNF and receptor-bound TNF, it will be difficult to know whether the antibody is localizing to cells secreting TNF or to target cells that have TNF bound to their surface. An alternative explanation of our observations might be that there is an increased stability of TNF mRNA within tumors, as has been reported with other cytokine mRNAs (41). If the turnover of TNF mRNA was slower in the tumor cells than the macrophages, this would be reflected in the in situ analysis by an increased probe localization to cells with a reduced mRNA turnover.

There is no detectable abnormality in the region of the TNF gene in this series of tumors at the level of analysis used in this study. Sequencing studies may reveal more discrete changes in the gene. The focal nature of the expression may imply that a local stimulus is in some way causing this aberrant expression. The distribution of macrophages relative to TNF expressing cells, and the observation that tumors with the highest macrophage density had the highest indices of TNF mRNA labeling, is suggestive that macrophage products may be responsible for this induction. It is widely known that activated macrophages secrete a number of factors that can induce TNF expression in adjacent cells including TNF itself (22, 42). Recent studies by Wu et al. (Prof. R. Bast, personal communication) has pro-

vided in vitro evidence that this may be what is occurring in ovarian cancer. They have found that malignant cells freshly isolated from ascitic fluid secrete TNF. This production declines on culture yet can be reinduced with TNF. In confirmation of this, studies in our laboratory have shown that treatment of ovarian cancer xenografts in vivo with TNF, results in TNF expression in the epithelial tumor cells. There is also increasing data emerging that tumors themselves secrete chemotactic factors that attract macrophages into the tumor including members of the CSF family (43) and the IL-8 family (44).

Our results appear to affirm the complex events that occur during TNF expression and secretion. It is likely that the ability of a cell to overide the normal suppression of the TNF gene determines its cell type–restricted specificity in production. Kruys et al. (45) have alluded to the existence of a transacting dominant factor that may overcome this repression. It may be that the ovarian epithelial cells acquire a similar factor as part of the transformation process.

TNF produced in ovarian cancers may be able to promote tumorigenesis by increasing local vasculature (11), and by inducing tissue remodeling (18). Our in situ hybridization studies indicate that TNF appears to be expressed by ascitic cells and high levels of TNF protein are found in these ascitic fluids. TNF may alter adhesion events in the ascitic stage of the disease that contribute to implantation as demonstrated in previous studies on our ovarian cancer xenografts (20, 21). Recombinant TNF has been used successfully in the resolution of cancer ascites (46), although there is no evidence that this treatment extends patient survival. Our previous studies (20, 21) indicate that the ascitic cells may not be eradicated but that the TNF causes a change in the biology of the tumor that leads to tumor implantation in these cases.

Soluble TNF receptors have been found at high levels in a number of disease states including ovarian cancer (38). While the cellular source of these TNF binding proteins has not been determined, our in situ results may provide the answer. In this study, the data does not show any significant increase in TNF receptor levels relative to normal samples, although there is clearly a need to look at ascites samples that would be a better indicator of local receptor shedding. The importance of looking in the ascitic fluid is underlined by our detection of high

levels of TNF in ascites that is not detected in matched serum. What remains to be determined is the neutralizing ability that TNF receptors have on the TNF produced by the tumor, for it is the balance of these, and probably many other local factors, that determine the biological activity of this cytokine. Both our immunohistochemistry and in situ results indicate that the expression of the two TNF receptors varies within the heterogeneous cell population of the tumor. The relative expression of these receptors by individual cell types is consistent in all cases studied. Infiltrating cells primarily express the p75 receptor, whereas the tumor epithelial cells appear to overexpress p55 with little p75 expression detected. Although mRNA for the receptors may not be detected in some cells, this may be a question of sensitivity of the assay. Other techniques such as ligand binding assays may identify receptors that are not detected by histological techniques. The data suggests that the relative levels of these receptors is cell type dependent. Studies by other groups have also demonstrated that the relative abundance of the two TNF receptors can vary in normal tissues depending on the cell populations within those tissues (47). Coexpression by the tumor cells of both TNF and its receptor suggests the existence of autocrine as well as paracrine mechanisms of action. The possibility still remains that in certain parts of the tumor environment TNF may function as an effective part of the host antitumor response and that this may be dependent on other factors produced in the locality. However, our analyses in the higher grade tumors would not suggest that an effective antitumor role is being mediated by TNF in this cancer.

Analysis of normal ovarian tissue indicated that TNF is not usually produced by nonneoplastic ovarian cells or stroma. It is interesting that a minority of cells in the thecal layer of the corpus luteum expressed TNF mRNA in 2 of 12 normal samples. The reason for TNF expression in normal ovary has yet to be determined, but it may reflect the role TNF has in the general wound healing response (9).

Production of TNF by ovarian cancer cells could result in resistance to the cytotoxic action of host derived TNF (22) or exogenous rhuTNF administered in a therapeutic setting. TNF has been used in several clinical trials in cancer patients and has resulted in no significant improvements in survival. Local production by tumor cells and their consequent resistance may in part explain these disappointing results.

Our study has demonstrated a high degree of expression and production of TNF and its receptors in ovarian cancer. This phenomenon appears to be peculiar to this cancer type in that the majority of expression is confined to the epithelial cells that do not normally express this cytokine in vivo. We have also observed a relationship with tumor grade and TNF expression in serous ovarian cancer. The higher incidence of TNF expression may be caused by the increased cell density in high grade tumors and the relative increase in infiltrating cells found in these cases. Increases in the proportion of macrophages may result in more TNF secreted into the tumor microenvironment with increased ability to induce TNF expression in the tumor epithelial cells. The relative distribution of macrophages suggests a role for these cells in the induction of TNF expression. The aberrant expression of this potent cytokine and its actions in promoting tumor spread may indicate that TNF therapy would be inappropriate for some ovarian cancers and that TNF antagonists would be more effective.

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