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Papers

In situ Detection of Tumour Necrosis Factor in Human Ovarian Cancer Specimens

M. Stuart Naylor, Saleem T.A. Malik, Gordon W.H. Stamp, Tom Jobling and Frances R. Balkwill

***In situ* hybridisation was used to study the local expression of tumour necrosis factor (TNF) mRNA in human ovarian tumours. In 8 of 14 ovarian cancers studied, a minority of cells in the epithelial areas of the tumour contained TNF mRNA. In individual high-power fields as many as 8% of cells were positive for TNF mRNA. Immunohistochemical studies on sequential sections and the morphology of the positive cells led to the conclusion that the ovarian tumour cells were transcribing the TNF gene. There was immunohistochemical evidence of the production of TNF protein by the tumour cells and TNF protein in a tumour lysate. The production of TNF by human ovarian cancer cells may influence the biology of the tumour, contribute to neoplastic progression and alter the response to therapy.**
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INTRODUCTION

WHEN THE gene encoding human tumour necrosis factor (TNF) was cloned in 1984 [1], there was great interest in this factor as an anticancer agent. Both the historical background [2] and preclinical studies with partly purified material [3] gave grounds for cautious optimism, and the recombinant cytokine caused necrosis of some experimental murine tumours [1, 4]. However, TNF was found to be a central member of the cytokine network and was implicated in the pathophysiology of, for instance, cachexia, endotoxic shock and cerebral malaria [5]. In contrast to the antitumour action of TNF in some experimental systems, studies in our laboratory have demonstrated that TNF can promote tumour progression. TNF therapy caused ascitic ovarian tumours to invade the peritoneal surface and form multiple solid tumours in xenograft models [6], and cells expressing a transfected TNF gene exhibited greatly enhanced metastasis and invasion in nude mice [7]. Since several human tumour cell lines have been reported to express TNF [8] we decided to look *in situ* for the production of this cytokine in human tumour specimens. We have already shown that in human colorectal cancers a few stromal cells (less than 0.1%) contained mRNA for TNF [9]. Morphological and immunohistochemical evidence suggested that these cells were activated macrophages. In contrast, we report here that in 8 of 14 untreated human ovarian cancers epithelial tumour cells expressed TNF mRNA and secreted TNF protein.

Correspondence to F.R. Balkwill.
M.S. Naylor, S.T.A. Malik, G.W.H. Stamp and F.R. Balkwill are at the Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX and T. Jobling is at the Department of Gynaecology, St. Bartholomew's Hospital, London, U.K.

MATERIALS AND METHODS

Antisense TNF was generated from the *Apa* I cleaved pGEM1-hTNF with T7 RNA polymerase (Promega Biotech, Madison). The negative control was sense TNF generated from *Bam* HI cleaved pGEM1-hTNF with SP6 RNA polymerase (Promega Biotech). *In vitro* transcriptions were done with Promega Biotech kits to incorporate ^{35}S-uridine triphosphate (Amersham). Restriction enzymes were from Pharmacia. The antisense probe identified a 17S species of mRNA in northern blots of RNA from phorbol 12-myristate 13-acetate (PMA) stimulated HL60 cells; the sense probe showed no signal.

In situ hybridisation was done on cryostat sections [9]. The positive control was HL60 cells stimulated with PMA at 50 ng/ml for 3 h.

Serial cryostat sections were stained by the standard peroxidase-antiperoxidase method [10] with the following antibodies: leucocyte common antigen (Dako) 1:20; T cell markers, UCHL1 (ICRF, London) 1:10; pan T, CD11 (Coulter Clone) 1:100; B cell markers, L26 (Dako) 1:50; macrophage/histiocyte antigens, E11 (ICRF) 1:5; HMFG-2 (ICRF) undiluted. For immunohistochemical detection of TNF protein, a 100-200 fold dilution of a murine anti-human TNF monoclonal antibody, CB0006 (Celltech) was used in a standard avidin–biotin peroxidase (Dako) method [11]. The specificity of staining was confirmed by positive staining of Chinese hamster ovary (CHO) cells transfected with the TNF gene, negative staining of control CHO cells and the absence of staining of parallel sections incubated with antibody that had been pre-absorbed with TNF.

Tumour tissue was placed in lysis buffer (10 mmol/l Tris HCl pH 7.4, 1% Nonidet P-40, 150 mmol/l NaCl, 1 mmol/l EDTA, Phenylmethylsulphonyl fluoride), homogenised and spun at 10000 *g* for 20 min at 4°C. TNF was measured in an immunoradiometric assay (Medgenix, High Wycombe).

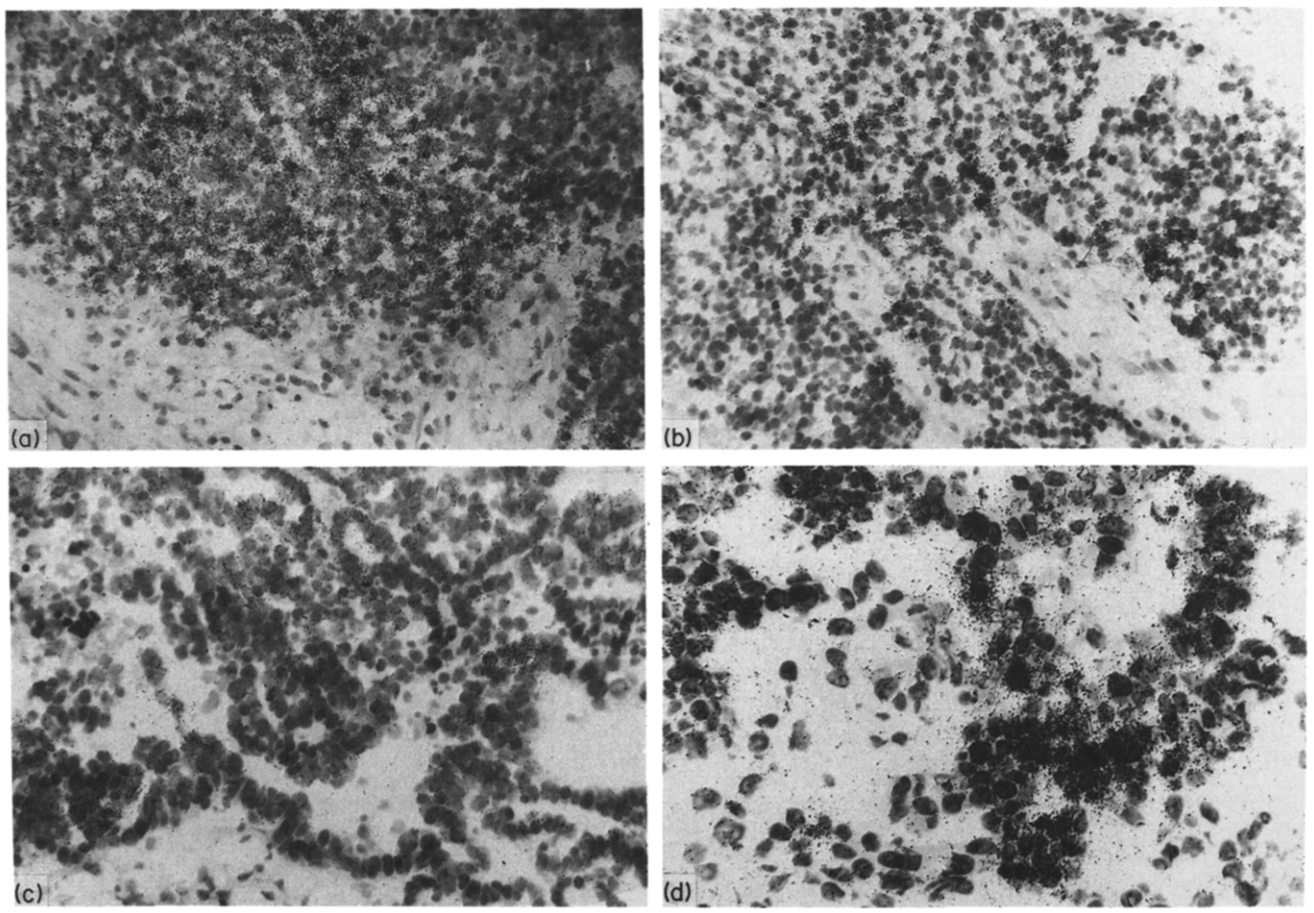

Fig. 1. ***In situ*** **hybridisation in human ovarian cancer sections with riboprobes to human TNF. (a) patient 6 (×66), (b) patient 6 (×74), (c) patient 2 (×67) and (d) patient 4 (×96).**

Table 1. *Cells positive for TNF mRNA in ovarian cancer tissue sections**

Patient	Mean stromal and epithelial nuclei per field	% epithelial cells per field	Mean % +ve cells in epithelial areas	Maximum % + ve cells in single field	Histology
1	480	84	0.2	1.0	PSC-MD
2	873	87	0.4	1.0	PSC-MD
3	428	65	0.5	8.0	SA-PD
4	605	76	0.4	4.5	PSC-PD
5	482	89	< 0.1	1.0	PSC-PD
6	868	88	1.4	4.7	SMA-PD
7	573	86	1.1	3.0	EA-PD
8	491	82	0.1	0.2	EA-PD

* At least 10 high-power fields (×400) were counted to assess cellularity and 20–30 fields for numbers of labelled cells. PSC = papillary serous cystadenocarcinoma, SA = serous adenocarcinoma, SMA = solid mucinous adenocarcinoma, EA = endometrioid adenocarcinoma and MC = mucinous cystadenocarcinoma; MD = moderately differentiated and PD = poorly differentiated. Histological diagnoses of the 6 patients (not shown here) who had no detectable TNF mRNA in their tumours were: MC-well differentiated, 1; SC-PD, 2; EA-MD, 1; and adenocarcinoma undifferentiated (not otherwise specified), 2.

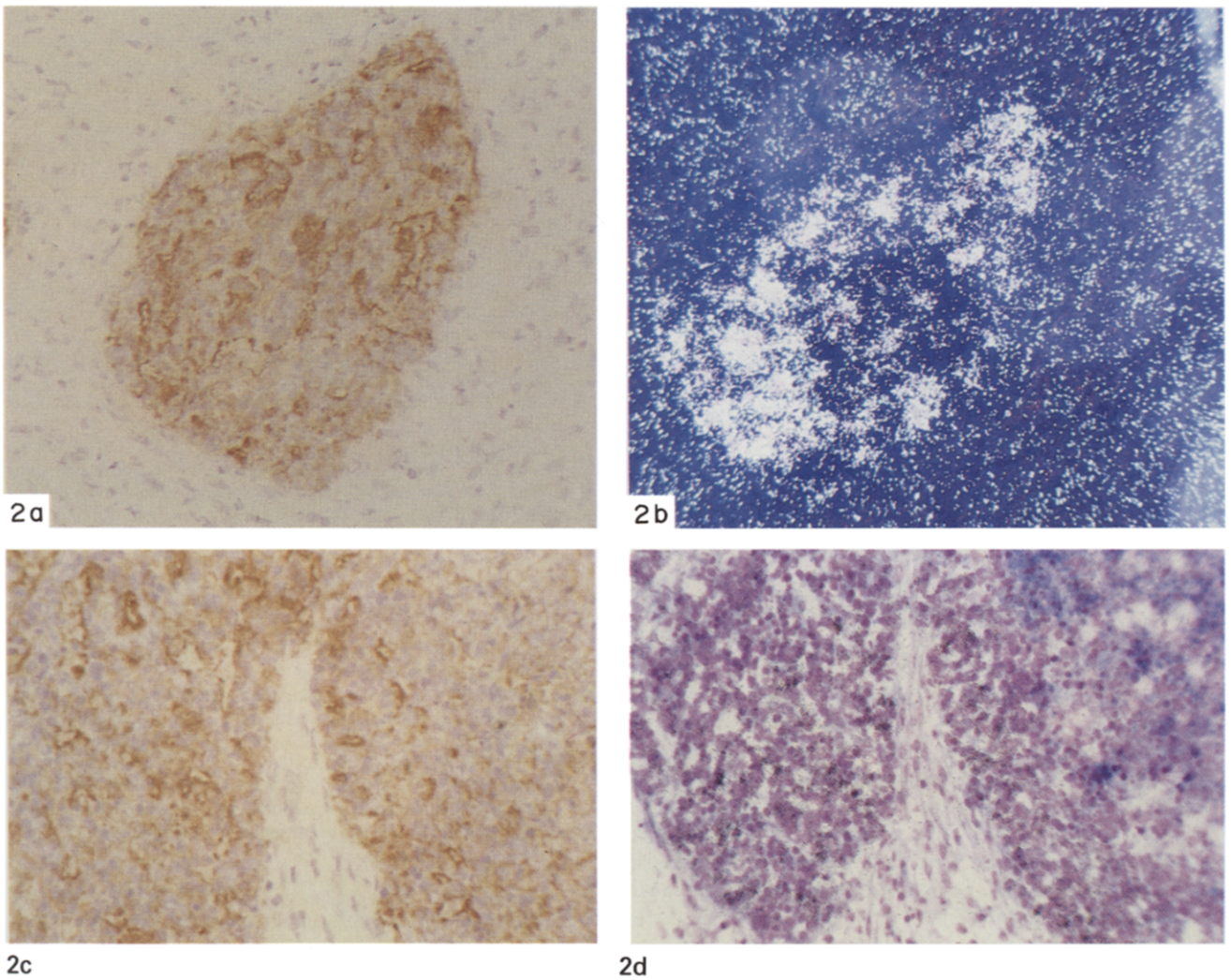

Fig. 2. Sequential ovarian cancer sections demonstrating localisation of TNF MRNA expression confined to areas expressing the tumour associated antigen HMFG 2. Patient 6: (a) section stained with HMFG 2 (×80), (b) *in situ* hybridisation of human TNF riboprobe, darkfield microscopy (×80). Patient 3: (c) section stained with HMFG 2 (×80), (d) *in situ* hybridisation of human TNF riboprobe, brightfield microscopy (×80).

RESULTS AND DISCUSSION

In situ hybridisation with a riboprobe for human TNF showed that there were cells expressing mRNA for TNF in 8 of 14 untreated human ovarian tumours. More than 99.9% of these were in the epithelial areas of the tumour. The proportion of cells in the epithelial areas of the tumour that expressed detectable TNF mRNA ranged from less than 0.1% to 1.4% when at least 20–30 high-power fields were examined in each section (Table 1). In individual fields as many as 8% of the cells in the epithelial areas expressed TNF mRNA. The positive cells were usually clustered. Examples of the results obtained with patients are shown in Fig. 1. In 4 of the 14 patients primary and metastatic lesions were examined. In 2 patients the primary tumours expressed TNF mRNA whereas the other 2 tumours were negative. There was no difference in TNF expression between the primary and secondary tumours.

The cells containing TNF mRNA had the morphological appearance of epithelial cells. Immunohistochemistry studies on sequential slides were done to characterise these cells further. Cells containing TNF mRNA were found in areas of the tumour that stained with HMFG-2 (Fig. 2), which recognises the human milk-fat globule membrane antigen and which is frequently expressed on ovarian epithelial neoplasms [12]. In 3 cases (patients 2, 4 and 6) the presence of $CD4^+$ and $CD8^+$ lymphocytes and activated macrophages was investigated. There was a mean number of 32, 34 and 19 $CD4^+$ cells per high-power field; 21, 7 and 13 $CD8^+$ cells per field; and 5, 8 and 2 activated macrophages per field in patients 2, 4 and 6, respectively. These cells were predominantly in the stroma with some occasionally seen at the margin of the epithelial areas. Less than 1 infiltrating $CD4^+$ cell per field was seen within the epithelial areas and no $CD8^+$ cells or activated macrophages. Thus the distribution of tumour and immune cells within the tumour and the morphological appearance of the positive cells indicated that the cells expressing TNF mRNA were epithelial tumour cells.

In 3 cases in which the cells expressed TNF mRNA, we looked for immunohistochemical evidence of production of protein with a monoclonal antibody against human TNF. A dense intracellular pattern of staining was observed (Fig. 3). In addition we made a lysate of tissue from patient 6. A 1/10 dilution of this lysate contained 95 pg/ml TNF, giving an overall content of 47 pg TNF per mg protein in the tumour tissue.

Our data show that a minority of human ovarian cancer cells actively produced TNF in primary and secondary lesions. This

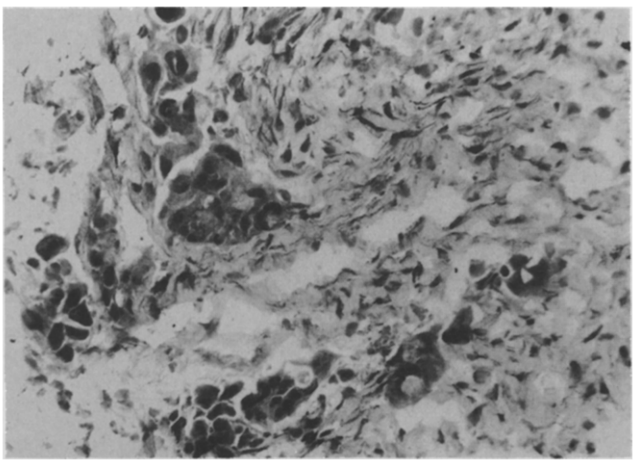

Fig. 3. Immunoperoxidase staining of TNF protein in ovarian cancer. Patient 4 (×100).

observation has important implications for the biology of this malignancy. Our animal studies suggest that these TNF-producing cells may show an increased ability to invade and metastasise. In addition, tumour cell production of TNF could promote angiogenesis in the surrounding area [13], stimulate the breakdown of connective tissue [14] and contribute to cachexia [5]. TNF could act as an autocrine and/or paracrine tumour growth factor [15] and TNF-producing tumour cells are likely to be resistant to any cytotoxic/cytostatic action of this cytokine [8]. The de-repression of the TNF gene leading to its constitutive expression may be one change that could contribute to disease progression in ovarian cancer. TNF production by a human tumour at the level demonstrated here may also affect response to chemotherapy or other cytokines.

In this small study we were not able to correlate between histological diagnosis, response to therapy or survival and the presence of TNF mRNA in the tumour cells. Further prospective studies are planned to investigate this. However, it is interesting that all four papillary tumours studied had cells expressing TNF mRNA.

The results presented here are in contrast to our work on human colorectal cancer specimens [9]. In such specimens, a few predominantly stromal cells expressed TNF mRNA, and from immunohistochemical studies we concluded that these cells were probably activated macrophages. Similar results have been reported by Beissert *et al.* [16].

During the past few years recombinant human TNF has been evaluated in several trials on advanced cancer patients. The results have not, so far, been encouraging, with an overall response rate to systemic therapy of less than 1% [17]. Production of TNF by a minority of tumour cells and its local action on surrounding cells may be one explanation for the insensitivity of solid tumours to TNF therapy.

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