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# Cells Secreting Tumour Necrosis Factor Show Enhanced Metastasis in Nude Mice

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**A tumour cell may acquire the ability to invade and metastasise via heritable changes in its genome and/or changes in the local environment. Chinese hamster ovary (CHO) cells transfected with the gene for human TNF (CHO/TNF cells) showed a greatly enhanced ability to invade peritoneal surfaces and metastasise in nude mice compared with cells transfected by the vector alone. *In situ* hybridisation with a riboprobe for human TNF showed that the CHO/TNF cells were actively transcribing this cytokine after *in vivo* injection. Neutralising antibodies to human TNF, both whole IgG and $F(ab)_2$ fragments, abrogated the enhanced metastatic activity of the TNF-secreting cells. Thus transfection of a cytokine/growth-factor gene can confer a metastatic phenotype on the recipient cell.**
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## INTRODUCTION

TUMOUR NECROSIS factor (TNF) was originally described as a macrophage-derived factor that caused haemorrhagic necrosis of murine tumours [1]. Recombinant TNF will cause regression of some animal tumours but has, to date, shown little efficacy in patients with advanced cancer [2]. TNF has pleiotropic actions [3], and its anticancer activity in animal models may be due to direct cytotoxicity/cytostasis [4], effects on tumour vasculature [5] or enhancement of specific anti-tumour immunity [6]. However, other activities of this cytokine could contribute to tumour progression. TNF can stimulate angiogenesis [7] and osteoclastic bone resorption [8], and contribute to cachexia [9, 10] and anaemia [11]. Studies in our laboratory have shown that TNF treatment can promote tumour invasion in ovarian cancer xenograft models [12]. We therefore investigated the *in vivo* behaviour of cells transfected with the human TNF gene and expressing its protein product.

## MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells were transfected with the TNF gene isolated from a human genomic library [10]. CHO cells were transfected with the expression vector alone (CHO/NEO cells) or with the plasmid containing the TNF gene (CHO/TNF cells). A single stable TNF producing clone was isolated (CHO/TNF clone 20) and used for the present experiments. $0.1 \times 10^6$ CHO cells in 2 ml α-MEM with 10% fetal calf serum were plated in triplicate wells in 6-well tissue culture plates (Flow) and incubated at 37°C in 5% $CO_2$. Cells were harvested in exponential growth phase after treatment with 0.2% trypsin and 0.05% EDTA, and counted in a Coulter counter.

6–12-week-old nu/nu mice of mixed genetic background (ICRF Breeding Unit) were used in all experiments. $5 \times 10^6$ cells in 0.5 ml α-MEM were injected intraperitoneally into nu/nu mice (8 per group). For histological studies mice were killed by $CO_2$ inhalation at appropriate times. The tissues were snap-frozen in liquid nitrogen or fixed in formol saline and in paraffin wax. Mice were killed when they developed gross abdominal distention, and the day of killing was equated to survival.

For radiolocalisation, CHO cells in exponential growth phase were labelled with 11 kBq $^{125}$I-iododeoxyuridine (IUdR)/ml (Amersham), harvested 18 h later after treatment with trypsin/versene, washed three times and counted. $5 \times 10^6$ cells in 0.5 ml α-MEM were injected intraperitoneally on day 1 (5 mice per group). On day 7, the mice were killed. A 3.0 × 1.5 cm area of the peritoneum was excised, plus the liver and lungs, and placed in 70% ethanol to remove free radiolabel. The ethanol was changed daily for 3 days, the tissues blotted dry and counted in a gamma counter. The total number of invading cells in each tissue was calculated by $5 \times 10^6 \times$ (cpm in tissue sample) ÷ (total cpm injected).

For antibody studies, 0.5 mg anti-TNF monoclonal antibody ($IgG_1$, Celltech), 0.5 mg $F(ab)_2$ fragments of the same antibody or 0.5 mg control antibody (SM3-$IgG_1$ provided by Dr J. Burchell, ICRF, London) were injected intraperitoneally with $5 \times 10^6$ CHO/TNF cells. A further 0.5 mg of antibody or $F(ab)_2$ fragment was injected intraperitoneally on day 4.

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 pGEM 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 sense and antisense riboprobes for TNF were used to probe northern blots of RNA from HL60 cells stimulated with phorbol-12-myristate 13-acetate as specificity controls. The antisense probe identified a 17S species of mRNA but the sense probe showed no signal at all. *In situ* hybridisation was done on 5–7 μm sections of snap-frozen samples [13].

The highly sensitive murine fibrosarcoma cell line, WEHI 164 clone 13, was used to assay biologically active TNF [14].

## RESULTS

CHO/TNF cells produced 2.6 μg TNF/$10^6$ cells per 24 h. CHO/NEO cells produced no detectable TNF. mRNA for human TNF was found by northern blotting in CHO/TNF but

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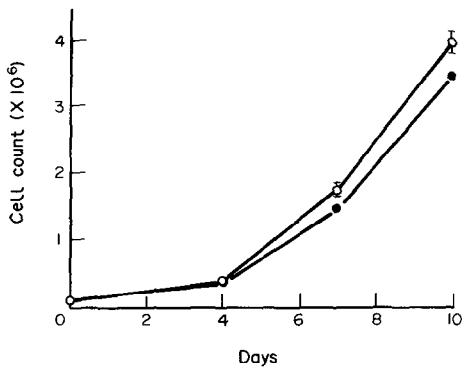


**Fig. 1. *In vitro* growth of CHO/TNF and CHO/NEO cells in liquid culture. Mean (S.D.) of triplicate wells. ○ = CHO/TNF and ● = CHO/NEO cells.**

not in CHO/NEO cells and human TNF protein was detected by immunohistochemistry and western blotting in CHO/TNF cells (data not shown). Figure 1 shows that the growth rate of the CHO/TNF transfectants in liquid culture was marginally greater than CHO/NEO cells at 7 and 10 days after culture (Wilcoxon two-sample test, $P < 0.025$).

Sequential postmortem histological analysis was done to assess the biological behaviour of the two cell lines. Differences were seen when the peritoneum and livers of mice were examined 3–14 days after tumour cell injection. The peritoneal surfaces of mice injected with CHO/NEO cells were normal, while tumour invasion was evident in mice injected with CHO/TNF cells (Fig. 2). Areas of tumour infiltration were highly vascularised, with evidence of a proliferative connective tissue response. *In situ* hybridisation with a riboprobe to human TNF showed that the tumour cells were transcribing TNF *in vivo* (Figs 3a and 3b). The level of TNF expression and the metastatic phenotype of the CHO/TNF cells was stable over 25 *in vitro* passages. Metastatic deposits were commonly seen on the surface of the liver in CHO/TNF injected mice. Histological examination revealed tumours that were infiltrating the liver parenchyma and often had a necrotic centre. Figure 3c shows *in situ* hybridisation with the TNF antisense riboprobe on one such tumour. The increased invasiveness of CHO/TNF cells was confirmed by assessing the localisation of $^{125}$ IUdR labelled CHO cells in the peritoneum, liver and lungs of nude mice (Fig. 4). This experiment was one of three similar experiments.

The enhanced invasiveness of the CHO/TNF clone 20 cells could have been due to another heritable change in this clone developed during the selection procedure. Therefore, to confirm that the behaviour of these cells was solely due to their acquired ability to secrete TNF, an anti-TNF specific monoclonal antibody or a control antibody of the same subclass was injected intraperitoneally with CHO/TNF cells. The anti-TNF antibody did not neutralise mouse TNF and had no cytotoxic/cytostatic effects on CHO/TNF cells *in vitro* at concentrations up to 1 mg/ml. The anti-TNF monoclonal antibody did, however, abrogate the metastatic behaviour of the CHO/TNF transfectants. A typical experiment is shown in Fig. 5. However, as immunohistochemical studies showed that the CHO/TNF cells express TNF protein, abrogation of metastasis could have been due to the destruction of the CHO/TNF cells by host-mediated antibody-dependent cellular cytotoxicity. We therefore included $F(ab)_2$ fragments of the anti-TNF antibody in the experiments shown in Fig. 5. These also significantly inhibited the metastatic behaviour of the CHO/TNF cells. We concluded that the enhanced metastatic behaviour of the CHO/TNF cells was due to their production of TNF and not due to selection of a highly metastatic variant of CHO cells during transfection and subsequent cloning.

We also studied the survival of mice injected with CHO cells in two experiments. At 60 days, 9 out of 16 mice in the CHO/TNF injected groups developed tumours compared with 6 out of 16 in the CHO/NEO group. The median survival of the mice developing tumours in the CHO/TNF group was 19 days compared with 37 days in the CHO/NEO group ($P < 0.05$). Necropsy revealed a large mass of intra-abdominal tumour in CHO/NEO mice, whereas CHO/TNF mice developed ascites and intra-abdominal tumours were rarely visible macroscopically.

## DISCUSSION

The mechanisms responsible for the enhanced metastatic capability of TNF-producing cells are likely to be complex. Histological examination of peritoneal specimens from CHO/TNF mice revealed an angiogenic response and deposition of connective tissue at the sites of tumour implantation (Fig. 2b), both of which may facilitate implantation and metastasis. Production of human TNF, which is cross-species reactive, in the peritoneal cavity leads to changes in the peritoneal cell

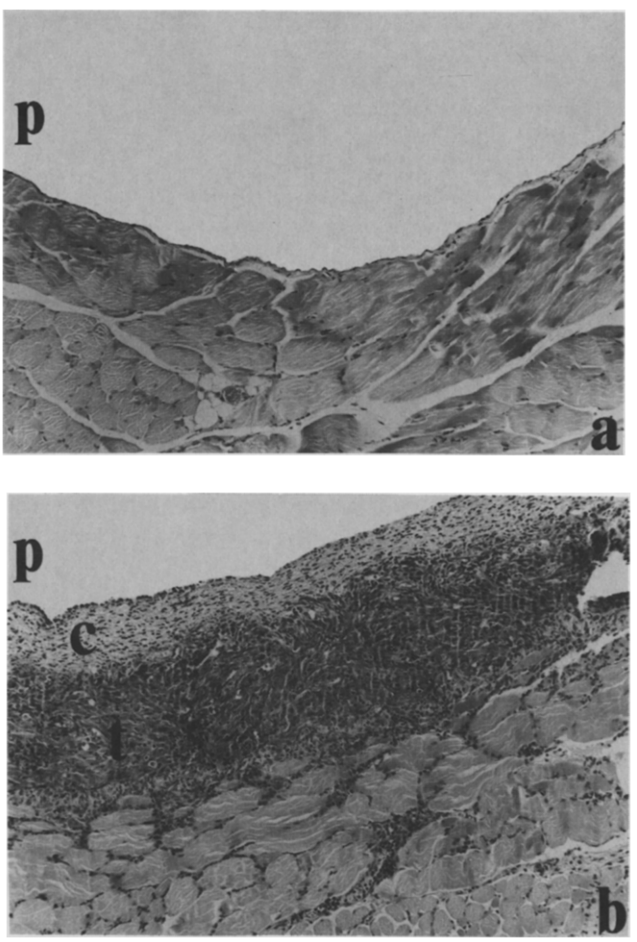


**Fig. 2. Peritoneal section 7 days after injection of nude mouse with (a) CHO/NEO cells or (b) CHO/TNF cells (×116). P = peritoneal cavity, C = connective tissue proliferation and T = CHO/TNF tumour.**

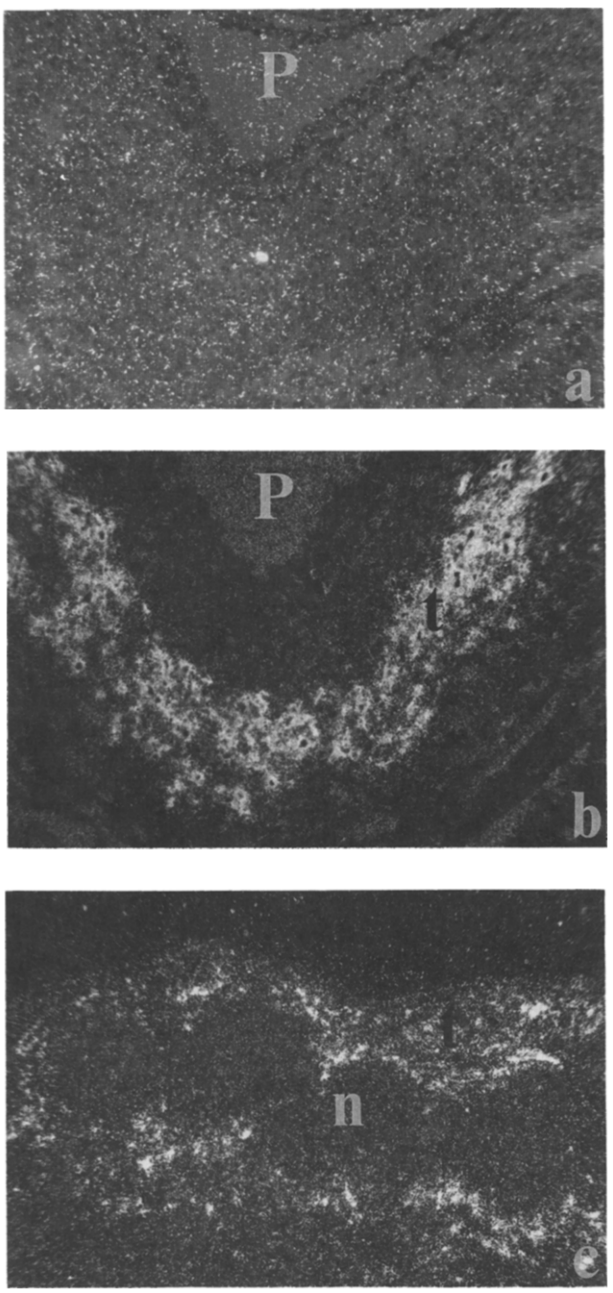


**Fig. 3. Dark-field microscopy of *in situ* hybridisation with human TNF riboprobe. Peritoneal and liver sections from mice 7 days after intraperitoneal injection of 5 × $10^6$ CHO/TNF cells. (a) = peritoneal specimen sense riboprobe (×170), (b) = peritoneal specimen antisense riboprobe (×170) and (c) = liver specimen antisense riboprobe (×130). P = peritoneal cavity, T = CHO/TNF tumour and N = necrotic area.**

populations, the most prominent being an increased infiltration of polymorphonuclear leucocytes (PMNs). Thus, 7 days after injection of CHO cells, although the total peritoneal cell count in the mice injected with CHO/NEO cells and the mice injected with CHO/TNF cells was not statistically different (14.9 [1.5] × $10^6$ vs. 13.4 [0.8] × $10^6$, respectively), total numbers of PMNs were different (0.2 [0.07] × $10^6$ vs. 4.02 [0.5] × $10^6$, respectively, $P < 0.01$). Production of enzymes by the PMNs may contribute to the increased implantation. However, inhibition of the TNF induced recruitment of myelomonocytic cells (57% reduction of total peritoneal cells and 76% reduction of

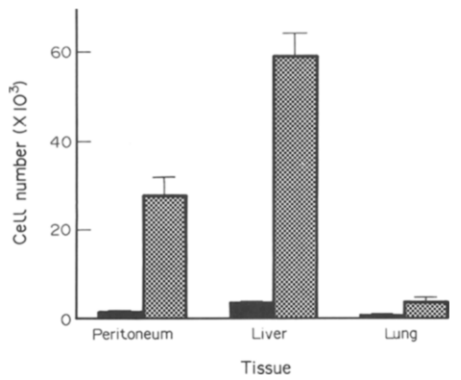


**Fig. 4. Localisation of radiolabelled CHO/NEO and CHO/TNF cells in peritoneum, liver and lungs of nude mice. Statistical differences between CHO/NEO and CHO/TNF groups were: peritoneum, $P = 0.01$; liver, $P = 0.01$; and lung $P = 0.06$. ■ = CHO/NEO cells and ▦ = CHO/TNF cells.**

PMN infiltration) by pretreatment of mice with intravenous injections of an antibody to the murine CR-3 receptor [15] did not inhibit the localisation of CHO/TNF cells in the peritoneum, liver or lung. TNF increases the adhesiveness of endothelium for tumour cells *in vitro* [16]. The peritoneum is lined with mesothelial cells which may be similarly affected, but we have been unable to demonstrate any significant differences in the adhesiveness of the CHO/NEO and the CHO/TNF cells to mesothelium or extracellular matrix components *in vitro* (data not shown). It is possible that TNF increases cell motility through tissues, and this activity may not be restricted to tumour cells. Production of TNF by activated macrophages may increase their ability to move to sites of inflammation.

Our results have at least two implications for the role of TNF in tumour biology. First, if tumour cells are not directly killed by TNF therapy, metastasis may be enhanced. Secondly, if tumour cells, or tumour infiltrating host cells, are secreting TNF, this would increase their invasiveness. Studies in our laboratory [16, 17] suggest that TNF mRNA can be readily detected in tumour biopsy specimens of patients with colorectal and ovarian adenocarcinomas. *In situ* hybridisation with TNF

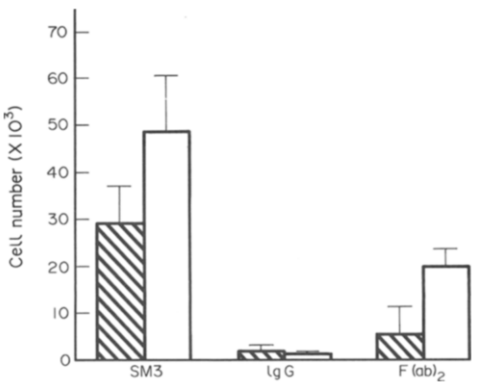


**Fig. 5. Effect of anti-TNF antibody (IgG) or fragments [F(ab)$_2$] on localisation of $^{125}$IUdR labelled CHO/TNF cells in nude mice. SM3 = control antibody. ■ = cell number in peritoneum and □ = cell number in liver. Statistical difference between tumour cell localisation in the anti-TNF IgG treated mice and control treated mice was $P < 0.005$ (in both peritoneum and liver), and that between F(ab)$_2$ treated mice and control treated mice was $P < 0.025$ (peritoneum) and $P < 0.05$ (liver). ▧ = cell number in peritoneum and ★ = cell number in liver.**

specific mRNA probes has revealed two sources of TNF mRNA in human tumour samples. In human colorectal tumours, a small proportion of predominantly stromal cells produce TNF, but in intraperitoneal human ovarian cancer the tumour cells themselves appear to transcribe TNF mRNA. In some areas more than 8% of the tumour cells were transcribing TNF mRNA. The production of TNF by tumour associated host cells may, at least in part, explain the enhancement of tumour metastasis by activated macrophages in other experimental tumour models [18], and the propensity for metastases to occur at sites of tissue damage [19]. Alternatively, if tumour cells acquire the ability to produce TNF, this may be one phenotypic change that could confer metastatic activity. Inhibition of TNF activity in the vicinity of tumours may be of therapeutic benefit.


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