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Functional and morphological effects of tumour necrosis factor α in an interleukin 6-producing pulmonary large cell carcinoma with sarcomatoid features

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Abstract We established a clonal cell line, HAT.MC8, derived from a human pulmonary large cell carcinoma with sarcomatoid features. This cell line was successfully maintained in a protein-free medium and exhibited sarcomatoid fibroblastic features in vitro. The cells constitutively produced a large amount of interleukin 6 (IL-6) in vitro. Tumour necrosis factor α (TNF-α) not only stimulated HAT.MC8 cells to produce IL-6, but also induced a morphological change from sarcomatoid fibroblastic to epithelial features. Although this change was related to actin and zonula adherens, there was no evidence that E-cadherin participated in the change. Interleukin 1β (IL-1β) had a stimulatory effect on IL-6 production by HAT.MC8 cells, but no influence on the morphology of the cells.

Key words Tumour necrosis factor · Interleukin 6
Biphasic tumour · Sarcomatoid tumour · Lung carcinoma

Introduction

Tumour necrosis factor-α (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6) are typical pleiotrophic cytokines (Oppenheim et al. 1986; Kishimoto 1989; Akira et al. 1990; Semenzato 1990); they form cytokine networks and interact with each other. For example, IL-6 production by human fibroblasts is enhanced by TNF-α (Kohase et al. 1987) and IL-1 (Eckes et al. 1992), and TNF-α and IL-1 are reported to enhance IL-6 production in some tumour cells (Isshiki et al. 1990; van Meir et al. 1990; Motoyama et al. 1993a, 1993b).

Biphasic tumours are uncommon in the lung. The carcinomatous component may be large cell, squamous cell or adenocarcinoma, while the sarcomatoid component usually shows a malignant fibrous histiocytoma-like or a fibrosarcoma-like pattern (Humphrey et al. 1988; Ro et al. 1992). Why these tumours exhibit a biphasic histological pattern is unknown.

We report here that TNF-α stimulated a cultured cell line, HAT.MC8, derived from a human pulmonary carcinoma with biphasic histological pattern, to produce IL-6, and that it influenced the morphology of these cells.

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Materials and methods

A clonal cell line, designated HAT.MC8, was derived from a pulmonary large cell carcinoma with sarcomatoid features (Fig. 1) that had arisen in the right upper lobe of a 58-year-old man. The patient underwent right upper lobectomy but died of pulmonary metastases 1 year after surgery. Serum levels of IL-6 and TNF-α were not determined. The HAT.MC8 cells were maintained in protein-free RPMI1640 medium (Nissui Seiyaku, Tokyo, Japan). For the determination of IL-6, IL-1β and TNF-α, 5×10^4 viable cells, suspended in 1.5 ml of protein-free RPMI1640 medium, were plated in 35 mm tissue culture dishes. Twenty-four hours after inoculation, and every 48 h thereafter, cells in 2 dishes were harvested to measure IL-6, IL-1β and TNF-α levels in the supernatant media and to calculate the cell number. As controls, we used cultured human skin fibroblasts, HFN3 and a gastric adenocarcinoma cell line, MKN74.C1 (Motoyama et al. 1986).

Levels of IL-6, IL-1β and TNF-α in the culture supernatants were measured by enzyme-linked immunosorbent assay.

For immunocytochemistry of the cytokeratins, epithelial membrane antigen (EMA), type I collagen, and intercellular adhesion molecule-1 (ICAM-1), cultured cells on Lab-Tek chamber slides (Miles, Naperville, Ill., USA) were fixed in acetone at –20° C for 15 min. Subsequently, the streptavidin-biotin-peroxidase complex method was applied. Mouse monoclonal antibodies to cytokeratin (PKK-1 Labsystem, Helsinki, Finland and KL-1 Immunotech, France), EMA (E29 Dakopatts, Glostrup, Denmark), procollagen type Ic-peptide (PC5-5 Takara, Kyoto, Japan), and ICAM-1 (BBIG-I1 Seikagaku Kogyo, Tokyo, Japan) were used. Immunofluorescence staining for E-cadherin on cultured cells was per-

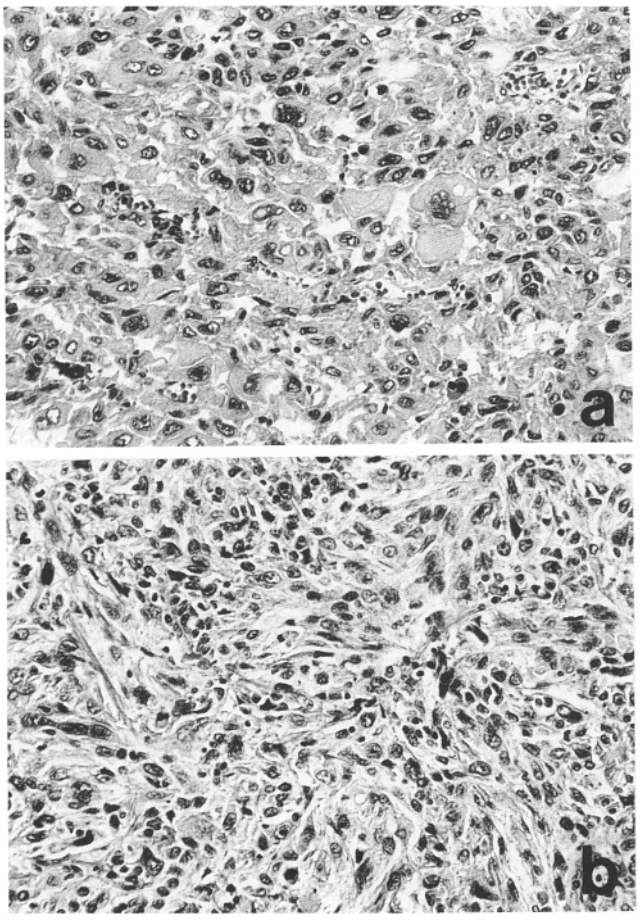

Fig. 1 Histology of the parent tumour. Large cell carcinomatous area (**a**) and sarcomatoid area resembling malignant fibrous histiocytoma or fibrosarcoma (**b**). The majority of the tumour was sarcomatoid. H & E, ×170

formed as previously described (Hirano et al. 1987). As negative controls, non-immune mouse, rabbit or rat serum was utilized in place of the primary antibody.

For electron microscopic examination the cultured cells were fixed in 2.5% phosphate-buffered glutaraldehyde, post-fixed in 1% osmium-tetroxide and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

The agents used for stimulation of IL-6 production were recombinant human interleukin 1β (rhIL-1β) (1–5 U) and recombinant human tumour necrosis factor α (rhTNF-α) (10–100 U) (both from Genzyme, Cambridge, Mass., USA), calcium ionophore A23187 (0.5–1 μg/ml), N^6, $O^{2'}$-dibutyryl adenosine 3′:5′ cyclic monophospholid acid (dbcAMP) (0.5–1 mM) and 12-O-tetradecanoyl phorbol-13-acetate (TPA) (10–100 ng/ml) (all from Sigma, St. Louis, Mo., USA). Bovine serum albumin (BSA) (Sigma), 0.1%, was used as a carrier protein. The effects were determined 24 h after the addition of the agents.

Induction of morphological change was achieved using fibronectin (1–10 μg/ml), laminin (1–10 μg/ml) and vitronectin (0.01–1 μg/ml) (all from Mallinckrodt, Imcera, Ind., USA), epidermal growth factor (1–100 ng/ml) (Sigma), and recombinant human IL-6 (rhIL-6) (10–100 U) (Genzyme), in addition to the above-mentioned agents. Media were renewed every day for 2 weeks. This experiment was performed in type I collagen-coated, gelatin-coated, fibronectin-coated and non-coated tissue culture dishes (Iwaki Glass, Tokyo, Japan).

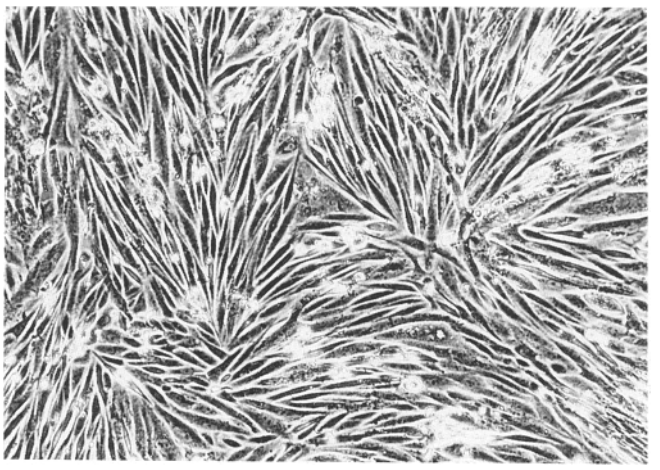

Fig. 2 HAT.MC8 cells cultured in protein-free medium show spindle-shaped fibroblastic features. Phase contrast microscopy

Total cellular RNA was prepared by guanidine thiocyanate/caesium chloride gradient centrifugation. Twenty micrograms of total RNA was denaturated and electrophoresed on a 1.3% agarose/formaldehyde gel and transferred to nitrocellulose filters. The filters were then hybridized with a ^{32}P-labeled IL-6 probe. To compare levels of mRNA expression, the same filters were rehybridized with a β-actin probe. An IL-6 exon 2 oligonucleotide probe and a β-actin oligonucleotide probe were obtained from British Bio-Technology (Oxford, England) and Clontech Lab (Palo Alto, Calif., USA), respectively.

Five to 6-week-old female athymic nude mice (nu/nu) with a BALB/c genetic background (Nihon Clea, Tokyo, Japan) were used for heterotransplantation. Approximately 10^7 cells in 0.3 ml of fresh culture medium were inoculated into the subcutaneous tissue of the back. The animals were killed, under anaesthesia, 2 months after transplantation.

Results

HAT.MC8 cells cultured in protein-free RPMI1640 medium exhibited spindle-shaped fibroblastic features (Fig. 2). Large amounts of IL-6 and small amounts of IL-1β and TNF-α were detected in the supernatant media. The total yields of each cytokine in the culture media increased logarithmically with culture time until the stationary phase, the maximal levels of IL-6, IL-1β, and TNF-α being 9790 pg/ml/48 h, 38 pg/ml/48 h, and 82 pg/ml/48 h, respectively (Fig. 3).

Table 1 shows the effects of various agents on IL-6 production by HAT.MC8 cells. RhIL-1β and rhTNF-α had the greatest stimulatory effect in this regard. Levels of IL-6 mRNA, determined by Northern blot analysis, those quickly following the stimulation of HAT.MC8 cells with either rhIL-1β or rhTNF-α. Maximal stimulation was seen 2 h after the addition of rhIL-1β or rhTNF-α. Levels of β-actin mRNA were markedly increased after the addition of rhTNF-α (Fig. 4). The increased level of β-actin mRNA was preserved by continuous stimulation with rhTNF-α (data not shown).

Although the calcium ionophore A23187 and TPA mildly enhanced IL-6 production, dbcAMP had no en-

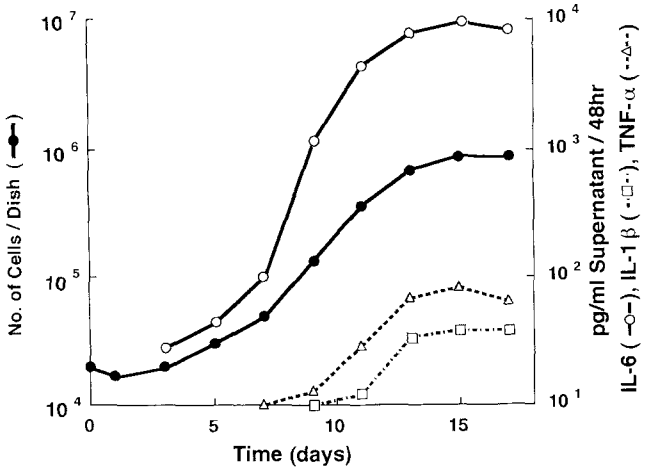

Fig. 3 Growth curves and IL-6, IL-1β and TNF-α levels in supernatant media. Large amounts of IL-6 and small amounts of IL-1β and TNF-α were detected

Table 1 Effects of various agents on release of IL-6 from HAT.MC8 cells

Incubation with		
Agent	Concentration	IL-6 level after 24-h incubation (pg/ml)[a]
None (protein free)	–	525±66
BSA (control)	0.1%	1253±33
BSA (0.1%)		
+rhIL-1β	1 U	2042±254*,[b]
	5 U	2983±491*
+rhTNF-α	10 U	2477±672*
	100 U	2509±257*
+Calcium ionophore	0.5 µg/ml	1444±104*
(A23187)	1 µg/ml	1657±332*
+dbcAMP	0.5 mM	713±83
	1 mM	841±14
+TPA	10 ng/ml	1530±109
	100 ng/ml	1472±188

[a] Mean±SD determined for triplicate samples
[b] Statistical significance was determined by the Student's *t* test
* $p<0.01$

hancing effect on this cell line. BSA, by itself, used as a carrier protein, had the capacity to enhance IL-6 production but to a lesser degree than rhIL-1β and rhTNF-α.

Morphological change from sarcomatoid fibroblastic to epithelial features in HAT. MC8 cells was induced only by rhTNF-α, irrespective of the coating used. HAT.MC8 cells treated with rhTNF-α became polygonal in shape and were arranged in a pavement-like fashion (Fig. 5). This change was completed in about 72 h, while the reverse change took about 10 days. Neither rhIL-1β nor rhIL-6 induced this change. BSA above also failed to induce such a morphological change.

Electron microscopic examination revealed the occasional presence of the zonulae adherens in HAT.MC8 cells with the epithelial features induced by rhTNF-α (Fig. 6). However, HAT.MC8 cells with sarcomatoid fibroblastic features had no zonulae adherens. The cultured human skin fibroblasts, HFN3, did not show any epithelial change on treatment with rhTNF-α.

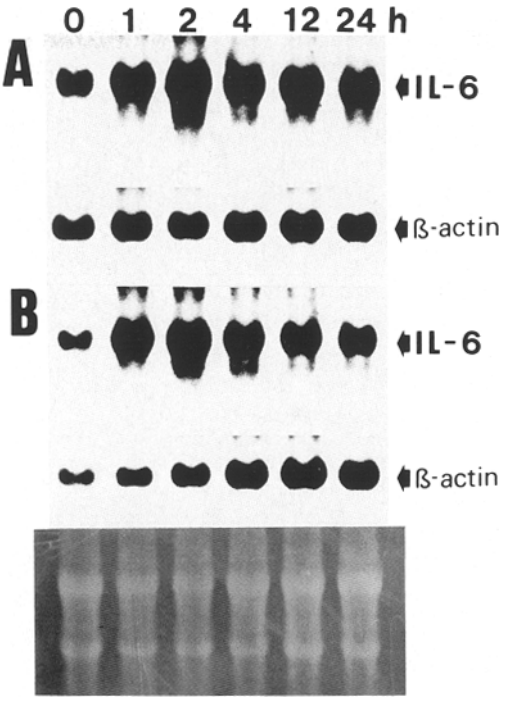

Fig. 4 Northern blot analysis of IL-6 mRNA. Expression of IL-6 mRNA in HAT.MC8 cells treated with rhIL-1β (**A**) and rhTNF-α (**B**). Although both agents enhanced the expression of IL-6 mRNA in a similar manner, only TNF-α markedly induced β-actin mRNA in the HAT.MC8 cells line

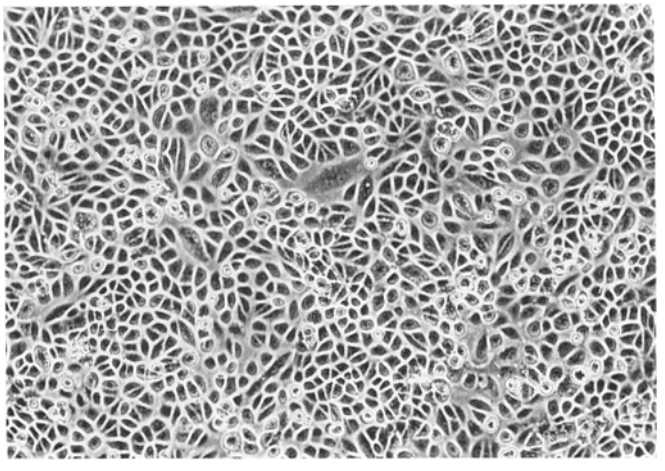

Fig. 5 HAT.MC8 cells treated with rhTNF-α. The cells have changed from sarcomatoid fibroblastic to epithelial; compare with **Fig. 2**. Phase contrast microscopy

The results of immunocytochemical examination of HAT.MC8 cells are summarized in Table 2. Both the sarcomatoid fibroblastic and epithelial HAT.MC8 cells frequently reacted with anti-low molecular weight cytokeratin antibodies. EMA was also detected in both types of HAT.MC8 cells. However, there were no E-cadherin-positive or ICAM-1-positive cells among either the

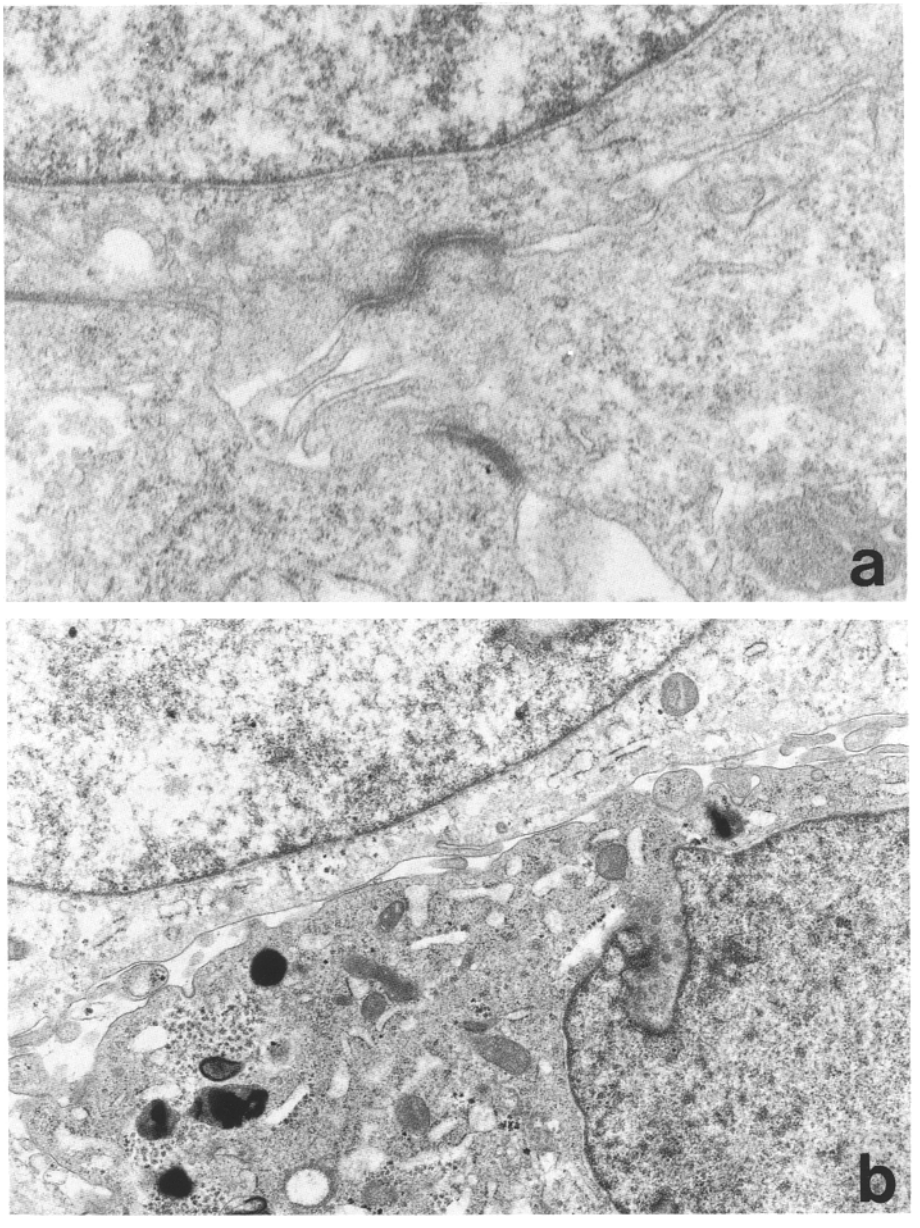

Fig. 6 Ultrastructural findings of HAT.MC8 cells. The epithelial HAT.MC8 cells induced by TNF-α frequently form zonulae adherens (**a**) while the sarcomatoid fibroblastic HAT.MC8 cells show only simple contact with each other (**b**). ×22500 (**a**) and ×11250 (**b**)

Table 2 Immunocytochemical phenotype of HAT.MC8 cells (*EMA* epithelial membrane antigen; *ICAM-1* intercellular adhesion molecule-1; – → *+++* denotes semi-quantitative scoring of the proportion of cells showing a positive reaction: *+* 1–9%; *++* 10–49%; *+++* 50–100%)

Antibody to: (clone)	HAT.MC8		HFN3 (Fibroblast)	MKN74.C1 (Adenocarcinoma)
	Fibroblastic form	Epithelial form		
Cytokeratin				
40, 45 and 52 kD (PKK-1)	+++	+++	–	+++
56 kD (KL-1)	+++	+++	–	+++
EMA	+	+	–	+
Type I collagen	–	–	+++	–
E-cadherin	–	–	–	++
ICAM-1	–	–	–	+

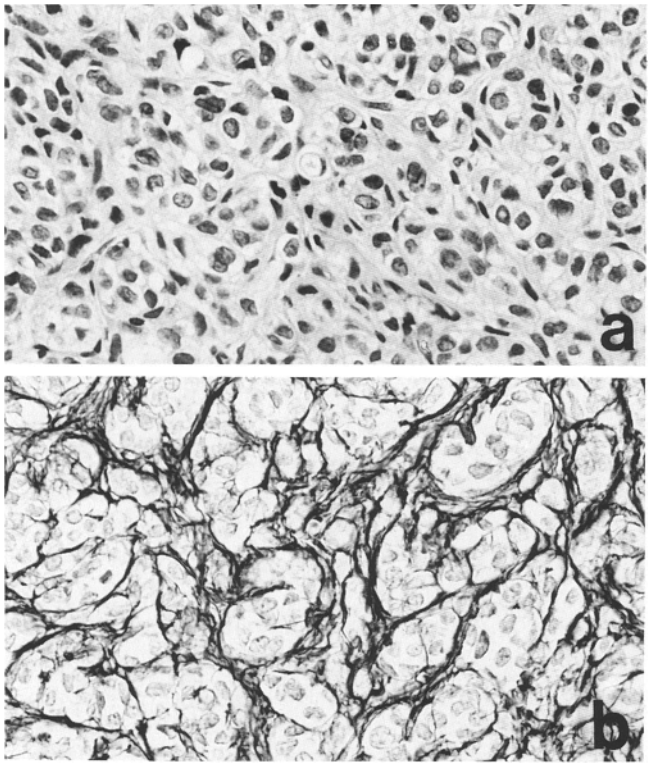

Fig. 7 Histology of transplanted tumour. All transplanted tumours show carcinomatous features. Polygonal tumour cells proliferate with an alveolar pattern. H & E (**a**) and silver impregnation (**b**), ×170

HAT.MC8 cells with sarcomatoid fibroblastic features or those with the epithelial features induced by rhTNF-α.

Both the HAT.MC8 cells cultured with protein-free medium and those cultured with rhTNF-α containing medium formed transplanted tumours with carcinomatous features (Fig. 7).

Discussion

The epithelial origin of HAT.MC8 cells was confirmed by the pattern of expression of immunocytochemical markers and by the histology of the transplanted tumours.

TNF (or TNF-α) was first identified as a protein whose function was thought to be modification or cytostatic/cytotoxic actions against tumour cells in culture and induction of haemorrhagic necrosis in some tumours in animals (Carswell et al. 1975; Old 1985). However, it is now accepted that this cytokine has pleiotrophic biological activity (inflammatory and immunomodulatory), in addition to its anti-tumour actions (Semenzato 1990). Moreover, some studies have clearly shown that TNF stimulates the growth of neoplastic cells (Lachman et al. 1987; Goillot et al. 1992), as well as non-neoplastic cells (Sugarman et al. 1985; Vilcek et al. 1986).

The production of both TNF-α and IL-6 by macrophages can be stimulated by the same factors, such as lipopolysaccharide (LPS) (Wollenberg et al. 1993). IL-6 production in fibroblasts is enhanced by TNF-α (Kohase et al. 1986), while that in T-cells is enhanced by phytohaemagglutinin and TPA, but not by TNF (Horii et al. 1988). TNF-α and IL-1β had enhance the production of IL-6 by tumors such as glioblastoma (van Meir et al. 1990; Isshiki et al. 1990), osteosarcoma (Motoyama et al. 1993) and malignant mesothelioma (Motoyama et al. 1993), which are non-epithelial and non-hematopoietic. IL-6 production in HAT.MC8 cells was not significantly enhanced by calcium ionophore A23187 or dbcAMP, both of which induce human fibroblasts to produce IL-6 (Shegal et al. 1987; Zhang et al. 1988).

The morphological change induced by TNF-α was observed only in HAT.MC8 cells, not in fibroblasts. Our findings regarding actin mRNA and the zonulae adherens suggest that TNF-α induced morphological changes are also related to actin. However, these effects were relatively prolonged, compared with the effects of other factors such as insulin-like growth factor I and epidermal growth factor. These other factors have been shown to induce rapid and striking morphological changes with membrane ruffling, in human epidermoid carcinoma KB cells, accompanied by the reorganization of actin structure (Kadowaki et al. 1986; Miyata et al. 1988). Moreover, we were unable to obtain any evidence that the morphological changes in HAT.MC8 cells were related to E-cadherin, which is also involved in actin bundles (Hirano et al. 1987). Although TNF-α is reported to induce expression of ICAM-1 in some human epithelial cells (Kvale and Brandtzaeg 1993), ICAM-1 was not expressed on either the epithelial or the sarcomatoid HAT.MC8 cells. We are now attempting to determine which molecule is related to the TNF-α induced morphological changes in HAT.MC8 cells, using subtractive hybridization.

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