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Stimulated kidney tubular epithelial cells express membrane associated and secreted TNFα

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Stimulated kidney tubular epithelial cells express membrane associated and secreted TNFα. Tumor necrosis factor-alpha (TNFα) is a pleiotropic, pro-inflammatory peptide cytokine which promotes immune renal injury, and participates in T cell activation. It is produced by macrophages, T cells, and some non-hematopoietic cells, and is cytotoxic in picogram quantities. As renal tubular epithelial cells (TEC) bearing MHC class II (Ia) antigens and adhesion molecules (ICAM-1) can act as immune accessory cells, the ability of TEC to produce costimulatory cytokines could augment TEC accessory capacity in vivo. We report that transformed TEC express low levels of TNFα in response to LPS or IL-1α as a secreted product and as a cytotoxic membrane associated molecule displayed on the cell surface. Surface labelling and immunoprecipitation studies of TEC detect a number of bands including a prominent 26 kD protein, which is the predicted size of TNFα precursor. TNFα mRNA transcripts were also detected by in situ hybridization in cortical tubules of C3H/FeJ mice injected with LPS, demonstrating the capacity of normal tubular epithelial cells to express TNFα in vivo. This report demonstrates for the first time the ability of kidney tubular cells to express TNFα protein and that membrane associated TNFα is not limited to hematopoietic cells. The function of small amounts of TNF displayed on the surface of tubular cells may be amplified by the abundance of these cells within the renal cortex, and may allow TEC to modulate immune responses within the kidney during inflammation.

Tumor necrosis factor-alpha (TNFα) is a potent cytokine produced by a variety of cell types. In addition to macrophages in which it was initially described, T cells, and several non-hematopoietic cells can also express mRNA for TNFα [1–8]. Minute quantities of this cytokine produce measurable effects in many cell types, and it is apparent that with the growing overlap that exists between cells which can produce TNFα and those affected by its presence, the physiology of TNFα is extremely complex [9].

The pro-inflammatory role of TNFα is well established and includes chemotactic attraction, prostaglandin release, synthesis of pro-coagulant factors, induction of other cytokines, and upregulation of MHC and adhesion molecules such as ICAM-1 [3, 7, 9–13]. Recent evidence has suggested that TNFα may also function as a costimulatory factor for T cells [14–16]. The expression of TNFα is normally tightly regulated to avoid systemic toxic injury. In some instances, most notably septic shock, dysregulation occurs, and the release of milligram quantities of TNFα systematically results in a deleterious or even fatal outcome for the host [9]. It is likely that most physiological effects of TNF result from very low levels of this cytokine. Several mechanisms may localize TNFα effects, including the production of small quantities, natural antagonists [17–19] or an association of TNFα with cell membranes [1, 2, 9, 20–22]. TNFα producing cells may employ one or more of these strategies to localize effects.

The primary function of renal tubular epithelial cells (TEC), the most abundant cell type in the kidney cortex, is solute and water transport. Recently we reported that TEC are also capable of immune accessory cell function since they can express MHC class II (Ia) antigens and adhesion molecules such as ICAM-1, and present antigen to T cells [7, 10, 23]. These studies prompted us to examine the ability of TEC to generate costimulatory cytokines. Our previous study reported that TEC can be stimulated by lipopolysaccharide (LPS) or interleukin-1α (IL-1α), to express mRNA for TNFα [7]. In the present study, we demonstrate for the first time that transformed TEC express secreted and membrane forms of TNFα. The membrane association and limited quantities of TNFα produced suggest a local role for this molecule within the kidney. The novel demonstration of cytotoxic membrane TNFα on TEC may have important implications in understanding the action of TNFα in renal disease.

Methods

Materials

Tissue culture media and reagents were obtained from Gibco (Grand Island, New York, USA), and chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Recombinant murine TNFα and IFN-γ were provided by Genentech (South San Francisco, California, USA). Recombinant murine IL-1α was donated by Hoffmann-LaRoche (Nutley, New Jersey, USA). Lipopolysaccharide (LPS, S. *minnesota*) was from

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Calbiochem (La Jolla, California, USA). Rabbit anti-mouse TNFα antisera was provided by Dr. A. Cerami (New York, NY, USA) [24] and Dr. S. Kunkel (Ann Arbor, Michigan, USA). Anti-mouse TNFα mAb (TN3-19.12) was provided by Dr. R.D. Schreiber (St. Louis, Missouri, USA) [25], and YN1/1.7.4 (anti-murine ICAM-1) was prepared as previously described [26].

Cell cultures and cell lines

The establishment of mouse C3H/FeJ (C1, C1.1) and MRL-*lpr* (M3.1) derived clonal cell lines by transformation of proximal tubular cells with origin-deficient SV40 DNA is described in detail elsewhere [7]. These epithelial cells were grown in modified K1 medium (50:50 mixture of DMEM and Ham's F12 medium), containing 5% fetal calf serum (FCS) and hormonal additives. P388D1 cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA) and grown according to the protocol provided.

RNA preparation and Northern blot analysis

Total RNA was isolated from confluent monolayers of transformed TEC with a single-step acid guanidium isothiocyanate-phenol-chloroform extraction method [27]. Total RNA (25 μg) was denatured with 1 M glyoxal and 50% dimethylsulfoxide, electrophoresed through a 1.2% agarose gel, and transferred onto Gene Screen Plus nylon membranes (New England Nuclear, Boston, Massachusetts, USA). The RNA was UV light crosslinked, and blots were prehybridized with 1 mM EDTA, 0.5 M NaH_2PO_4 (pH 7.2) and 7% sodium dodecyl sulfate (SDS) at 65°C. A specific 250 bp cDNA insert encoding murine TNFα was isolated by plasmid digestion with RsaI [28, 29]. Probes were radiolabeled by primer extension using random hexanucleotides [30]. Blots were hybridized for 16 to 24 hours at 65°C and washed twice at 65°C with 1 mM EDTA, 40 mM NaH_2PO_4 (pH 7.2), and 5% SDS for 30 to 60 minutes, and twice with 1 mM EDTA, 40 mM NaH_2PO_4 (pH 7.2), and 1% SDS for 30 to 60 minutes at 65°C. After hybridization the blots were exposed to Kodak X-AR film at −70°C for 4 to 10 days. Blots were reprobed with β-actin to ensure that approximately equal amounts of RNA were loaded in each lane. Steady-state mRNA transcript levels were quantified using scanning densitometry (Biosoft, Milltown, New Jersey, USA). In blots with high background due to long autoradiography times, background counts were subtracted in each lane from corresponding specific TNF signal. The ratio of TNFα/β-actin was then calculated to standardize RNA levels and allow quantitative comparison of values between stimulated and unstimulated cells.

In situ hybridization

C3H/FeJ mice aged four to five months were given 50 μg of LPS i.v. via the tail vein, and tissue was obtained two hours later. Kidneys were fixed in 4% paraformaldehyde, perfused with 30% sucrose and then snap frozen. To synthesize riboprobes, a 700 bp EcoR1/Sst1 fragment of cDNA for murine TNFα (Genentech) was subcloned into a pBluescript vector (Stratagene, La Jolla, California, USA) allowing the generation of both sense and antisense RNA probes. Probes were labelled using ^{35}S-rUTP (Amersham). Kidney cryosections (6 μm) were digested with proteinase K (1 μg/ml), refixed with 4% paraformaldehyde, and acetylated with acetic anhydride. Probes (5×10^5 cpm) were added to a hybridization solution (containing final concentrations of 10% dextran sulfate, 0.24 M NaCl, 4 mM Tris-Cl, pH 7.5, 0.8 mM EDTA, 0.4× Denhardt's, 200 μg/ml yeast tRNA, 40% formamide), which was added to each slide, and incubated at 42°C overnight. Unhybridized probe was washed from the slides at 42°C, and then digested with RNase. Dried slides were dipped in NTB2 emulsion (Kodak), developed 7 to 10 days later, fixed, and counter stained with hematoxylin-eosin. Antisense probes hybridized to TNFα mRNA, while sense probes served as negative controls. In some experiments, grains were counted over six fields using a 100× oil immersion lens and 10 squares of a grid marking a total area of 1 mm^2 for each field, on coded slides by two investigators.

Flow cytometry

Unstimulated C1 cells and cells treated with IL-1α (100 ng/ml), LPS (1 μg/ml) and/or IFN-γ (250 U/ml) were pipetted vigorously to detach from culture plates, washed with HBSS, and then co-labelled with rabbit anti-mouse TNFα antisera and biotinylated rat anti-mouse ICAM-1 (YN1/1.7.4) as primary antibodies. Fluorescein-conjugated affinity-purified goat anti-rabbit IgG $F(ab')_2$ fragment and phycoerythrin-streptavidin (Becton-Dickinson, San Jose, California, USA) were used as secondary steps. Cells were analyzed with a Becton-Dickinson flow cytometer.

Western blot

Recombinant mouse TNFα was electrophoresed on an SDS polyacrylamide gradient gel (10 to 20%) under reducing conditions [31] and blotted onto nitrocellulose using standard techniques. Blots were incubated with rabbit anti-mouse TNFα antisera (1:600), followed by goat anti-rabbit alkaline phosphatase (1:3000) and developed with 0.3 mg/ml p-nitro blue tetrazolium chloride plus 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt.

Radioiodination and immunoprecipitation of cell surface TNFα

Cells were grown in K1 media, stimulated with LPS (1 μg/ml) for 12 hours, and washed with HBSS. Approximately 1×10^7 cells in phosphate buffered saline (PBS) were surface labelled with 1 mCi of $Na^{125}I$ (Amersham, Arlington Heights, Illinois, USA) using immobilized lactoperoxidase conjugated to Sepharose-4B beads. The reaction was stopped with 1 mM cysteine, and all steps were then carried out at 4°C. Cells were then lysed in 1 ml of extract buffer containing 1% Nonidet P-40 (NP-40), 50 mM KCl, 10 mM NaCl, 1 mM EDTA, 50 mM KH_2PO_4 buffer (pH 7.5), 2 mM phenylmethylsulfonyl fluoride (PMSF), and 2 μg/ml of antipain, leupeptin and pepstatin A. Debris was removed by centrifugation at 15,000 rpm for 20 minutes and the lysate was precleared with Protein-A Sepharose for 16 hours. The extract was divided into two equal aliquots and 20 μl of rabbit anti-mouse TNFα antisera or preimmune rabbit serum was added for one hour, followed by precipitation with Protein-A Sepharose beads for 30 minutes. The immunoprecipitate was washed three times with lysis buffer and then twice with PBS pH 7.2. Buffer containing dithiothreitol (DTT) and SDS was added to the samples, and after boiling for five minutes, 5 μl was run in

a 12% polyacrilamide gel. The gels were fixed, dried and autoradiography with an intensifying screen was carried out at −70°C.

Metabolic labelling of TEC with ^{3}H-leucine

Confluent C1 cells were incubated for 16 hours in leucine free RPMI-1640 media supplemented with ^{3}H-leucine (New England Nuclear, Boston, Massachusetts, USA) at 16 μCi/ml, in the presence or absence of LPS (1 μg/ml) or IL-1α (100 ng/ml). Cells were removed from the plates by the addition of HBSS without Ca^{++}/Mg^{++} and vigorous pipetting, and lysed as above. Precleared lysates were equally divided and treated with 20 μl of anti-TNFα antisera alone, or antisera which had been pre-incubated with 1 μg of unlabelled recombinant murine TNFα. Immunoprecipitation and electrophoresis were as above, the gel was fixed, treated with Amplify (Amersham Corp) for fluorography before drying and autoradiography.

TNFα L929 cell bioassay

TNF activity in culture supernatants was determined in a standard cytotoxicity assay using L929 mouse fibroblasts (ATCC, Rockville, Maryland, USA). Briefly, 1.5-2 × 10^4 L929 cells/well were plated in 96-well flat bottom plates (Costar, Cambridge, Massachusetts, USA). Supernatants were added in triplicate to wells containing an equal volume of RPMI media with 5 μg/ml of actinomycin D. Plates were incubated for 18 to 24 hours at 37°C, media was removed, and 50 μg of MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) solution was added to each well. After an additional four hours of incubation, an equal volume of 10% SDS was added to the wells. Optical densities were read on a 96-well microtiter plate reader at 570 to 650 nm (Dynatech). Serial dilutions of murine recombinant TNFα (0.9 pg/well to 250 pg/well) were used to produce a standard curve [32, 33].

To determine cell surface associated TNF cytotoxicity, we modified the L929 cell method. TEC were grown in 96-well flat bottom plates to confluence in groups containing triplicate wells, washed and incubated in the presence or absence of LPS (1 μg/ml) or IL-1α (100 ng/ml) for 4 to 12 hours. Monolayers were then fixed with 1% paraformaldehyde for one hour. Cells were incubated for 12 hours after fixation, and washed three times with media prior to use in experiments. Plates were examined with an inverted phase contrast microscope (Nikon) to exclude disruption of the monolayers. In some experiments, monolayers were further incubated at 37°C with rabbit anti-TNFα antisera, pre-immune serum (1:20) or fresh media for one hour, and washed before L929 cells (2 × 10^4) were layered onto the monolayers. MTT was added for four hours as above, and a cytotoxicity index was derived from $1/OD_{570\text{-}650nm}$. The antisera used for blocking experiments were able to neutralize 250 to 500 pg/ml of recombinant mouse TNFα activity at a dilution of 1:180 [24].

Limulus endotoxin assay

Samples of medium including all additives, were assayed for LPS contamination by the Limulus amebocyte assay (Associates of Cape Cod, Wood's Hole, Massachusetts, USA) according to the protocol provided. The lower limit of detection for this assay was 0.125 EU/ml and the lowest concentration of LPS standard used that provided a positive result contained 25 pg of LPS (*E. coli* 0113).

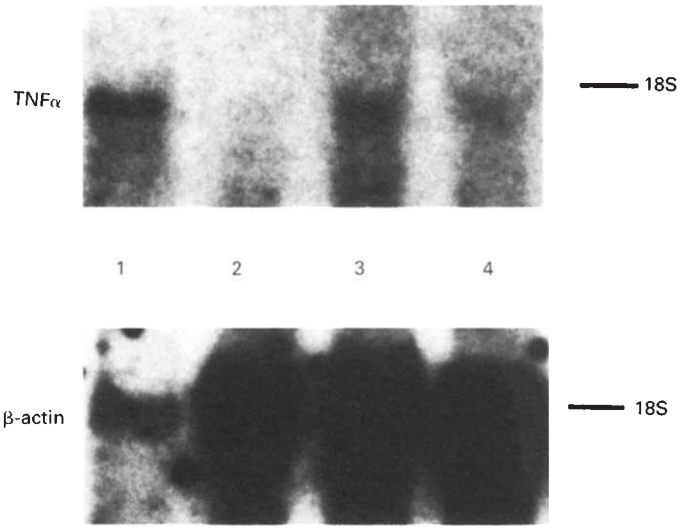

Fig. 1. *TNFα mRNA is induced in C1 cells by IL-1α and LPS.* Lane 1: unstimulated P388D1 cell RNA as positive control (0.507); lane 2: unstimulated C1 cells (0.005); lane 3: IL-1α (100 ng/ml) for 4 hours (0.204); lane 4: LPS (1 μg/ml) for 4 hours (0.150). Lane 1 contains 5 μg and lanes 2 to 4 contain 25 μg of total RNA. Autoradiography was for 10 days accounting for increased background. The blot was also reprobed for β-actin. The ratio of TNFα/β-actin indicated in parentheses was calculated by scanning densitometry, as in methods.

Statistics

Statistical analysis, where applicable, was performed using Statview SE+ (Abacus Concepts, Berkley, California, USA). Differences between groups were compared by one way analysis of variance and unpaired t tests. All results are expressed as mean ± SEM.

Results

TEC expression of TNFα mRNA is less than macrophages

C1 cells were incubated with IL-1α or LPS for four hours and total RNA was probed for TNFα. RNA for a positive control was also extracted from P388D1 cells which are macrophage derived tumor cells known to express TNFα mRNA [34]. Figure 1 demonstrates C1 cells have detectable TNFα mRNA within four hours of stimulation. However, considering C1 lanes have been loaded with 25 μg of total RNA, and that 5 μg of total RNA extracted from P388D1 cells demonstrated a markedly larger TNF/β-actin ratio, it is apparent that TEC express TNFα mRNA at much lower levels than macrophages.

TEC expression of TNFα mRNA is increased by cycloheximide

We have previously shown that TNFα can be specifically induced in TEC by either LPS or IL-1α, and not by IFN-γ which can activate TEC and increase the expression of Ia antigens and ICAM-1 [7, 10]. In some cell types, the expression of TNFα mRNA may be increased by blocking protein synthesis [8]. To determine if protein synthesis was required for transcription of TNFα mRNA in TEC, we treated C1 cells with

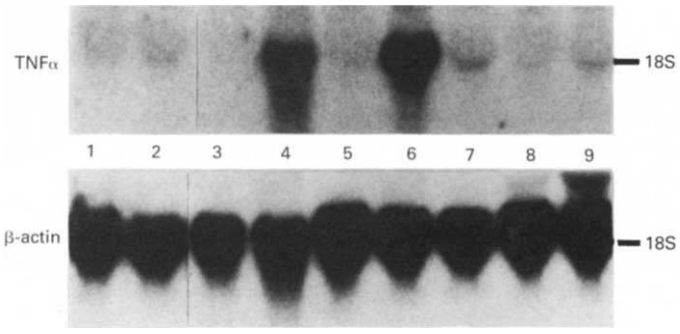

Fig. 2. *TNFα mRNA is superinduced in TEC by cycloheximide.* Lane 1: unstimulated C1 cells (0.003); lane 2: IL-1α (100 ng/ml) for 3 hours (0.034); lane 3: IL-1α for 12 hours (0.009); lane 4: IL-1α and cycloheximide (5 μg/ml) for 12 hours (0.457); lane 5: LPS (1 μg/ml) for 12 hours (0.156); lane 6: LPS and cycloheximide for 12 hours (0.538); lane 7: cycloheximide alone for 12 hours (0.203); lane 8: indomethacin (10^{-5} M) for 4 hours followed by fresh media for 12 hours (0.021); lane 9: indomethacin followed by IL-1α for 12 hours (0.042). Autoradiography was for 4 days. Results are representative of 3 experiments. The blot was also reprobed for β-actin, and the ratio of TNFα/β-actin indicated in parentheses was calculated for each sample by scanning densitometry.

cycloheximide [35]. In this experiment, TNFα mRNA was seen with IL-1α but not LPS stimulation at three hours (Fig. 2). However, concurrent stimulation with IL-1α or LPS and treatment with 5 μg/ml of cycloheximide caused a marked increase of TNFα mRNA, indicating that blocking protein synthesis caused increased mRNA transcription, or that stabilization of transcripts had occurred. Cycloheximide alone resulted in a slight increase of mRNA. This "superinduction" was not unique to C1 cells as similar results were observed for M3.1 cells concurrently treated with cycloheximide (not shown).

The levels of TNFα mRNA at 3 to 12 hours in Figure 2 are reduced from what we have observed in the past with TEC stimulated by individual cytokines. While this may have been due in part to shorter autoradiography exposure times that we used to emphasize "superinduction", we have noted a generalized decreased inducibility of TNFα in some of our cell lines with prolonged passaging. There is no evidence by morphology or growth characteristics that these transformed cells have undergone senescence. One possibility was inhibition of TNF by endogenous production of prostaglandins (PG). As TEC are capable of PGE_2 synthesis [7] which has been shown to downregulate TNFα production in other cell types [36], we pretreated C1 cells with indomethacin (10^{-5} M) for four hours prior to stimulation to block prostaglandin release (Fig. 2) [37]. However we observed only a slight augmentation in TNFα expression in pretreated IL-1α stimulated cells. Another possibility is densitization of C1 cells by chronic exposure to undetectable but functional levels of LPS [38, 39]. Since cyclooxygenase inhibition may not totally overcome LPS desensitization in monocytes, it is possible that low levels of endotoxin in the media were sufficient to cause desensitization of tubular cells and subsequent reduced TNFα expression.

Tubular epithelial cells express TNFα mRNA in vivo

To examine if tubular epithelial cells could similarly express TNFα mRNA in vivo, C3H/FeJ mice were injected with LPS

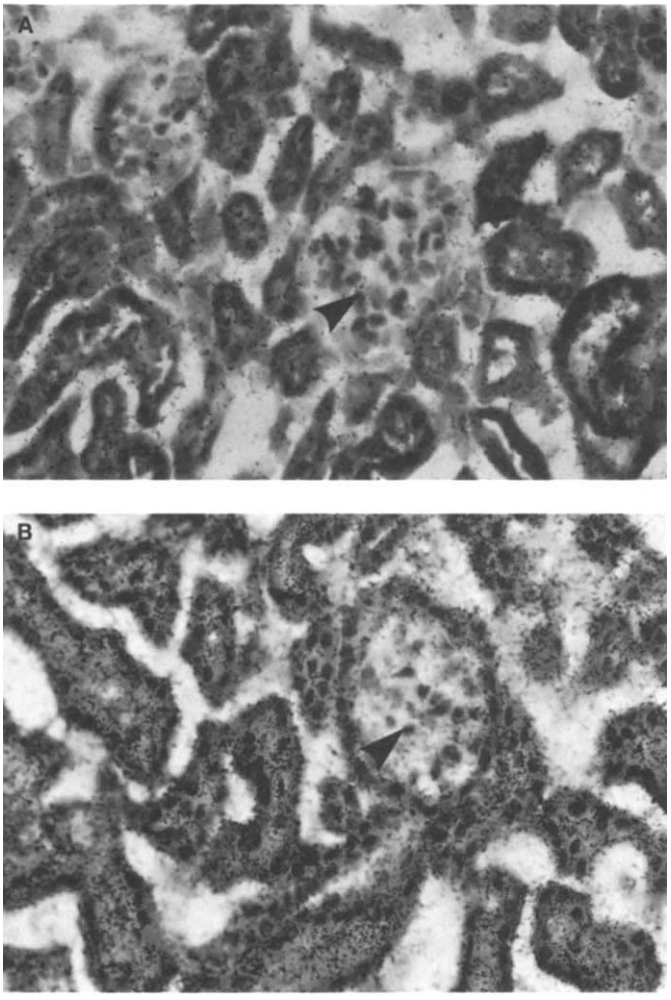

Fig. 3. *Cortical tubular cells of C3H/FeJ mice express TNFα mRNA in response to LPS.* In situ hybridization was with TNF specific ^{35}S antisense riboprobes. Background grain density was variable between experiments and results shown are from an experiment separate from those detailed in Table 1. **(A)** Grain density in tubules was low in untreated mice and was equivalent to non-hybridizing sense controls in treated mice. Glomeruli are indicated by arrows. **(B)** Following LPS treatment, cortical tubules had marked increases in grain density, indicating TNFα transcripts. Autoradiography was 10 days. Magnification is approximately 500×.

and cortical tubular expression of TNFα mRNA was assessed by in situ hybridization (Fig. 3 A,B). To quantify this response, grain counts were obtained in two additional experiments. Grain counts using sense riboprobe controls which should not hybridize to mRNA, were comparable to background counts over non-tissue areas or counts over medulla. Treatment of the tissue with RNAse prior to hybridization reduced grain counts to background in stimulated animals (not shown). Even with variation in the background counts, LPS stimulation increased grain counts over cortical tubular cells 2- to 10-fold as compared to unstimulated controls or sense controls ($P < 0.01$; Table 1). In sections where medulla could clearly be identified, there was a twofold difference in cortical areas ($P < 0.01$), suggesting that TNFα expression may be related more to tubular cells than collecting ducts. Firm conclusions would require co-localiza-

Table 1. LPS induces cortical tubular expression of TNF mRNA by in situ hybridization in C3H/FeJ mice

LPS treatment	Grain counts[a]			
	Cortical tubules[b]	Glomeruli	Medulla	Background[c]
Exp 1				
Antisense[d] −	19 ± 2	17 ± 2	NA[e]	18 ± 2
Sense −	24 ± 2	22 ± 2	NA	21 ± 2
Antisense +	212 ± 15[f]	83 ± 7[f]	NA	16 ± 2
Sense +	22 ± 2	22 ± 2	NA	21 ± 2
Exp 2				
Antisense +	113 ± 7[f]	113 ± 9[f]	78 ± 3	56 ± 3
Sense +	71 ± 2	71 ± 2	57 ± 2	58 ± 2

C3H/FeJ mice were given LPS IV and kidney tissue was probed with antisense and sense TNF specific riboprobes. Grain counts were compared between treated and non treated mice, and between different areas of the kidney. Results are from 2 experiments.

[a] grains were counted over cells in a 1 mm^2 grid, in 6 separate areas

[b] periglomerular cortical tubules counted

[c] grain counts over non-tissue areas was variable between experiments

[d] antisense = complementary to TNF mRNA

[e] NA = medulla not visible in coronal sections of kidney pole

[f] $P < 0.01$ from background, medulla, and unstimulated mice

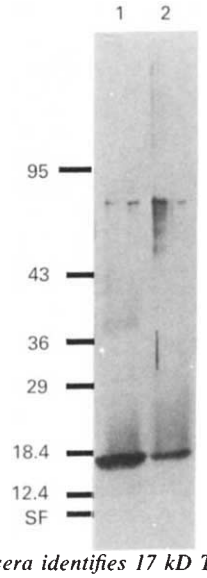

Fig. 4. *Anti-TNFα antisera identifies 17 kD TNFα protein.* Recombinant mouse TNFα was loaded in lane 1 (10 ng) and lane 2 (5 ng), and analyzed by Western blot as in **Methods**. Size markers (kD) and solvent front (SF) are indicated.

Table 2. TEC supernatants contain cytotoxic TNF activity

Sample	Time *hours*	TNFα activity *pg/ml*
Media alone		0
Unstimulated		8.9 ± 1
IL-1α	6	21 ± 5
	12	33 ± 6
LPS	6	28 ± 4
	12	15 ± 2
IL-1α and LPS	6	32 ± 2

L929 cells were incubated with fresh media, culture supernatants from unstimulated C1.1 cells, and supernatants from cells stimulated with either LPS (μg/ml) or IL-1α (100 ng/ml). Results are means of triplicate samples from two experiments.

tion with a proximal tubular cell marker, which is technically difficult with low level mRNA's.

Stimulated TEC secrete low levels of TNFα into culture supernatants

To examine whether mRNA was translated into biologically active protein, supernatants were tested for activity in a standard L929 cell TNF cytotoxicity bioassay. TEC stimulated with IL-1α or LPS for 4 to 12 hours resulted in significant ($P < 0.05$) but low levels of TNF activity (Table 2). Quantification of the specific TNFα activity present in the supernatants indicated that the maximum levels of approximately 35 pg/ml produced by TEC were 25 to 30 times less than what has been reported for macrophages, and is consistent with our TNFα mRNA results [36]. Not all non-hematopoietic TNF producing cells are capable of expressing large amounts of this cytokine. Paneth cells in gut epithelium have detectable mRNA levels but similarly produce low levels of TNFα, indicating that there is a wide spectrum of synthesis capacity among various cells types [5]. Interestingly, the 24-hour culture supernatant from unstimulated TEC in one experiment (Table 2), demonstrated cytotoxic activity which was above the media controls. The significance of this is unclear but may suggest a basal secretion of TNFα, which can be further increased with stimulation. In this regard, influence by media LPS levels below the limits of detection by Limulus assays has not been excluded.

TEC express cytotoxic membrane associated TNFα

Since we could detect only low levels of TNFα in culture supernatants, we were interested if TNFα produced by TEC remained associated with the cell membrane. Anti TNFα antisera which specifically identifies 17 kDa recombinant mouse TNFα was used for FACS analysis and immunoprecipitation studies (Fig. 4). In FACS analysis, C1 cells co-labelled with anti-TNFα and anti-ICAM-1 antibodies showed some basal expression of both molecules in unstimulated cells (Fig. 5). After 16 hours of stimulation with IL-1 or LPS, there was a predominant increase in TNFα surface expression, in contrast to IFN-γ stimulation, which is known to cause a predominant increase in TEC ICAM-1 [10]. Interestingly, co-incubation of IFN-γ with IL-1α or LPS markedly increased ICAM-1 expression but TNFα expression remained at basal levels.

To determine if the surface proteins identified by anti-TNFα antisera had biological activity, we examined TEC monolayers, rigorously fixed with paraformaldehyde for one hour, for their ability to lyse TNF-sensitive L929 cells (Fig. 6). Monolayers offer an advantage over using membrane fragments, as only the external cell surface portion of the TNFα molecule can interact with target cells. In four experiments, monolayers stimulated with IL-1α or LPS for 4 to 12 hours had a greater cytotoxicity index (inverse of optical density) than unstimulated cells ($P < 0.05$). Furthermore, when the monolayers were incubated with anti-TNF antisera prior to the L929 cells, cytotoxicity was reduced to baseline values ($P < 0.05$). The data shown are representative of two experiments performed using blocking

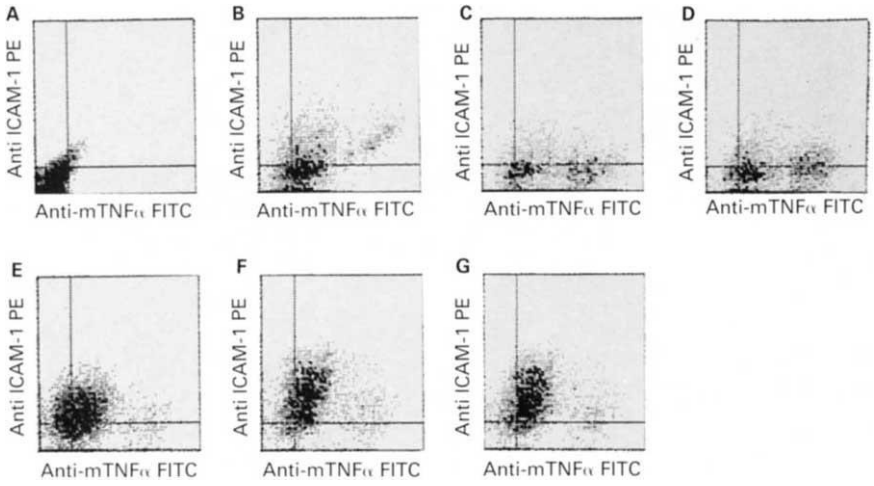

Fig. 5. *TEC express surface TNFα in response to IL-1α (100 ng/ml) and LPS (1 μg/ml) but not IFN-γ (250 U/ml).* C1 cells were co-labelled with anti-mouse TNFα antisera and anti ICAM-1 antibodies (b-g), and assessed for surface expression by FACS as in methods. Panels: **(A)** without primary antibodies, **(B)** unstimulated cells, **(C)** IL-1α, 16 hours, **(D)** LPS, 16 hours, **(E)** IFN-γ, 16 hours, **(F)** IFN-γ and IL-1α, 16 hours, **(G)** IFN-γ and LPS, 16 hours.

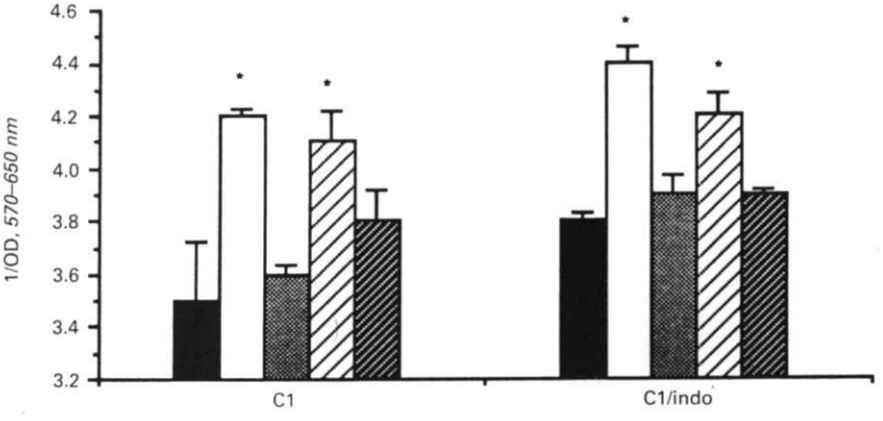

Fig. 6. *TEC express cytotoxic membrane associated TNF.* Symbols are: (■) unstimulated; (□) LPS + non-immune serum; (▩) LPS + anti-TNF; (▨) IL-1 + non-immune system; (▨) IL-1 + anti-TNF. C1 and M3.1 cell monolayers were fixed after incubation with LPS (1 μg/ml), IL-1α (100 ng/ml) or fresh media for 4 hours. Monolayers were then treated with rabbit anti-mouse TNFα antisera or pre-immune rabbit sera and then assessed for their ability to lyse L929 cells. Results are means of triplicate samples and are representative of two experiments. Similar cytotoxicity was seen when monolayers were incubated with indomethacin (10^{-5} M) for 4 hours prior to stimulation. Values marked by * represent differences ($P < 0.05$) from unstimulated controls.

antisera. Similar results were obtained with M3.1 cell monolayers (not shown). In contrast, incubation of the monolayers with pre-immune rabbit serum did not reduce the level of cytotoxicity and was equivalent to adding fresh media alone. Interestingly, the addition of excess amounts of antisera to stimulated monolayers never reduced cytotoxicity to below baseline values. This suggests that any basally-expressed surface form of TNF was not cytotoxic to L929 cells. To reduce the potential effect of endogenous PGE synthesis and overcome desensitization by low levels of endotoxin, monolayers were also pre-treated with indomethacin prior to layering of L929 cells (Fig. 6). Although the general level of cytotoxicity was increased with this pre-treatment, a similar pattern of cytotoxicity was obtained with cells stimulated with IL-1 or LPS, and anti-TNF antisera blocked cytotoxicity to baseline.

C1 cells express multiple forms of TNFα with stimulation

Labelling with ^{3}H-leucine and immunoprecipitation of lysates from TEC stimulated with IL-1α or LPS (Fig. 7) showed prominent bands at approximately 40 to 45 kD. The presence of reduced but similar bands in unstimulated cells again suggests there is a basal expression of some form of TNFα in vitro. The bands that were detected could be competitively blocked by excess unlabelled recombinant murine TNFα, indicating that the immunoprecipitate was antigenically related to TNFα. With a ^{3}H-leucine label, we did not detect any bands corresponding to the 26 kD membrane associated TNF found in other cells [2, 20, 24]. To clarify the nature of TNFα related surface proteins on TEC, C1 cells were stimulated with LPS for 12 hours and cells were surface radioiodinated (Fig. 8). Using this technique, a major band was seen at 26 kD, and as well, bands at ~45, and ~70 kD. No bands were observed using pre-immune rabbit serum in a control immuno-precipitation. A TNF related band of ~17 kD size could not be clearly distinguished in the gel front of either surface or metabolically-labelled cell lysates.

Discussion

Tumor necrosis factor (TNFα) is a pro-inflammatory cytokine which is elevated during renal allograft rejection and murine autoimmune lupus nephritis [40, 41]. Furthermore, the administration of exogenous TNFα accelerates kidney damage in NZB/W mice [42], and thus the production of TNFα by intrinsic renal cells could contribute to immune renal injury. We now report that TEC synthesize a biologically active, cytotoxic form of TNFα, which is both secreted and associated with the cell surface as an integral membrane protein. Given the direct apposition of TEC to other intrinsic renal cells, the pleiotropic nature of TNFα, and the fact that 80 percent of the renal cortex is comprised of proximal TEC, we believe that TNFα produced

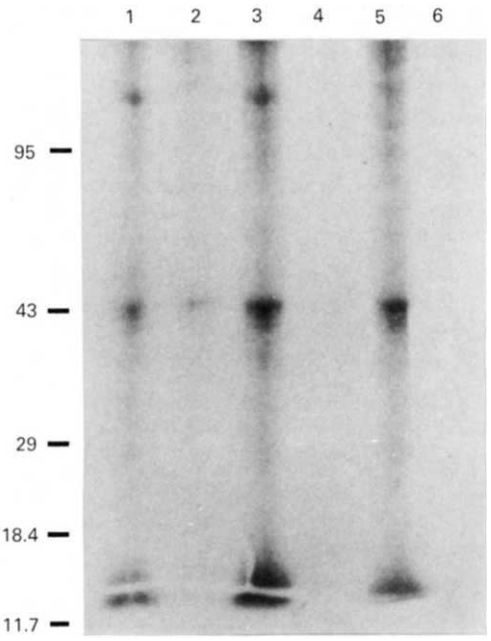

Fig. 7. *Immunoprecipitation of C1 cell extracts is blocked by rTNFα.* C1 cell monolayers were incubated with media alone (lanes 1, 2), LPS (1 μg/ml) (lanes 3, 4) or IL-1α (100 ng/ml) (lanes 5, 6) and labelled with ^{3}H-leucine. Cell extracts were then analyzed with SDS-PAGE (10 to 20%). Lanes 1, 3 and 5 were precipitated with anti-TNFα antisera. Lanes 2, 4, and 6 were precipitated with anti-TNFα antisera which was previously incubated with an excess of unlabelled rTNFα. Size markers (kD) are indicated. Autoradiography was for 12 days.

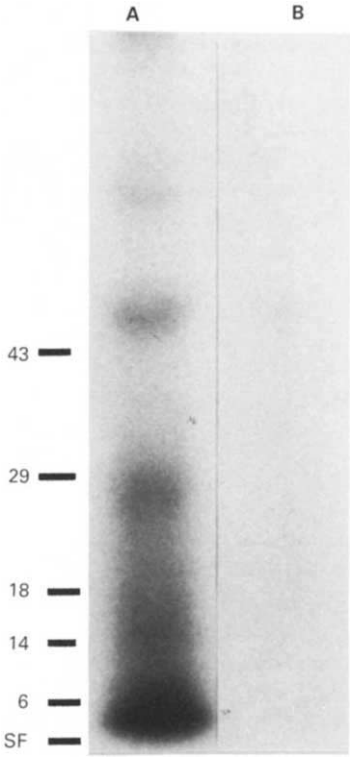

Fig. 8. *C1 cells express a prominent 26 kD surface molecule related to TNFα.* C1 cells were incubated with LPS (2 μg/ml) for 12 hours, and surface labelled with ^{125}I. Cellular extracts were immuno-precipitated with anti-TNFα antisera (lane 1), or preimmune serum (lane 2). Size markers (kD) and solvent front (SF) are indicated.

by TEC may play an important role in modulating immune responses within the kidney.

The expression of TNFα by TEC can be increased by both LPS and IL-1α, which also cause TNFα release by other cell types [7, 9, 12]. The response to IL-1α is of note in relation to autoimmune nephritis, since IL-1 produced by infiltrating mononuclear cells is elevated in the kidneys of mice with lupus nephritis [40, 43, 44]. Overall, the amount of TNFα synthesized by TEC appears to be small, when compared to macrophages which can produce massive amounts [6, 36]. However, the demonstration of cytotoxicity using the L929 cell bioassay which is sensitive to picogram quantities, emphasizes the potent biological effect of even small quantities of TNFα. The presence of membrane TNF on TEC may serve to increase bioactivity locally by focusing its effect on contacting cells. The overall importance of secreted and membrane TNFα by TEC, may be further amplified by the abundant presence of these cells within the renal cortex of the kidney.

In addition to a secreted form, we have found that TEC also express a cytotoxic surface molecule which is related to TNFα. This finding is consistent with similar data in macrophages [1, 20], but more interestingly indicates that membrane associated cytokines are not limited to cells of hematopoietic origin. There has been recent controversy concerning the membrane association of IL-1, and the adequacy of fixation procedures [45]. To avoid potential artifact, we chose a rigorous fixation for one hour with paraformaldehyde, followed by a prolonged incubation and extensive washes. Thus it is very unlikely that any of the effects we observed with fixed cells were due to simple leakage of cytoplasmic proteins.

The observation that the membrane associated cytotoxicity on TEC is related to TNFα is supported by several findings. Firstly, cytotoxicity is increased by LPS or IL-1α, which individually can induce TNFα mRNA [7, 12]. Secondly, cytotoxicity is blocked specifically by anti-TNFα antisera which identifies recombinant mouse TNFα. Thirdly, cell surface labelling of TEC identifies a prominent 26 kD molecule, which has been shown to represent the surface form of TNFα in other cells, and is the size predicted by the cDNA sequence if the leader peptide is not cleaved [20]. Several larger molecular weight (M_r > 40 kD) bands seen with our surface or metabolic labelling, have been noted in similar studies of T cells and may represent multimeric forms of TNFα [2, 46]. The presence of prominent bands at 40 to 45 kD using ^{3}H-leucine metabolic labelling, which were blocked with unlabelled TNFα, suggests that they are also antigenically related to TNF. It is interesting that using a ^{3}H metabolic label, we observed bands similar to that observed with ovarian epithelial tumor cells using a similar method [47], and that in contrast to ^{125}I surface labelling, a 26 kD band was not seen. It is not clear if this discrepancy is due to an increased stability of the 26 kD surface form using lactoperoxidase or to a difference in the sensitivity of the methods.

The regulation of TNFα is complex and involves both pre- and post-transcriptional mechanisms. In non-transformed smooth muscle cells, the expression of TNFα mRNA can only

be induced by co-incubation with cycloheximide and LPS, suggesting that repressor mechanisms normally prevent expression in these cells or that TNFα mRNA is rapidly degraded [8]. The short lived nature of many cytokine mRNA's is generally due to A-U rich sequences in the 3′ untranslated region [9]. In contrast, macrophages express constitutive levels of TNFα mRNA, but must be stimulated with LPS to secrete product. In this regard, our data showing low level induction with LPS or IL-1α, and superinduction with IL-1α or LPS plus cycloheximide suggests that TEC may be more similar to smooth muscle cells. These data also indicate that transcriptional regulatory mechanisms exist in TEC following SV40 DNA transformation.

To allow long-term studies of pure populations of cells, we have used immortalized and cloned TEC transformed with origin deficient SV40 DNA [7]. Since epithelial tumor cells may express TNFα mRNA, the question arises if SV40 DNA transformation alters normal cell function and imparts TNFα producing capabilities to TEC [47–49]. However, in situ hybridization studies of C3H/FeJ mice indicate non-transformed TEC have the capacity to express TNFα mRNA in response to LPS stimulation in vivo. In our experience, immunohistochemistry techniques have not been able to demonstrate tubular cell TNFα in kidney cryosections from mice with autoimmune nephritis or stimulated with LPS (unpublished observations). This is not surprising considering the potentially low levels expressed, the lability of this cytokine and the high levels of endogenous peroxidase activity in kidney tubules which could obscure detection of low levels by standard techniques. It is also possible that additional post-transcriptional factors control translation of TNFα mRNA in these cells in vivo.

Since the expression of TNFα is influenced by LPS, we screened our media for endotoxin contamination by Limulus assays. Although these assays are sensitive, the potential contribution of trace amounts of LPS to TNFα production basally or in response to IL-1α, cannot be entirely excluded [38]. However, we have not detected other endotoxin inducible TEC products, such as MHC and adhesion molecules (ICAM-1), at concentrations below what can be detected by Limulus screening (<25 pg/ml). Also, TNFα is not induced in TEC by other cytokine preparations (TNFα, IFN-γ, IL-4), which would be equally susceptible to endotoxin contamination [7]. Alternatively, low levels of endotoxin contamination may have caused some desensitization of tubular cells to further stimulation and TNFα production. This may account for the decreased TNFα inducibility we have noted in cell lines that have been extensively passaged [39].

The physiological relevance of secreted or membrane associated cytokine production by TEC is unknown, particularly when the amounts are small. TNFα may modulate immune responses locally and act as a costimulatory factor for T cells [14, 15]. It is interesting to note that an earlier report identified a "IL-1-like" thymocyte stimulating factor in the supernatant of LPS stimulated TEC [23], and that TNFα has recently been demonstrated to have thymocyte co-stimulatory actions [16]. Alternatively, TNFα may indirectly function as a growth factor by increasing EGF receptors which are present on TEC [50]. Several groups have also noted that an inverse relationship exists between TNF production, and TNF sensitivity [48, 49]. This raises the intriguing possibility that in addition to focussing its pro-inflammatory action on adjacent cells, the expression of TNFα by TEC, may confer some protection against its own toxic properties. These results suggest that a role exists for TNFα production by non-hematopoietic cells within the kidney and highlight the complexity of TNFα involvement in mediating renal injury.

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