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Tumor necrosis factor modulation of expression of the cystic fibrosis transmembrane conductance regulator gene

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Based on the knowledge that expression of the cystic fibrosis transmembrane conductance regulator (CFTR) gene can be modulated at the transcriptional level, and that the CFTR gene promoter contains sequences homologous to elements in other promoters that respond to tumor necrosis factor-α (TNF), we evaluated the hypothesis that TNF might modulate CFTR gene expression in epithelial cells. Studies with HT-29 cells, a colon epithelium-derived tumor cell line known to express the CFTR gene, demonstrated that TNF downregulated CFTR mRNA transcript levels in a dose- and time-dependent fashion. Interestingly, nuclear run-on analyses demonstrated that TNF did not affect the rate of transcription of CFTR gene, but exposure of the cells to TNF did modify the stability of CFTR mRNA transcripts, resulting in a mRNA half-life that was reduced to 65% of the resting level. These observations suggest that CFTR gene expression can be modulated by TNF, at least in part, at the post-transcriptional level.

Cystic fibrosis transmembrane conductance regulator; Gene expression; Tumor necrosis factor-α; mRNA transcript; Transcription; mRNA stability

1. INTRODUCTION

Cystic fibrosis (CF), a fatal recessive hereditary disorder characterized by abnormalities of electrolyte transport in organs such as the lungs, sweat glands, pancreas and intestines, results from mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1–4]. Several lines of evidence suggest that the CFTR gene product is a cyclic AMP-regulated Cl^- channel on the apical surface of epithelial cells [5–11]. Although the link between mutations of CFTR gene and the pathogenesis of CF is not completely defined, the available evidence supports the concept that it is expression of the abnormal CFTR gene in the epithelial cells of affected organs that leads to the profound clinical manifestations of CF [1–11].

There is a growing body of evidence that expression of the CFTR gene is regulatable. Although the 5′-flanking region of exon 1 of the CFTR gene has the characteristics of a housekeeping gene, it contains a number of known putative regulatory sequences [12,13]. Further, phorbol esters [12,14] and agents that mobilize intracellular calcium [15] downregulate CFTR gene expression at the level of transcription, resulting in decreased CFTR mRNA transcript levels.

In the present study, we evaluated the ability of tumor necrosis factor-α (TNF) to modulate the expression of the CFTR gene in epithelial cells. We choose TNF as a 'model' stimulus based on the recognition that the 5′-flanking region of CFTR gene includes a 28 bp sequence that shows an 89% homology with a sequence within the promoter of the human α1(I) collagen gene [16], and that TNF downregulates the rate of α1(I) collagen gene transcription [17]. Surprisingly, although the data demonstrate that TNF downregulates CFTR gene expression, it does not influence transcription, but does affect CFTR mRNA stability.

2. MATERIALS AND METHODS

2.1. *Cell culture*

The HT-29 colon epithelium-derived adenocarcinoma cell line (American Type Culture Collection [ATCC], HTB 38) is known to express both the CFTR gene and the TNF receptor gene [12,18]. Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Whittaker Bioproducts) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (all from Biofluids). In the studies to investigate the modulation of CFTR gene expression by TNF, experiments were carried out in both serum supplemented and serum-free conditions. HT-29 monolayers were washed with medium, incubated for 12 h, 37°C under the same conditions, and then incubated alone or with human recombinant TNF (20 U/ng, Genzyme) for specified times and doses. The cells were then harvested for evaluation (see below). No differences were observed in serum-supplemented or serum-free conditions and thus all data shown are derived from the serum-free conditions.

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Abbreviations: TNF, tumor necrosis factor-α; CFTR, cystic fibrosis transmembrane conductance regulator

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2.2. *Evaluation of CFTR mRNA transcripts*

The levels of CFTR mRNA transcripts and, as a endogenous control, β-actin mRNA transcripts, were determined by Northern analysis [19]. Total cellular RNA was isolated by the guanidine thiocyanate-CsCl gradient method [20], RNA (15 μg/lane) was subjected to formaldehyde-agarose gel electrophoresis, transferred to a nylon membrane (Nytran, Schleicher and Schuell), hybridized with a ^{32}P-labeled CFTR or β-actin cDNA probe generated by the random priming method [21,22], and evaluated by autoradiography. The CFTR cDNA probe (pTG4964) was prepared as previously described [14]. The cDNA clone pHFβA-1 was used for the β-actin probe [23].

2.3. *Modulation of CFTR gene expression in HT-29 cells by TNF*

To evaluate the dose-dependency of TNF-induced modulation of CFTR gene expression, HT-29 cells were exposed to various concentrations of TNF (0–100 ng/ml) for 8 h. To determine the time-dependency of CFTR gene expression, cells were incubated for various times (0–24 h) in the presence of TNF at the final concentration of 10 ng/ml. Following the incubations, total cellular RNA was isolated, and the levels of CFTR and, as a endogenous control, β-actin mRNA transcripts were evaluated by Northern analysis as described above. The autoradiographic signals were quantified using a laser densitometer (Ultroscan Laser Densitometer, Pharmacia LKB). In the dose-dependent study, CFTR and β-actin mRNA transcript levels were expressed as percent of the unstimulated level defined as 100%. In the time-course study, CFTR mRNA transcript levels in the absence or presence of TNF were expressed as percent of the level at time zero defined as 100%.

The rate of CFTR gene transcription was examined by nuclear transcription run-on analysis [12,21]. Nuclei were isolated from 5×10^7 resting or TNF-stimulated (10 ng/ml; 4 and 8 h) cells, and incubated (20 min, 37°C) with 5 mM ATP, 2 mM CTP, 2 mM UTP, 250 μCi [α-^{32}P]GTP (> 400 Ci/mmol; Amersham) and 700 U/ml RNase inhibitor (RNasin, Promega) to label actively transcribed RNA. RNA was recovered by the acid guanidinium thiocyanate–phenol–chloroform method [24], purified by Sephadex G-50 column chromatography (5 Prime→3 Prime) and hybridized to excess amounts (5 μg) of DNA targets (see below) immobilized on Nytran [12,21]. The membranes were then washed, exposed to RNase A (5 μg/ml) and RNase T_1 (5 U/ml), followed by proteinase K (50 μg/ml) (all from Boehringer Mannheim Biochemicals), and evaluated by autoradiography. The DNA targets included plasmids containing a CFTR cDNA (pTG4964), a human β-actin cDNA (pHFβA-1), or, as a negative control, the plasmid pBlueskript II SK+ (pBS, Stratagene) containing no human DNA. Interleukin-8 (IL-8) cDNA (pPB248) was used as a positive control for TNF stimulation (the rate of IL-8 gene transcription is upregulated by TNF in epithelial cells [25]). To determine the relative rate of CFTR gene transcription after TNF stimulation compared to the resting rate, the autoradiograms were quantified using a laser densitometer, expressed as% of the transcription rate of β-actin endogenous control (defined as 100%) and the values normalized to relative length of the mRNA coding sequences within DNA targets (CFTR 4.5 kb; β-actin 2.0 kb, respectively).

To evaluate the stability of CFTR mRNA transcripts, HT-29 cells at rest and after incubation with TNF (10 ng/ml; 8 h) were exposed to actinomycin D (5 μg/ml, US Biochemicals) for 0 to 24 h to block further transcription. In three separate experiments, total cellular RNA was extracted, CFTR mRNA levels were evaluated by Northern analysis as described above, and quantified by laser densitometry.

3. RESULTS

3.2. *Modulation of CFTR mRNA levels by TNF*

Northern analyses demonstrated that the HT-29 colon carcinoma cell line expressed 6.5 kb CFTR mRNA transcripts, but the CFTR transcript level decreased markedly after exposure to TNF (Fig. 1; lanes 1,2). In contrast, the level of β-actin mRNA transcripts was similar in amount in resting cells and cells exposed to TNF (lanes 3,4).

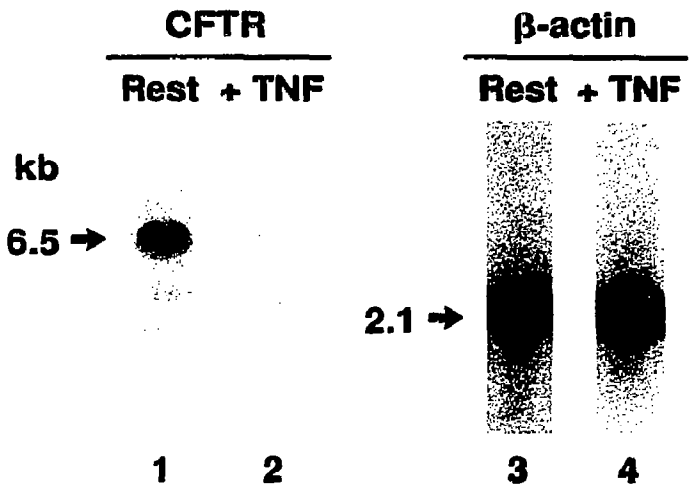

Fig. 1. Modulation of CFTR mRNA levels by TNF in HT-29 epithelial cells. Shown are Northern analyses of RNA (15 μg/lane) from resting cells and cells exposed to TNF (10 ng/ml, 8h) hybridized with ^{32}P-labeled CFTR (lanes 1 and 2) or β-actin probes (lanes 3 and 4). The sizes of mRNA transcripts are indicated.

TNF exposure decreased the levels of CFTR mRNA transcripts in a dose-and time-dependent fashion (Fig. 2). TNF-induced downregulation of CFTR gene expression was observed at very low concentrations of TNF (0.1 ng/ml) and the CFTR transcript level was further decreased with increasing concentrations of TNF up to 100 ng/ml. In contrast, the level of β-actin mRNA transcripts in the same cells did not change significantly with the dose of TNF. With a fixed dose of TNF (10 ng/ml), CFTR mRNA transcript levels declined over time with a minimum at 8 h. By 12 h, the levels had increased, but still remained below the levels of the resting cells at 24 h. In contrast, resting HT-29 cells had mildly increasing levels of CFTR mRNA transcripts throughout the same period. This data was confirmed with two additional experiments.

3.2. *Effects of TNF on the rate of transcription of the CFTR gene*

Isolated nuclei from resting HT-29 cells demonstrated a low rate of transcription of the CFTR gene (Fig. 3). Interestingly, at 4 and 8 h following TNF exposure, the rate of CFTR gene transcription did not change compared to the resting rate (Fig. 3 for 8 h data; 4 h data not shown). The transcription rate of the β-actin gene also did not change with TNF. The relative transcription rate of the CFTR gene compared to that of the β-actin gene was 11% (resting) and 10% (TNF 8h). In contrast, the rate of the transcription of the IL-8 gene was dramatically increased after TNF stimulation.

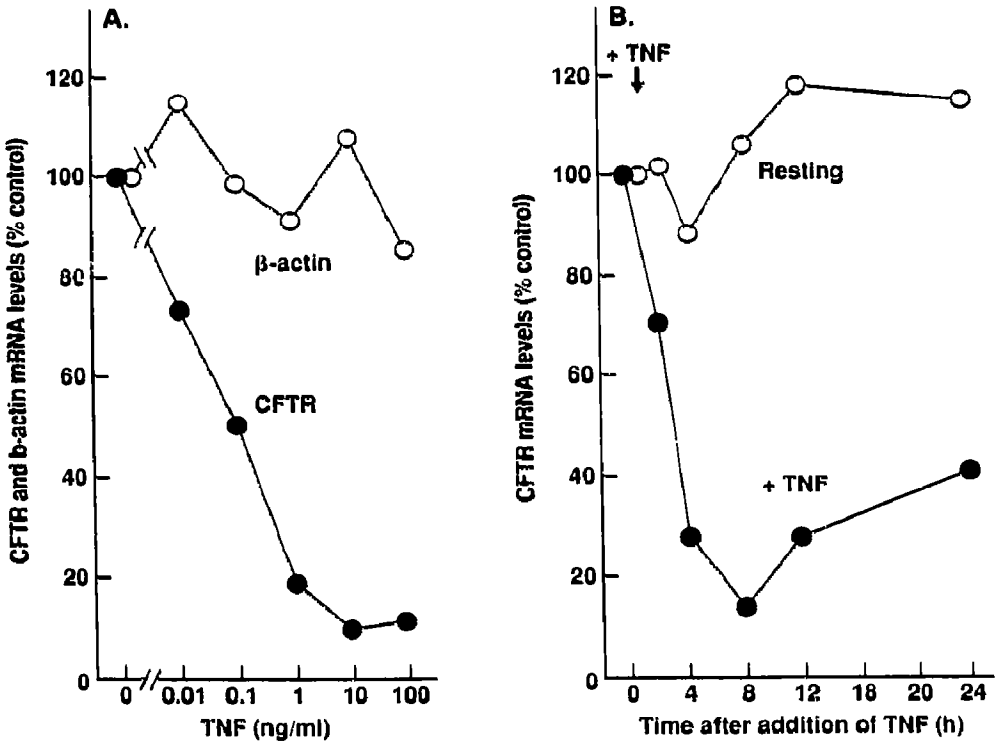

Fig. 2. CFTR mRNA levels in HT-29 cells following exposure to TNF. A. CFTR (●) and β-actin (○) mRNA transcript levels in response to increasing concentrations of TNF (0–100 ng/ml, 8h). B. Time course of CFTR mRNA expression in resting HT-29 cells (○) or cells exposed to TNF (10 ng/ml) for various periods of time (●). The data represent densitometric scanning of the autoradiograms of Northern analyses, and each data point is presented as % of the resting level (A) and level at time zero (B) respectively (defined as 100%). Shown are representative data from two separate experiments.

3.3. *Stability of CFTR mRNA transcripts*

Following inhibition of RNA synthesis with actinomycin D, CFTR mRNA levels in resting HT-29 cells fell relatively slowly with a half-life of 18.7 h, suggesting that CFTR mRNA transcripts were relatively stable in the resting state (Fig. 4). In contrast, the addition of TNF to HT-29 cells decreased the half-life of the CFTR mRNA transcripts (12.2 h), suggesting that downregulation of CFTR transcript levels following TNF stimulation is modulated, at least in part, by changes in transcript stability. Three separate experiments were carried out to confirm these results.

4. DISCUSSION

The critical role of the CFTR gene product in many tissues with epithelial surfaces is highlighted by the profound clinical manifestations of cystic fibrosis in the gastrointestinal and respiratory tracts [1]. The CFTR gene is expressed at low levels in these sites, as evidenced by the low abundance of CFTR mRNA in epithelial cells examined [3,12,26]. There is evidence, however, that expression of the CFTR gene can be modulated by mediators such as phorbol esters [12,14] and calcium ionophores [15], and by the state of differentiation and/or proliferation state of the cell [27,28]. In those examples where the level of modulation has been identified, control of CFTR expression appears to be focused at the level of transcription [12,15]. Consistent with these observations, analysis of the promoter of the CFTR gene shows it has a variety of putative control sequences capable of binding regulatory proteins, despite its overall characteristics as a housekeeping-type gene and its relatively low level of expression in epithelial cells [12].

The concept that TNF might modulate CFTR mRNA levels arose from the recognition that a segment of the CFTR promoter (−168 to −141 from the major CFTR transcription start site in T84 and HT-29 colon carcinoma cells) had an 89% homology with a sequence of the α1(I) collagen gene promoter that responds to TNF by downregulating the rate of gene transcription, although the direct linkage between the sequence and modulation by TNF is unknown [12,16,17]. Together, the knowledge that TNF is produced by inflammatory cells frequently populating epithelial surface in CF [29–36], and the observation of elevated TNF levels in plasma and respiratory epithelial lining fluid of individuals with CF [37,38] leads to the hypothesis that TNF may play a role in modulating CFTR gene expression.

Interestingly, the data demonstrates that although

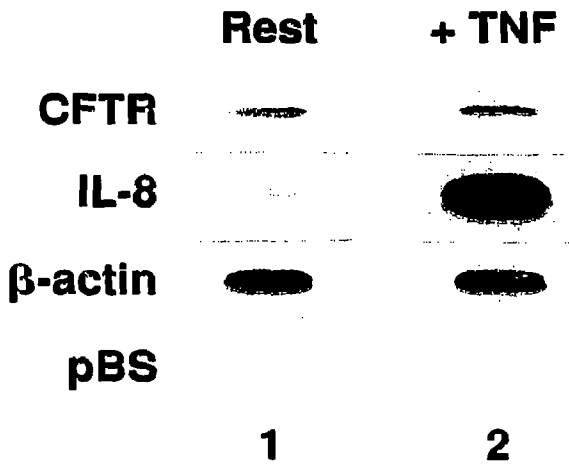

Fig. 3. Effect of TNF on the transcription rate of the CFTR gene in HT-29 cells. The cells were incubated in the absence or presence of TNF (10 ng/ml) for 8 h. ^{32}P-labeled nascent nuclear RNA was hybridized to nylon membrane-bound DNA plasmid targets (5 μg each) including CFTR cDNA, β-actin cDNA, pBluescript II SK+ (pBS, an irrelevant plasmid control) and IL-8 cDNA. Shown is an autoradiogram representative of three individual experiments. Lane 1, Resting HT-29 cells; lane 2, HT-29 cells after TNF exposure. Note that no significant change was observed in transcription levels of the CFTR gene as well as endogenous control β-actin gene after incubation with TNF, while IL-8 gene transcription increased markedly.

TNF causes CFTR mRNA levels to decline in epithelial cells, it does so, at least in part, by reducing the half-life of CFTR mRNA transcripts, not by suppressing the rate of transcription of the CFTR gene. However, since CFTR mRNA levels are profoundly suppressed as soon as 4 h after exposure and the overall decrease in CFTR mRNA transcript levels surpass the decrease in the mRNA half-life, it is likely that other mechanism(s), such as processing of heterogenous nuclear RNA following transcription of the CFTR gene in nuclei resulting in instability, or different unstable population of CFTR mRNA transcripts in the presence of TNF, contribute to the TNF-induced down regulation of CFTR gene expression. Of genes known to be regulated by TNF, the modulation occurs primarily at the transcriptional level [34,35]. Post-transcriptional modulation by TNF has been observed for the gene for macrophage-specific colony stimulating factor, but unlike CFTR, this gene is also modulated by TNF at the transcriptional level [39].

The fact that TNF can modulate CFTR gene expression at the post-transcriptional level has implications for designs for the gene therapy of CF. Based on the evidence that the human CFTR gene can be transferred the epithelia of the lung in vivo, it is now possible to conceptualize such an approach [40]. One of the important questions in designing gene therapy for CF is whether or not the transferred gene will have to be controlled at the transcriptional level. Likely, this question will have to be answered empirically, but the decision to include regulatory elements will partly depend on the list of known modulators of the CFTR gene, and their theoretical relevance for in vivo modulation of the gene. In the context that TNF is present in elevated levels in the plasma and respiratory epithelial lining fluid of individuals with CF [37,38], the fact that TNF regulates CFTR mRNA levels at the post-transcriptional level suggests that concerns about transcriptional control (at least for this mediator) are irrelevant for gene therapy, as long as the normal CFTR gene that is transferred has the appropriate (as yet unknown) recognition sequences for TNF to modulate CFTR mRNA stability [41].

As regards to cystic fibrosis, theoretically, TNF could play a modulating influence on the disease state. In this regard, there is anecdotal evidence that compound 'null' heterozygotes with stop codon mutations of CFTR protein coding sequence have milder lung disease than do individuals homozygous for the common ΔF508 mutation [42]. Thus, it is conceivable that downregulation of expression of the CFTR gene in ΔF508 homozygotes may have a moderating influence in the disease, i.e. by converting the ΔF508 homozygous state to the equivalent of the homozygous null state.

Finally, the fact that TNF can modulate the expression of the normal CFTR gene has implications for the modulation of this gene by inflammatory mediators in individuals who do not have cystic fibrosis. TNF is

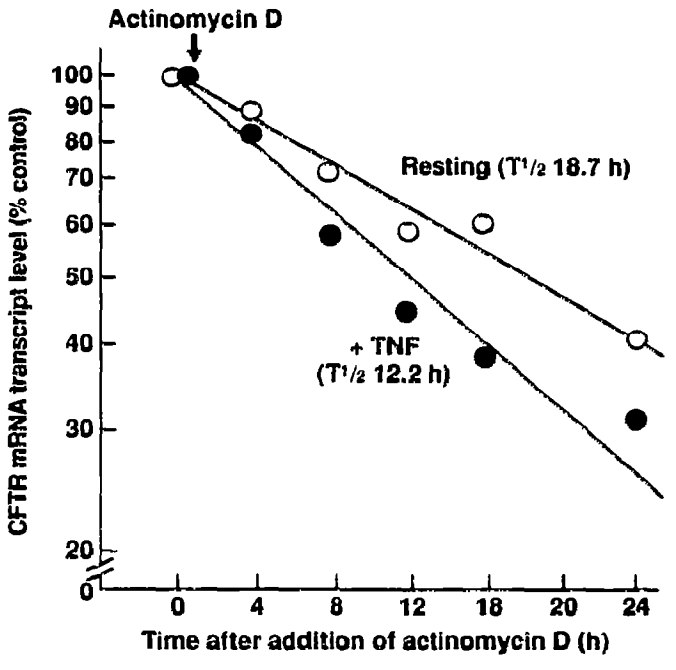

Fig. 4. Change in stability of CFTR mRNA transcripts in HT-29 cells in response to TNF. Shown are effects of inhibition of RNA synthesis on CFTR mRNA levels in resting (○) or TNF-treated (10 ng/ml, 8 h) HT-29 cells (●). Cells were harvested at the indicated times after the addition of actinomycin D (5 μg/ml) and extracted RNA was evaluated by Northern analysis. Data shown are representative of three individual experiments.

released by activated macrophages and neutrophils, cells commonly associated with epithelial inflammation [29–36]. In this regard, it is conceivable that local downregulation of the CFTR gene, and induces 'localized cystic fibrosis' in the affected region, i.e. inflammation on the surface of epithelial cells may signal downregulation of the CFTR gene, with the consequent equivalent of a mild cystic fibrosis phenotype in the local milieu.

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